Defective Release of Corepressor by Hinge Mutants of the Thyroid Hormone Receptor Found in Patients with Resistance to Thyroid Hormone*

(Received for publication, June 2, 1998, and in revised form, August 12, 1998)

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On positive thyroid hormone response elements (pTREs), thyroid hormone receptor (TR) binding to DNA in the absence of ligand (thyroid hormone, T₃) decreases transcription (silencing). Silencing is due to a family of recently described nuclear corepressor proteins (NCoR and SMRT) which bind to the CoR box in the hinge region of TR. Ligand-dependent activation of TR is associated with displacement of corepressors and recruitment of coactivating proteins. Resistance to thyroid hormone (RTH) is due to mutations in the β isoform of the thyroid hormone receptor (TR-β). To date, three RTH mutations reported with near-normal T₃ binding (A234T, R243Q, and R243W) have been described in or near the CoR box. To determine the mechanism of RTH caused by these mutants, the interaction of wild type (wt) and mutant TRs with the corepressor, NCoR, and the coactivator, SRC-1, was tested in gel-shift assays. As expected, NCoR bound wt TR in the absence of T₃ and dissociated from TR with increasing T₃ concentration. SRC-1 failed to bind wt TR in the absence of T₃, but bound to TR with increasing avidity as T₃ concentrations rose. At no T₃ concentration did both NCoR and SRC-1 bind to wt TR, indicating that their binding to TR was mutually exclusive. Hinge mutants bound NCoR normally in the absence of T₃, however, dissociation of NCoR and recruitment of SRC-1 was markedly impaired except at very high T₃ concentrations. Importantly, hinge mutant TRs when complexed to DNA bound T₃ poorly despite their near-normal T₃ binding in solution. These binding studies correlated with functional assays showing defective transactivation of pTREs by hinge mutants except at high T₃ concentrations. Thus, we describe a novel mechanism of RTH whereby TR hinge mutants selectively affect T₃ binding when complexed to DNA, and prevent NCoR dissociation from TR. Our data also suggest that solution T₃ binding by RTH mutants may not accurately reflect physiologically relevant T₃ binding by TR when bound to DNA.

Thyroid hormone receptor (TR) is a member of the steroid/receptor superfamily and regulates the expression of many target genes through its ability to bind to thyroid hormone response elements (TREs) (1, 2). TR contains a variety of protein domains involved in DNA binding, hormone binding, receptor dimerization, and interaction with the basic transcription machinery. On positive response elements (pTREs), TR binding to DNA in the absence of ligand (thyroid hormone, T₃) decreases transcription (silencing) (3). Silencing is due to binding of the TR to a family of recently described nuclear corepressor proteins (TRACs, NCoR (RIP 13), SMRT, SUN-CoR) (4–15). Corepressors are thought to silence transcription by promoting a closed chromatin configuration through histone deacetylation. The CoR box (amino acids 211–240 of human TR-β1), located in the hinge region of TR, binds corepressor proteins (16). Addition of T₃ results in displacement of corepressors and a return of transcription to a basal rate (4, 5). In the presence of ligand, proteins termed coactivators are recruited to mediate the ligand-dependent response (10, 12). A large number of nuclear receptor-interacting or coactivating proteins have been isolated including SRC-1 (ERAP 160, p160), RIP 160, ERAP 140 (p140), R1P140, TIF1, TIP2 (GRIP1), TRIP1 (SUG-1), RAP 46, hRPF 1, ARA 70 (RFG), CBP (p300), pCAF, P120, ACTR (AIB1, TRAM-1, pCIP), and GRIP 170 (10, 12–29).

Resistance to thyroid hormone (RTH) is the result of mutations in the carboxyl terminus of the β thyroid hormone receptor (TR-β) (30–33). Individuals with the disorder require greater thyroid hormone (T₃) concentrations in order to achieve T₃-dependent actions in tissues. RTH is a dominant disorder in which most individuals are heterozygous for a mutant TR-β allele. In a phenomenon called dominant negative activity, the mutant allele interferes with the activity of the normal allele (34–37). RTH mutations congregate in two major “hot spots” in the ligand binding domain of TR-β (38, 39). Recently, mutations have also been reported in the hinge region, suggesting a third hot spot for mutations within the TR-β locus (41–43).

To date, six naturally occurring RTH mutations have been described in or near the hinge region (amino acids 174–237 (44)) of TR: A234T, R243Q, R243W, V264D, T277A, and R282S (40, 42, 43). The former three mutations are located in or near the CoR box. Based on the location of corepressor binding, RTH mutations in or near the CoR box might be expected to alter corepressor binding and affect TR function. While the A234T mutation is reported to have mildly decreased T₃ binding, R243Q and R243W are reported to bind T₃ normally in solution.

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Experimental Procedures

Site-directed mutagenesis (CLONTECH, Palo Alto, CA) was used to create hinge mutations in the context of the human TR-β1 isoform (45). All mutations were confirmed by DNA sequencing of the TR-β1 C terminus. The expression vector used for transfections in this study was pSG5, containing either wild type (wt) or mutant TR-β cDNAs as EcoRI fragments. The human TR-β1 cDNA was used as the wt TR-β1. Expression vector plasmid preparations used in this study were carefully quantitated by agarose gel electrophoresis. To confirm the integrity and quality of each expression vector plasmid DNA preparation, in vitro translation with [35S]methionine was performed using T7 polymerase and the products analyzed by SDS-polyacrylamide gel electrophoresis.

The SRC-1 nuclear binding domain (NBD-1) fragment was generated by polymerase chain reaction of amino acids 594–780 of an F-SRC-1 clone generously provided by William W. Chin. The generation of the binding domain of NCoR, NCoR-I, has been previously described (9).

Reporter constructs included two copies of idealized pTREs, direct repeat with a four-base pair interval (DR 1) is not significantly impaired with each mutant displaying only a 20–40% reduction in T3 binding. These results confirm the mechanism by which they cause RTH.

Defective Corepressor Release by Hinge Mutant TRs

Gel-shift studies were performed with 32P-labeled DR 1 probe, the indicated TR, and T3. Concentrations of T3 were used as noted. Data were pooled from at least three independent experiments and displayed as mean ± S.E.

Gel-shift studies were performed using constructs of the wt and mutant TRs placed in a pGEM vector. To study corepressor binding, a construct was fashioned inserting the TR interacting domains of NCoR (9) into the pKCR2 expression vector. TRs and the binding domain of NCoR were in vitro translated using rabbit reticulocyte lysate (Promega, Madison, WI). The nuclear binding domain (NBD-1) of SRC-1 (21, 49) was inserted into a procaryote glutathione S-transferase (GST) expression vector resulting in a GST fusion protein. TR-SRC interactions were evaluated accordingly. A polyclonal anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA) which specifically disrupted the TR/SRC interaction was used to more clearly identify TR-SRC-1 interactions.

The binding affinities for wt and mutant TRs in solution were assessed with a filter binding assay utilizing 125I-labeled T3 (50). Ks values were assigned after Scatchard analysis. T3 binding on DNA was also assessed following a standard gel-shift protocol except that 125I-labeled T3 replaced 32P-labeled DR +4 for visualization.

RESULTS

TR-β Mutations Used in This Study—As shown in Fig. 1, three TR-β mutations were evaluated in this study (A234T, R243Q, and R243W). These mutations were introduced into a TR-β1 cDNA in pGEM 3, using site-directed mutagenesis and transferred to the expression vector pSG5 for use in transient transfection studies. Mutant TR binding to T3 in solution (Table I) is not significantly impaired with each mutant displaying only a 20–40% reduction in T3 binding. These results confirm those of other laboratories studying the T3 binding properties of these mutant receptors (40, 42, 43).

Ligand-dependent Dissociation of Corepressor from Mutant TRs Is Impaired—We first evaluated mutant TR binding to the nuclear corepressor, NCoR, using a gel-shift assay. The three hinge mutations noted above (A234T, R243Q, and R243W), a severe GRT mutation (Δ377T) (51), and an artificial hinge mutant known to not interfere with corepressor binding (E220R) (7) were tested in this assay. A portion of NCoR, containing the TR interacting domains but lacking the repressing domains, termed NCoR-I, was employed in the assay (9).
vitro translated TRs formed a dimer (D) in the absence of NCoR-I on a DR1-4 radiolabeled element. Addition of in vitro translated NCoR-I caused the dimer to be shifted to a new position and serial dilution of the amount of NCoR-I added to the binding reaction reduced the intensity of this complex. Ligand-independent binding of NCoR-I to each of the mutants was similar to wt TR (Fig. 2). Addition of RXR-α or RXR-β to

**Fig. 3.** TR hinge mutants require more T3 to dissociate NCoR than does wt TR. A 32P-labeled DR+4 gel shift as in Fig. 2 was done with reticulocyte lysate generated TRs incubated with NCoR binding domain across a range of T3 concentrations as noted: A, wt; B, E220R; C, A234T; D, R243Q; E, R243W; and F, Δ337T. Note that NCoR-I cannot be dissociated from the Δ337T mutant at any of the T3 concentrations tested.

**Fig. 4.** Shown is a 32P-labeled Lys gel shift as in Fig. 2 of TRs incubated with NCoR binding domain across a range of T3 concentrations as noted. Hinge mutant TRs required more than 10-fold more T3 to achieve the same degree of dissociation as wt. TRs are A, wt; B, E220R; C, A234T; D, R243Q; E, R243W; and F, Δ337T.

**Fig. 5.** Hinge mutant recruitment of SRC-1 is only mildly impaired relative to wt. A 32P-labeled DR+4 gel shift as in Fig. 2 is demonstrated. Reticulocyte lysate generated TRs were incubated with GST fusion protein generated SRC-1 (NBD-1) across a range of T3 concentrations as noted: A, wt; B, A234T; C, R243Q; and D, R243W. The R243Q mutant is nearly normal in SRC-1 (NBD-1) recruitment; the A234T and R243W mutants require a T3 concentration twice that for wt to recruit SRC-1.
the binding reaction resulted in the formation of a strong heterodimeric complex, and a significant reduction in the TR\(\mathrm{z}\)NCoR-I complex (data not shown).

In contrast, as shown in Fig. 3, T\(_3\) dissociation of NCoR-I from certain mutant TRs was much different than from wt TR on the DR\(_{1-4}\) element. With wt TR, 5 nM T\(_3\) resulted in an almost complete dissociation of the TR/NCoR-I complex (panel A). The artificial hinge mutant with normal T\(_3\) binding, E220R (panel B), had a similar NCoR-I dissociation pattern. The A234T mutant (panel C), however, required about 2-fold higher concentration of T\(_3\) to dissociate NCoR-I than did wt TR. The R243Q and R243W mutants (panels D and E) required about 20-fold greater T\(_3\) concentration relative to wt TR to dissociate this complex. Finally the Δ337 mutant, which cannot bind T\(_3\), could not be dissociated from NCoR-I even at the 100 nM T\(_3\) concentration. Similar observations were made on the chicken lysozyme TRE (Lys, Fig. 4) and palindromic TRE (data not shown). On the Lys element, a higher T\(_3\) concentration was necessary to completely dissociate NCoR-I from wt TR (100 nM). Dissociation of NCoR-I from E220R and Δ234T was similar to wt, while the R243Q and R243W mutant TRs required a 10-fold higher T\(_3\) concentration (1000 nM) than did wt TR for dissociation. As expected, NCoR-I could not be dissociated from the Δ337T mutant even at this very high T\(_3\) concentration.

Recruitment of SRC-1 by Mutant TRs Is Mildly Impaired Relative to wt TR—To test whether hinge mutants could normally recruit SRC-1, the NBD-1 of SRC-1 (21, 49) was fused to the GST protein and expressed in bacteria. Purified GST and GST-SRC-1(NBD-1) were then employed in a gel-shift study of wt and mutant TR binding to a DR\(_{1-4}\) probe (Fig. 5). In the absence of T\(_3\), SRC-1 did not form a complex with wt TR (panel A). A wt TR-SRC-1 complex was first noted at 2.5 nM and was maximal at 100 nM T\(_3\). These results were unexpected since T\(_3\) is known to dissociate the TR homodimer and prevent its binding to DNA. The presence of a TR\(\mathrm{z}\)SRC-1 complex indicates that the SRC-1(NBD-1) must, in some way, stabilize the binding of TR on DNA in the presence of T\(_3\). The three hinge mutants also recruited the NBD-1 of SRC-1 with increasing T\(_3\) concentrations. The R243Q mutant (panel C) appeared qualitatively similar to wt TR in its ability to recruit SRC-1 over the T\(_3\) concentrations tested. The A234T and R243W mutants (panels D and E) required about 2-fold greater T\(_3\) concentrations, relative to wt TR, to begin to recruit SRC-1(NBD-1). In Fig. 6, these experiments were repeated in the presence of RXR. SRC-1(NBD-1) was able to bind to both wt TR homodimers and wt TR/RXR heterodimers (panel A) as evidenced by the decrease in intensity of both complexes and the presence of two closely spaced more slowly migrating complexes on a lighter exposure of this autoradiogram (data not shown). Qualitatively, how-
ever, the defect in SRC-1 association caused by the hinge mutants was similar to the data obtained without RXR. Thus, hinge mutants display a significantly greater impairment in T₃-mediated dissociation from NCoR-I than T₃'-mediated association to the NBD-1 of SRC-1.

TR Binding to NCoR Prevents SRC-1 Recruitment—To determine whether NCoR binding interfered with SRC-1 recruitment, the gel-shift assays were repeated using TR interacting domains from both cofactors. In Fig. 7 gel-shifts obtained using the wt TR and two hinge mutants, R243Q and R243W are shown. Although the TR-NCoR-I and TR-SRC-1(NBD-1) complexes migrated at a similar position, closer inspection revealed that the TR-SRC-1(NBD-1) complex migrated slightly faster than the TR-NCoR-I complex. We used this difference in migration position and an antibody directed against the GST protein to distinguish the two complexes. In the absence of T₃ where only NCoR-I would be expected to bind to the wt TR, one distinct complex was formed and the location and intensity of the complex was not affected by addition of the GST antibody (panel A). Conversely at high T₃ concentration (100 nM) where only SRC-1(NBD-1) should bind to TR, a faster migrating complex was formed which was completely eliminated by the addition of the GST antibody. A gradual shift from the TR-NCoR-I to the TR-SRC-1(NBD-1) complex was noted over the T₃ concentrations tested (panel A).

Under no condition was a complex formed consistent with binding both NCoR-I and SRC-1(NBD-1) to wt TR. Rather, as the T₃ concentration rose, the shifted band migrated from the
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**Solution and DNA Complex Binding of Mutant TR Do Not Correlate**—It is unclear why the hinge mutants displayed such significant impairment in dissociation from NCoR-I in the absence of significant defects in solution binding to T₃. Moreover, the R243Q and R243W mutants required significantly greater T₃ concentrations to dissociate NCoR-I than did the A234T mutant despite the fact that these receptors had very similar T₃ binding profiles. To investigate the reason for this discrepancy, gel-shift assays were performed in parallel using either a ³²P-labeled DR¹⁺ probe or ¹²⁵I-T₃ to identify the shifted complexes. Fig. 8 is such an experiment comparing wt and mutant TRs. In vitro translated RXR-α and the indicated TR-β were added to each column except columns 1 and 8 where an equal volume of unprogrammed lysate was used (UP). In columns 2–7, reticulocyte lysate generated TRs (wt, E220R, A234T, R243Q, R243W, and Δ337T, respectively) were incubated with RXR-α and ³²P-labeled DR¹⁺, resulting in heterodimeric and homodimeric complexes of equal intensity. In columns 9–14 the same paradigm was followed except that the ³²P-labeled DR¹⁺ pTRE was replaced by a nonradioactive DR¹⁺ pTRE and ¹²⁵I-T₃ was added (10 nM final concentration).

As found by others (52), the wt TR/RXR heterodimer was clearly labeled by ¹²⁵I-T₃ (lane 9) and the homodimer was not labeled, despite equal amount of these complexes on the ³²P-labeled gel-shift. We interpret this result as indicating that occupancy of one TR molecule by radiolabeled T₃ marks the heterodimer which is not dissociated by T₃, while T₃ bound to one TR molecule is sufficient to dissociate the homodimer and eliminate a labeled band at that position. Because of the migration position of free ¹²⁵I-T₃ on the gel, we could not determine if the wt TR/NCoR-I complex was labeled. The heterodimeric complex containing the A234T mutant was labeled 3-fold less well by T₃ (as determined by densitometry) and heterodimers containing the R243Q, R243W, and Δ337T mutants were not labeled. Clearly the Δ337T mutant would not be expected to bind T₃; however, results with the hinge mutants were unexpected given their solution T₃ binding. These results indicate that solution and DNA-complex binding of T₃ by hinge mutants of the TR do not correlate.

**Mutant TR Function Is Diminished at T₃ Concentrations Where NCoR Remains Bound and Is Not Reversed by Transfection of an NCoR Inhibitor (NCoR-I)**—As a model for thyroid hormone action, two copies of a positive thyroid hormone response element were fused upstream of a heterologous promoter luciferase construct (pTK109-Luc) for use in transient transfection assays of CV-1 cells. On the DR¹⁺ element were fused upstream of a heterologous promoter luciferase reporter gene.

The net result of the impaired NCoR-I dissociation from hinge mutants is also shown in Fig. 7 (panels B and C). Only at 100 nM T₃ was NCoR-I sufficiently dissociated from the hinge mutants to permit significant SRC-1(NBD-1) recruitment, based on the position of the protein-DNA complex. The GST antibody blocks formation of this complex, confirming that at this T₃ concentration, the hinge mutants bound exclusively to SRC-1(NBD-1). At 10 nM T₃, SRC-1(NBD-1) bound somewhat to the hinge mutants based on partial dissociation of the shifted complex by the GST antibody (R243Q > R243W). This result is consistent with the other data showing that the R243Q mutant recruited SRC-1(NBD-1) at lower T₃ concentrations than the R243W mutant. Similar data was obtained with the A234T mutant (not shown). Thus, an impairment in NCoR dissociation by T₃, as observed with the hinge mutants, prevents SRC-1 recruitment.

**Fig. 10.** A, an NCoR inhibitor (NCoR-I) reverses ligand-independent repression by wt TR. Shown are the results of transient transfection of CV-1 cells with plasmids containing wt TR, NCoR-I, and the luciferase reporter gene. The net result of the impaired NCoR-I dissociation from hinge mutants is also shown in Fig. 7 (panels B and C). Only at 100 nM T₃ was NCoR-I sufficiently dissociated from the hinge mutants to permit significant SRC-1(NBD-1) recruitment, based on the position of the protein-DNA complex. The GST antibody blocks formation of this complex, confirming that at this T₃ concentration, the hinge mutants bound exclusively to SRC-1(NBD-1). At 10 nM T₃, SRC-1(NBD-1) bound somewhat to the hinge mutants based on partial dissociation of the shifted complex by the GST antibody (R243Q > R243W). This result is consistent with the other data showing that the R243Q mutant recruited SRC-1(NBD-1) at lower T₃ concentrations than the R243W mutant. Similar data was obtained with the A234T mutant (not shown). Thus, an impairment in NCoR dissociation by T₃, as observed with the hinge mutants, prevents SRC-1 recruitment.

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the two 243 mutant TRs was first noted at 10 nM T3. At very high T3 doses, however, transactivation by all mutants except D337T equaled that of wt TR. Each hinge mutant had strong dominant negative activity against wt TR on the DR1 element (data not shown). Qualitatively similar results were noted with the everted palindrome element derived from the chicken lysozyme gene promoter, Lys (Fig. 9, panel B).

Since transfection of an NCoR inhibitor (NCoR-I) expression vector can reverse TR-mediated repression on pTREs (9), we wanted to determine if cotransfection of this plasmid could correct the defect in transactivation observed with the hinge mutants. In Fig. 10A, cotransfection of NCoR-I completely reversed ligand-independent repression by the wt TR on the DR+4 element and yielded similar levels of reporter gene activation in the presence of T3. Since endogenous corepressors (NCoR and SMRT) and NCoR-I are released from wt TR at physiological T3 concentrations, transcriptional repression by endogenous corepressors is selectively removed by NCoR-I without affecting T3-mediated coactivator recruitment and transactivation. In contrast, in Fig. 10B are data obtained with one of the hinge mutants, R243W. Note that NCoR-I cotransfection completely eliminated TR-mediated repression but did not allow for normal T3-mediated transactivation except at 100 nM T3. Importantly, however, we determined the ability of these mutants to bind to T3 when complexed to DNA. To our surprise, these mutants as RXR heterodimers bound radiolabeled T3 poorly, if at all, in gel shift studies. Our results are limited to the heterodimer complex since TR homodimers were not labeled in this assay, perhaps due to the fact they would be dissociated when bound by T3, and free radiolabeled T3 obscured the TR-NCoR-I complex. Regardless these data suggest that binding of T3 in solution and on DNA do not always correlate as highlighted by mutations in the hinge region of TR.

Based on the crystal structure of TR-a, hinge mutants might affect T3 binding (53). These mutations are near helix two, which is believed to stabilize the polar pocket which binds T3. Since regions of the hinge domain are known to contact DNA (54, 55) and to mediate ligand binding (56) it is possible that DNA-binding induces a conformation change in the mutant hinge domain so as either to prevent ligand access to the heterodimeric bound TR or prevent stabilization of ligand-binding on the TR. The net result is that corepressor continues to bind to TR and prevent transactivation despite the presence of T3 in solution. It is unclear whether this discrepancy between solution and DNA-complex binding of T3 is specific for hinge mutants or will also be observed with RTH mutants in other locations on the TR-β.

**FIG. 11. Model of resistance caused by hinge mutants of the TR.** When wt TR is bound to DNA, addition of T3 results in dissociation of corepressor, permitting recruitment of coactivator and transcriptional activation. When hinge mutant TR is bound to DNA, T3 cannot bind and NCoR is not dissociated. SRC-I recruitment is prevented and DNA transcription remains repressed.
In evaluating TR cofactors and their role in RTH, the question of the relative importance of corepressors and coactivators in the genesis of RTH has arisen. For example, impaired ligand-dependent activity could be associated either with impaired (13) coactivator release or with defective coactivator recruitment (52, 57). Our data suggest that impaired corepressor release is the primary defect in RTH patients with hormone mutations and probably for most other patients with RTH. This is based on two lines of evidence. 1) Most RTH mutations affect T₃ binding to TR in solution or on DNA, as is the case with hinge mutants. This would result in impaired release of corepressor by physiological T₃ concentrations and continued gene silencing. 2) Both NCoR and SRC-1 do not bind simultaneously to TR, suggesting at least two discreet steps are necessary for transactivation. Addition of T₃ must first cause dissociation of NCoR before recruitment of SRC-1 can take place. Either the binding locations of the two cofactors must overlap such that both cannot be present on TR or the TR conformation when NCoR is bound is such that SRC-1 binding is not favorable. This result is most clearly seen using the NCoR inhibitor in transfection studies. Transfection of NCoR-I competes with endogenous corepressors, resulting in a loss of ligand-independent repression on pTREs caused by the R243W mutation. Remarkably the mutant TR is still unable to activate transcription except at very high T₃ concentration. Since mutant TR binding to NCoR-I is also resistant to T₃ dissociation, SRC-1 or other coactivators are not recruited to the TR due to lack of binding to the corepressor occupied TR, yielding a lack of transactivation.

We propose the following model (Fig. 11) based on our results. wt TR, in the absence of ligand, binds corepressor and transcription is repressed. Addition of T₃ results in dissociation of corepressor, permitting recruitment of coactivators and transactivation activation. Although, the hinge mutant TRs bind corepressor normally in the absence of ligand resulting in normal silencing activity, T₃ fails to dissociate the corepressor and thus coactivator recruitment is prevented. While T₃ binding by the hinge mutant TRs is normal or near-normal in solution, their T₃ binding is clearly defective when complexed to DNA. Our data indicate that DNA-binding modifies the ability of hinge mutants to bind T₃, yielding this novel mechanism of resistance by this class of RTH mutants.

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