Rearrangements in Thyroid Hormone Receptor Charge Clusters That Stabilize Bound 3,5',5-Triiodo-L-thyronine and Inhibit Homodimer Formation*

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In this study, we investigated how thyroid hormone (3,5',5-triiodo-L-thyronine, T₃) inhibits binding of thyroid hormone receptor (TR) homodimers, but not TR-retinoid X receptor heterodimers, to thyroid hormone response elements. Specifically we asked why a small subset of TRβ mutations that arise in resistance to thyroid hormone syndrome inhibit both T₃ binding and formation of TRβ homodimers on thyroid hormone response elements. We reasoned that these mutations may affect structural elements involved in the coupling of T₃ binding to inhibition of TR DNA binding activity. Analysis of TR x-ray structures revealed that each of these resistance to thyroid hormone syndrome mutations affects a cluster of charged amino acids with potential for ionic bond formation between oppositely charged partners. Two clusters (1 and 2) are adjacent to the dimer surface at the junction of helices 10 and 11. Targeted mutagenesis of residues in Cluster 1 (Arg₃₃₈, Lys₃₄₂, Asp₃₅₁, and Asp₃₅₅) and Cluster 2 (Arg₄₂₉, Arg₃₈₃, and Glu₃₁₁) confirmed that the clusters are required for stable T₃ binding and for optimal TR homodimer formation on DNA but also revealed that different arrangements of charged residues are needed for these effects. We propose that the charge clusters are homodimer-specific extensions of the dimer surface and further that T₃ binding promotes specific rearrangements of these surfaces that simultaneously block homodimer formation on DNA and stabilize the bound hormone. Our data yield insight into the way that T₃ regulates TR DNA binding activity and also highlight hitherto unsuspected T₃-dependent conformational changes in the receptor ligand binding domain.

Thyroid hormone receptors (TRα and TRβ)¹ are conditional transcription factors that play important roles in development, metabolism, and homeostasis (1–4). TRs regulate gene transcription in the presence of 3,5,3′-triiodo-L-thyronine (T₃) and in the absence of ligand (5). Current efforts to modulate TR activities have focused on development of selective agonists that mimic the beneficial effects of T₃ upon circulating cholesterol and body weight without producing unwanted effects of the hormone on heart rate (6). However, there is also a need for TR antagonists, which could represent improved and faster acting treatments for hyperthyroidism and cardiac arrhythmias (6, 7). Furthermore observations from TRα/β knock-out mice suggest many clinical manifestations of hypothyroidism are due to actions of unliganded TRs (8, 9). Thus, drugs that specifically reverse actions of unliganded TRs could be useful for treating hypothyroidism and would avoid risk of thyroid hormone excess (7). Improved understanding of unliganded TR structure and ways that unliganded TRs rearrange in response to T₃ will facilitate development of all of these drugs.

Presently the organization of unliganded TR is only partly understood (10, 11). X-ray structures of liganded TR C-terminal ligand binding domains (LBDs) reveal a canonical α-helical structure with T₃ buried in the core of the protein (12–16), but there are no equivalent structures of unliganded TRs. It has proven possible, however, to use a combination of x-ray structural information and targeted mutagenesis to learn about the organization of unliganded TRs. For example, T₃ blocks transcriptional and transrepression activities of unliganded TRs by promoting release of corepressors such as N-CoR and SMRT (silencing mediator of retinoid and thyroid receptors) (5) and induces a T₃-dependent activation function (AF-2) that binds coactivators such as the p160s (17). Functional analysis of TR mutants reveals that AF-2 is comprised of surface-exposed residues from helices (H) 3, 5, and 12 and that the corepressor binding surface overlaps AF-2 but extends below the position of H12 in the liganded state (18–21). Thus, it is possible to infer that H12 is displaced in the unliganded state and that T₃ binding leads to repositioning of H12 over the lower part of the corepressor binding surface, simultaneously promoting corepressor release and completing the coactivator binding site (5).

T₃ also regulates TR DNA binding activity (1). TRs utilize their DNA binding domain to recognize specific thyroid hormone response elements (TREs) comprised of AGGTCA repeats and bind these elements either as heterodimers with the closely related retinoid X receptor (RXR) or as homodimers and monomers. T₃ does not affect RXR-TR interactions with TREs but does promote release of TR homodimers from some TREs (inverted palindromes (F2/IP-6) and direct repeats (DR-4)) but not from TREs at which TRs bind as monomers or paired monomers (palindromes, TREpal) (22). TR homodimers bind N-CoR more strongly than RXR-TR heterodimers (23, 24), and the extent of TR homodimer binding to different TREs in vitro correlates with the extent of repression from these elements in

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The abbreviations used are: TR, thyroid hormone receptor; T₃, 3,5',5-triiodo-L-thyronine; RXR, retinoid X receptor; TRE, thyroid hormone response element; RTH, resistance to thyroid hormone syndrome; H, helix; LBD, ligand binding domain; N-CoR, nuclear receptor corepressor; AF, activation function; DR, direct repeat; IP, inverted palindrome; GRIP1, glucocorticoid receptor-interacting protein 1; ANOVA, analysis of variance; PPAR, peroxisome proliferator-activated receptor; NR, nuclear hormone receptor.
vivo (25, 26). Thus, it is thought that T₃-dependent inhibition of homodimer formation relieves transcriptional repression by unliganded TRs. Nevertheless the mechanisms involved in coupling of T₃ binding to inhibition of DNA binding are not clear; TRs utilize the same surface at the junction of H10 and H11 in homodimer and heterodimer formation on DNA (27). The structural elements that render homodimers sensitive to T₃ are not known.

In this study, we utilized targeted mutagenesis to explore elements of the TR that are specifically required for homodimer formation on TREs and tested the hypothesis that the same surface at the junction of H10 and H11 in homodimer and heterodimer formation on DNA (27). The structural elements that render homodimers sensitive to T₃ are not known.

**MATERIALS AND METHODS**

**TR Mutants**—The pCMX vector was used for expression of the full-length human TRβ (17). Mutations within TR-encoding sequences were created using the QuikChange XL site-directed mutagenesis kit (Stratagene). Mutation of target sequences was verified by automated DNA sequence (Eli Lilly Biopharmaceuticals, Inc., Hayward, CA).

**Transfections**—HeLa cells were maintained in Dulbecco’s modified Eagle’s H-21 4.5 g/liter glucose medium containing 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin. For transfection, cells were collected and resuspended in Dulbecco’s phosphate-buffered saline (0.5 mM/4.5 × 1⁰⁻³ cells) containing 0.1% dextran and typically 4 µg of reporter, 1 µg of T3 expression vector or empty vector control, and 2 µg of pCMV-beta-galactosidase (17). Cells were electroporated at 240 V and 960 microfarads, transferred to fresh media, and plated into 12-well plates. After incubation for 24 h at 37 °C with T3 or vehicle, cells were collected, and pellets were lysed by addition of 150 µl of 100 mM Tris-HCl, pH 7.8, containing 0.1% Triton X-100. The reporters contained two copies of each TRE (DR-4, F2, and TREpal) upstream of the herpes simplex virus thymidine kinase promoter-TATA box linked to luciferase coding sequence. Luciferase and β-galactosidase activities were measured by using a luciferase assay system (Promega) and Galacto-Light Plus β-galactosidase reporter gene assay system (Applied Biosystems).

**Glutathione S-Transferase Pull-down Assays**—Full-length human TRβ was expressed in a coupled transcription translation system (TN'T, Promega). N-CoR (amino acids 563–1121) were expressed in *Escherichia coli* strain BL21 as a fusion protein with glutathione S-transferase according to the manufacturer’s protocol (Amersham Biosciences). Bindings were performed by mixing glutathione-linked Sepharose beads containing 4 µg of glutathione S-transferase fusion proteins (Coomasie Blue protein assay reagent, Pierce) with 1–2 µl of 35S-labeled human TRβ in 150 µl of binding buffer (20 mM HEPES, 150 mM KCl, 25 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors) containing 20 µg/ml bovine serum albumin for 1.5 h. Beads were washed three times with 200 µl of binding buffer, the bound proteins
were resuspended in SDS-PAGE loading buffer, and proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

**T₃ Binding Assay**—TRs were expressed using the TnT T7 quick coupled transcription translation system (Promega). The affinities of T₃ binding were determined using a saturation binding assay. Briefly, 15 fmol of each in vitro translated protein were incubated overnight at 4 °C with varying concentrations of [¹²⁵I]T₃ (PerkinElmer Life Sciences) in 100 μl of E400 buffer (400 mM NaCl, 20 mM KPO₄, pH 8.0, 0.5 mM EDTA, 1 mM MgCl₂, 10% glycerol), and 50 μg of calf thymus histones (Calbiochem). The bound [¹²⁵I]T₃ was isolated by gravity flow through a 2-ml Sephadex G-25 (Amersham Biosciences) column and quantified using a γ-counter (COBRA, Packard Instruments). Off rate (kₒ) was determined by adding a 1000-fold molar excess of un labeled T₃ to a mixture containing TR and 1 μM [¹²⁵I]T₃, incubated previously overnight at 4 °C; aliquots were taken at the indicated time points to determine how rapidly the labeled ligand dissociates from TR. These aliquots were applied to Sephadex G-25 columns, and TR-bound [¹²⁵I]T₃ was quantified using a γ-counter. As each T₃-TR complex dissociates at a random time, the amount of specific binding follows an exponential dissociation equation: Y = Y₀ e⁻kt + Plateau where x is time (min), Y is the binding (cpm), Span is the difference between binding at time 0 and plateau (cpm), and k is the dissociation rate constant (kₒ, expressed in min⁻¹). Binding curves were fit by nonlinear regression, and dissociation constant (Kₒ) and kₒ values were calculated using the one-site saturation binding, one-phase exponential decay, and one-phase exponential association models, respectively, contained in the Prism version 3.03 program (GraphPad Software, Inc., San Diego, CA).

**Gel Shift**—Binding of TR to DNA was assayed by mixing 20 fmol of TRs produced in a reticulocyte lysate system, TNT T7 (Promega), with varying concentrations of [¹²⁵I]T₃ (PerkinElmer Life Sciences) in a running buffer containing 45 mM Tris borate (pH 8.0) and 1 mM EDTA. The gel was then fixed, dried, and exposed for autoradiography. The mixture was loaded onto a 5% nondenaturing polyacrylamide gel that was previously run for 30 min at 200 V. To visualize the TR-DNA complexes, the gel was run at 4 °C for 120–180 min at 200 V in a running buffer containing 45 mM Tris borate (pH 8.0) and 1 mM EDTA. The gel was then fixed, dried, and exposed for autoradiography.

**Statistical Analysis**—All data are presented as means ± S.D. One-way ANOVA with Tukey’s post-test or t test was performed using GraphPad Prism version 3.03 for Windows. Data analyzed referred to at least three independent experiments. A p value of <0.05 was considered statistically significant.

**RESULTS**

**RTH Mutations That Inhibit T₃ and DNA Binding Reside in Charge Clusters**—RTH mutations that inhibit homodimer formation on DNA affect positively charged Arg residues (R338W, R429Q, and R316H) (30, 31, 33–35). In addition, we found that another RTH mutation that affects a positively charged Lys residue (K342I) also inhibits homodimer formation on DNA (not shown). Investigation of TR structural models revealed that each of these amino acids lies within separate clusters of closely juxtaposed charged residues (Fig. 1A and Table I). The TRβ LBD contains only one similar charge cluster that is not known to be affected by RTH mutations (Cluster 4, see Table I).

Clusters 1 and 2 are comprised of residues that are exposed or partially exposed on the surface of the LBD and are both adjacent to the classical dimer surface at the junction of H10 and H11 (Fig. 1A and Table I). Unlike many residues that are affected by RTH mutations, none of the residues in the clusters directly contacts T₃ or comprises part of a known coregulator binding surface.

The residues in Clusters 1 and 2 have the potential to engage in electrostatic interactions with each other. Cluster 1 includes Arg338 and Lys342 on H7 and two negatively charged residues on H8, Asp351 and Asp355, and is completely surface-exposed. We originally suggested that Arg338 and Lys342 engage in parallel ionic pairings with Asp351 and Asp355, respectively, based on analysis of x-ray crystal structures of the TRβ LBD (12). Reinvestigation of TRβ-LBD structures (13) suggested another arrangement: Arg338 and Lys342 both pair with Asp351 and Asp355 is not directly engaged in the cluster (Fig. 1B). Cluster 2 includes Arg429 on H11 and Arg383 on H9, both of which are also mutated in RTH but reported not to affect DNA binding (36), and is partially surface-exposed. Here x-ray structures of
TRα and TRβ indicated that both Arg residues pair with Glu
t on H6 in the LBD core (Fig. 1C).

Residues in Clusters 1 and 2 show considerable conservation.
They are conserved in TRs throughout vertebrate species (not shown).
Residues equivalent to those in charge Cluster 1 are conserved on H7 and H8 in other NRs, including retinoic acid receptors and PPARs (Fig. 1D). Residues in Cluster 2 show even better conservation (Fig. 1E). Together all of these considerations indicate that the charge clusters play an important, and unappreciated, role in TR activities. Furthermore the fact that mutations in the clusters affect T3 binding and homodimer formation indicates that the clusters must play a role in activ-
ties associated with liganded and unliganded TRs.

Cluster 1 Is Required for Optimal T3 Binding—

Fig. 2 shows effects of mutations on activity of transfected TRβ in mammalian cells. TRβ Cluster 1 mutants did not affect maximal activation of transcription from a TRE-driven re-
porter (F2) in the presence of saturating T3 or repression of basal transcription in the absence of T3 (Fig. 2A).

Nevertheless several TRβ Cluster 1 mutants displayed altered T3 concentration dependence (Fig. 2B and Table II) both in HeLa cells (shown here) and in other cells (U2-OS and CV-1, not shown).

Mutations in two residues (Arg338 and Asp351) led to reduced T3
sensitivity. In contrast, different mutations at
Asp355 did not reduce T3 sensitivity.

Finally mutations at Asp355 did not reduce T3 sensitivity.

TRβD355A and TRβD355R either exhibited T3 sensitivity com-
parative effects of mutations in Cluster 1 on activities of liganded TRs in vitro. A, mutations in Cluster 1 do not affect coregulator binding.

Shown are autoradiograms of SDS-polyacrylamide gels used to separate labeled TRs bound to bacterially expressed GRIP1 (amino acids 563–1121) and N-CoR (amino acids 1944–2453) in pull-down assays. The result is representative of three experiments. B, Kd, equilibrium dissociation constant. Mutants are compared with values obtained with wild type TR, which was 161.4 ± 12 M and set to 100%. Values represent the averages of at least three determinations. C, kinetics of ligand dissociation from wild type and mutant TRs, koff. Values represent the averages of at least three determinations. In B and C, different letters over bars indicate statistical difference (p < 0.05) according to ANOVA and Tukey’s test. WT or wt, wild type; GST, glutathione S-transferase.

**Fig. 2.**

<table>
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<tr>
<th>TRE/TR</th>
<th>TR</th>
<th>R338A</th>
<th>K342A</th>
<th>D351A</th>
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<td>F2 (IP-6)</td>
<td>100±1</td>
<td>357 ± 149.1±1</td>
<td>73 ± 9.5±1</td>
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<td>DR-4</td>
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<td>TREpal</td>
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</table>

**Table II**

Average EC50 for T3 response obtained with TRs bearing Cluster 1 mutants at different TREs. Values are compared to wild type TR set at 100%. Mean values ± S.D. are the average of at least three experiments. Different letters in the same horizontal row indicate statistical difference (p < 0.05), and different numbers in the same vertical column indicate statistical difference (p < 0.05) according to ANOVA and Tukey’s test.
parable to wild type TRβ or enhanced T3 sensitivity at some reporters (Fig. 2B and Table II).

None of the Cluster 1 mutations impaired binding to a coactivator (GRIP1, Fig. 3A) or to a corepressor (N-CoR, Fig. 3A) in pull-down assays in vitro. This is consistent with the results that show no impairment in the maximal effect of the hormone in transfection assays. By contrast, the same mutations that show no impairment in the maximal effect of the hormone in transfection assays performed in HeLa cells with an F2-driven reporter gene as in Fig. 3. B, Arg338 is required for optimal homodimer formation. Shown are electrophoretic mobility shift assays to determine binding of Cluster 2 mutants to an F2 oligonucleotide as in Fig. 4. A, different letters over bars indicate statistical difference (p < 0.05) according to ANOVA and Tukey’s test. WT or wt, wild type.

Mutations in Cluster 2 Residues Differentially Affect T3 Activation and DNA Binding—Mutations in Cluster 2 (Arg383, Arg429, and Glu311) also exhibited differential effects on activity of liganded TRs and DNA binding. Fig. 5A shows that TRβK342A exhibited a much larger reduction in T3 sensitivity than TRs bearing mutations at Arg429 and Arg383 (TRβR338W and TRβR342A) in the absence of T3, whereas TRβD335R and TRβK342D showed enhanced DNA binding in the presence of T3. TRβD355R exhibited enhanced DNA binding in the presence of T3, reversing the usual effects of T3 on TR DNA binding activity (Fig. 4C). More surprisingly, the charge reversal mutants exhibited enhanced DNA binding (Fig. 4C) even though most of these mutations inhibit T3 binding (Fig. 2B). The precise effect of the charge reversals varies; TRβR338W and a dimer RTH mutant. How-

FIG. 4. Mutations in charge Cluster 1 inhibit or enhance homodimerization on DNA. A–C, autoradiograph of gel shift assays of labeled F2 (A–C) and DR-4 (A and B) element oligonucleotides with wild type TR (TRwt) and various TR mutants in the presence or absence of both T3 and RXR. A, comparison of TRβ with TRβR338W and a dimer surface mutant (L422R). B, comparison of TRβ with Ala substitution mutants as indicated. C, comparison of TRβ with charge reversal mutants. WT, wild type; Retic., reticulocyte lysate.

abolished both homodimer and heterodimer formation. In parallel, TRs bearing Ala substitution mutants at Arg338 and Asp355 (both required for optimal T3 binding) exhibited reduced homodimer but not heterodimer formation at F2 and DR-4 elements (Fig. 4B) just like the TRβR338W RTH mutant. However, TRβK342A displayed reduced homodimer formation even though it did not inhibit T3 binding (compare Figs. 2B and 4B). More surprisingly, the charge reversal mutants exhibited enhanced DNA binding (Fig. 4C) even though most of these mutations inhibit T3 binding (Fig. 2B). The precise effect of the charge reversals varied; TRβR338W exhibited DNA binding dose-response (Fig. 4C) even though most of these mutations inhibit T3 binding (Fig. 2B). The precise effect of the charge reversals varies; TRβR338W and a dimer RTH mutant. How-

FIG. 5. Mutations in Cluster 2 differentially affect T3 response and DNA binding. A, Glu311 is required for optimal T3 response. Shown is a summary of relative EC50 values for T3 response obtained in transfection assays performed in HeLa cells with an F2-driven reporter gene as in Fig. 3. B, Arg338 is required for optimal homodimer formation. Shown are electrophoretic mobility shift assays to determine binding of Cluster 2 mutants to an F2 oligonucleotide as in Fig. 4. A, different letters over bars indicate statistical difference (p < 0.05) according to ANOVA and Tukey’s test. WT or wt, wild type.
Cluster 1 Is Dispensable for Ligand TR Activity—Because Lys\textsuperscript{342} and Asp\textsuperscript{355} interfere with TR\(\beta\) activity and T\(_3\) binding when the putative Arg\textsuperscript{338}-Asp\textsuperscript{351} ionic bond is reversed (Fig. 6), we asked whether Lys\textsuperscript{342} and Asp\textsuperscript{355} might also interfere with TR\(\beta\) activity and T\(_3\) binding in the context of wild type TR\(\beta\). To do this, we examined effects of multiple Ala substitutions in Cluster 1.

Mutations at Lys\textsuperscript{342} and Asp\textsuperscript{355} rescued effects of mutations at Arg\textsuperscript{338} and Asp\textsuperscript{351}. Fig. 7A shows that a TR\(\beta\) double mutant bearing Ala substitutions at residues that are required for optimal T\(_3\) binding (TR\(\beta\)R338A,D351A) displayed reduced T\(_3\) sensitivity and T\(_3\) binding and increased dissociation rates of bound T\(_3\). Furthermore a TR\(\beta\) double mutant bearing Ala substitutions at residues that are not required for optimal T\(_3\) binding (TR\(\beta\)K342A,D355A) did not affect TR\(\beta\) activity. These results confirm that Arg\textsuperscript{338} and Asp\textsuperscript{351} are needed for optimal hormone binding, and Lys\textsuperscript{342} and Asp\textsuperscript{355} are not. More surprisingly, a double mutant that eliminated both positive charges in Cluster 1 (TR\(\beta\)R338A,K342A) exhibited a phenotype that was similar to wild type TR\(\beta\). Furthermore a double mutant that removed both negative charges (TR\(\beta\)D351A,D355A) exhibited a phenotype that was intermediate between TR\(\beta\)D351A, reduced affinity for T\(_3\), and TR\(\beta\)D355A, similar to wild type TR\(\beta\). Thus, Ala substitution mutations at Lys\textsuperscript{342} and Asp\textsuperscript{355} rescue effects of similar mutations at Arg\textsuperscript{338} and Asp\textsuperscript{351}.

The fact that some mutations in Cluster 1 rescue effects of others was underscored by the observation that elimination of all charge within Cluster 1 with a quadruple Ala substitution (TR\(\beta\)A4) failed to inhibit T\(_3\) binding or liganded TR\(\beta\) function. TR\(\beta\)A4A displayed enhanced T\(_3\) sensitivity in transfections (Fig. 7B), slightly increased affinity for T\(_3\) (Table III), and normal levels of coactivator and corepressor binding (not shown). Nevertheless TR\(\beta\)A4A exhibited strongly reduced homodimer formation on DNA (Fig. 7C). This reduction in homodimer formation, the largest obtained with any Cluster 1 mutation in this study (not shown), was paralleled by impaired repression at a TR-regulated reporter without T\(_3\) (Fig. 7B, inset).

Together our results show that, whereas two individual residues in the cluster (Arg\textsuperscript{338} and Asp\textsuperscript{351}) are required for optimal T\(_3\) response and T\(_3\) binding, Cluster 1 itself is dispensable for the function of liganded TR. Nevertheless Cluster 1 is required for activities associated with unliganded TRs: homodimer formation on DNA and transcriptional repression (see “Discussion”).

**DISCUSSION**

In this study, we examined how TR DNA binding activity is regulated by its LBD and by ligand. To begin to understand this issue, we asked why some RTH mutations (R316H, R338W, K342I, and R429Q) that reduce the affinity of TR\(\beta\) for T\(_3\) also inhibit binding of TR homodimers, but not heterodimers, to TREs (30, 31). We reasoned that these mutations might affect structural elements that are involved in coupling T\(_3\) binding to inhibition of DNA binding activity. We report here that each of these RTH mutations affected amino acids that lie within clusters of charged residues with potential for electrostatic interactions between individual residues in the cluster. Two of these clusters (1 and 2) are adjacent to the TR dimer/heterodimer surface (Table I and Fig. 1). The importance of the clusters is underscored by their conservation both in TRs across evolution (not shown) and in other NRs (Fig. 1, D and E) and by our studies, which revealed that mutations in Clusters 1 and 2 lead, variously, to increases and decreases in T\(_3\) binding and/or DNA binding.

The existence of functionally important clusters of charged residues on the TR LBD surface was surprising because proteins are largely stabilized by hydrophobic effects in
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FIG. 7. Cluster 1 is dispensable for liganded TR action. A, mutations at Lys$^{342}$ and Asp$^{351}$ rescue effects of mutations at Arg$^{338}$ and Asp$^{351}$. Shown is a comparison of $EC_{50}$, $K_d$, and $k_{off}$ values obtained with TR mutants with single and double Ala substitutions in Cluster 1; data are presented as in Figs. 2 and 3. B, Cluster 1 can be eliminated without loss of function for liganded TR. Shown are hormone activation profiles for human TRβ wild type and a quadruple Ala mutant (4xA) at an F2-TRE-regulated reporter. The data represent a single transfection assay in which standard errors are derived from multiple wells, representative of several experiments. The inset shows maximal (Max.) activation and repression obtained with TRβ and TRβ4A (4xA). C, elimination of Cluster 1 inhibits DNA binding. Shown is a gel shift comparing binding of TRβ and TRβ4A (4xA) on an F2-TRE. In A, different letters over bars indicate statistical difference (p < 0.05) according to ANOVA and Tukey’s test. WT or wt, wild type.

TABLE III

<table>
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<tr>
<th></th>
<th>$K_d$</th>
<th>$k_{off}$</th>
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<td>$\times10^{-12}$ M</td>
<td>$\times10^5$ min$^{-1}$</td>
</tr>
<tr>
<td>TRβ4A</td>
<td>134.5 ± 44.6$^a$</td>
<td>2.32 ± 0.72$^a$</td>
</tr>
<tr>
<td></td>
<td>67.1 ± 23.6$^a$</td>
<td>1.92 ± 0.56$^a$</td>
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which hydrophobic residues form the interior of the protein and charged side chains are surface-exposed, freely solvated with water (37). Nevertheless electrostatic interactions between oppositely charged side chains have been shown to provide additional stability to proteins in several contexts, including particular conformers of allosteric proteins, protein-protein interaction surfaces, and proteins in thermophilic organisms (37–40). For TRs, two RTH mutations that disrupt ionic bonds, one in Cluster 3 (TRβR316H) and a single surface-exposed ionic bond between Arg$^{243}$ in H3 and Glu$^{322}$ at the base of H6 (TRβR243Q), lead to broadening of experimental electron density in the lower part of the LBD in x-ray structures (14, 15). This confirms that electrostatic interactions between oppositely charged TRβ residues can stabilize the liganded TRβ-LBD.

Our mutational analysis supports the notion that Clusters 1 and 2 are stabilizing elements for liganded TR. Mutations that disrupted the predicted ionic bond arrangements in Clusters 1 and 2 led to reduced $T_3$ sensitivity, reduced affinity for $T_3$, and increased $T_3$ dissociation rates. These phenotypes resemble those of aforementioned TRβ RTH mutations that destabilize the TR LBD by breaking electrostatic interactions, R316H and R243Q (14, 15). In addition, three lines of evidence indicate that Arg$^{338}$ and Asp$^{351}$ form an ionic bond required for stable $T_3$ binding. 1) Placement of like, repelling charges at Arg$^{338}$ and Asp$^{351}$ severely inhibited $T_3$ binding (Fig. 2). 2) Arg$^{338}$ and Asp$^{351}$ could be reversed without significant disruption of $T_3$ binding, albeit only in the absence of charge at Lys$^{342}$ and Asp$^{351}$ (Fig. 6). 3) TRs with double mutations at Arg$^{338}$ and Asp$^{351}$ exhibited phenotypes similar to single mutants, suggesting that both residues are parts of the same structural element (Figs. 6 and 7).

Nevertheless our results also suggest that the clusters adopt a different organization in unliganded TRs. Distinct arrangements of charge are required for optimal $T_3$ binding and for DNA binding by unliganded TR homodimers (Figs. 2–5). Thus, the TRβK342A mutation inhibited DNA but not $T_3$ binding. Furthermore and more strikingly, charge reversal mutations at Arg$^{338}$ (R338D), Asp$^{351}$ (D351R), and Lys$^{342}$ (K342D) all inhibited $T_3$ binding but not DNA binding, and a charge reversal mutation at Asp$^{355}$ (D355R) did not affect $T_3$ binding yet enhanced TR homodimer formation on DNA in the presence of $T_3$ (Fig. 4).

Other results are hard to reconcile with the simple notion that Clusters 1 and 2 act as static stabilizing elements for liganded and unliganded TRs. Cluster 1 was dispensable for optimal $T_3$ response and $T_3$ binding (Fig. 7) even though Arg$^{338}$ and Asp$^{351}$ were required for $T_3$ binding (Figs. 2, 3, and 7). Furthermore two Cluster 1 residues (Lys$^{342}$ and Asp$^{355}$) must inhibit $T_3$ binding to some extent as judged by the fact that TRβK342A and TRβD355A mutants exhibited enhanced sensitivity to $T_3$ in transfections and increased affinity for $T_3$ in vitro and that Ala substitutions at both positions rescued effects of similar mutations at Arg$^{338}$ and Asp$^{351}$ (Fig. 7).

Our hypothesis to explain these observations is outlined in Fig. 8. We propose that Clusters 1 and 2 are hormone-dependent stabilizing elements for the TR LBD. We suggest that, in the unliganded state, the clusters adopt an unspecified organi-
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We recognize that our model cannot yet be verified directly because apoTR dimer structures are not available. Nevertheless, analysis of liganded and unliganded RXR crystal structures revealed evidence that is consistent with the basic predictions of our model. First, charged residues in the region of RXR that is equivalent to H8 rearrange in response to ligand binding (Fig. 9). RXR Glu352 and Lys356 form an ionic bond within the interior of the unliganded LBD. Binding of 9-cis-retinoic acid twists the helix, exposing the charged side chains on the protein surface where they can pair with PPARs in RXR-PPAR heterodimers (41–45). We suggest that TRβ charge clusters (on H7, H8, and H11) must undergo similar ligand-dependent rearrangements. This model implies that functionally important conformational rearrangements that accompany T3 binding are not restricted to H12 and that T3 induces reorganization of the opposite face of the TR near the dimer surface.

Finally our results also lend support to the notion that TR homodimers are highly active in mediating transcriptional repression in vivo (25, 26). We observed that a TRβ mutant that strongly inhibited homodimer formation on TREs (TRβ4A) impaired the ability of unliganded TRs to suppress transcription in the absence of hormone (Fig. 7B). We predict that mutations such as those described here that either specifically inhibit or stabilize particular oligomeric forms of TR will help us to further dissect the relative roles of RXR-TR heterodimers and TR homodimers in vivo.

REFERENCES