THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 27, Issue of July 8, pp. 25665–25673, 2005
Printed in U.S.A.

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

Received for publication, February 11, 2005, and in revised form, May 9, 2005
Published, JBC Papers in Press, May 10, 2005, DOI 10.1074/jbc.M501615200

Marie Togashi‡, Phuong Nguyen‡, Robert Fletterick§, John D. Baxter‡¶, and Paul Webb‡‖

From the ‡Diabetes Center and the §Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143-0540

In this study, we investigated how thyroid hormone (3,5′,5-triiodo-L-thyronine, T_3) 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 T_3 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 T_3 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 (Arg^{338}, Lys^{342}, Asp^{351}, and Asp^{355}) and Cluster 2 (Arg^{429}, Arg^{383}, and Glu^{311}) confirmed that the clusters are required for stable T_3 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 T_3 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 T_3 regulates TR DNA binding activity and also highlight hitherto unsuspected T_3-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 (T_3) 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 T_3 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/TRβ 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 T_3 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 T_3 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, T_3 blocks transactivation 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 T_3-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 T_3 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).

T_3 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. T_3 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*

* This work was supported by National Institutes of Health Grants DK41482 and DK51281 (to J. D. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶Deputy director and consultant to Karo Bio AB, a biotechnology company with commercial interests in nuclear receptors.

‖ To whom correspondence should be addressed: Diabetes Center, University of California School of Medicine, HSW1210, 513 Parnassus Ave., San Francisco, CA 94143-0540. Tel.: 415-476-6789; Fax: 415-564-5813; E-mail: webbp@itsa.ucsf.edu.

[1] The abbreviations used are: TR, thyroid hormone receptor; T_3, 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.

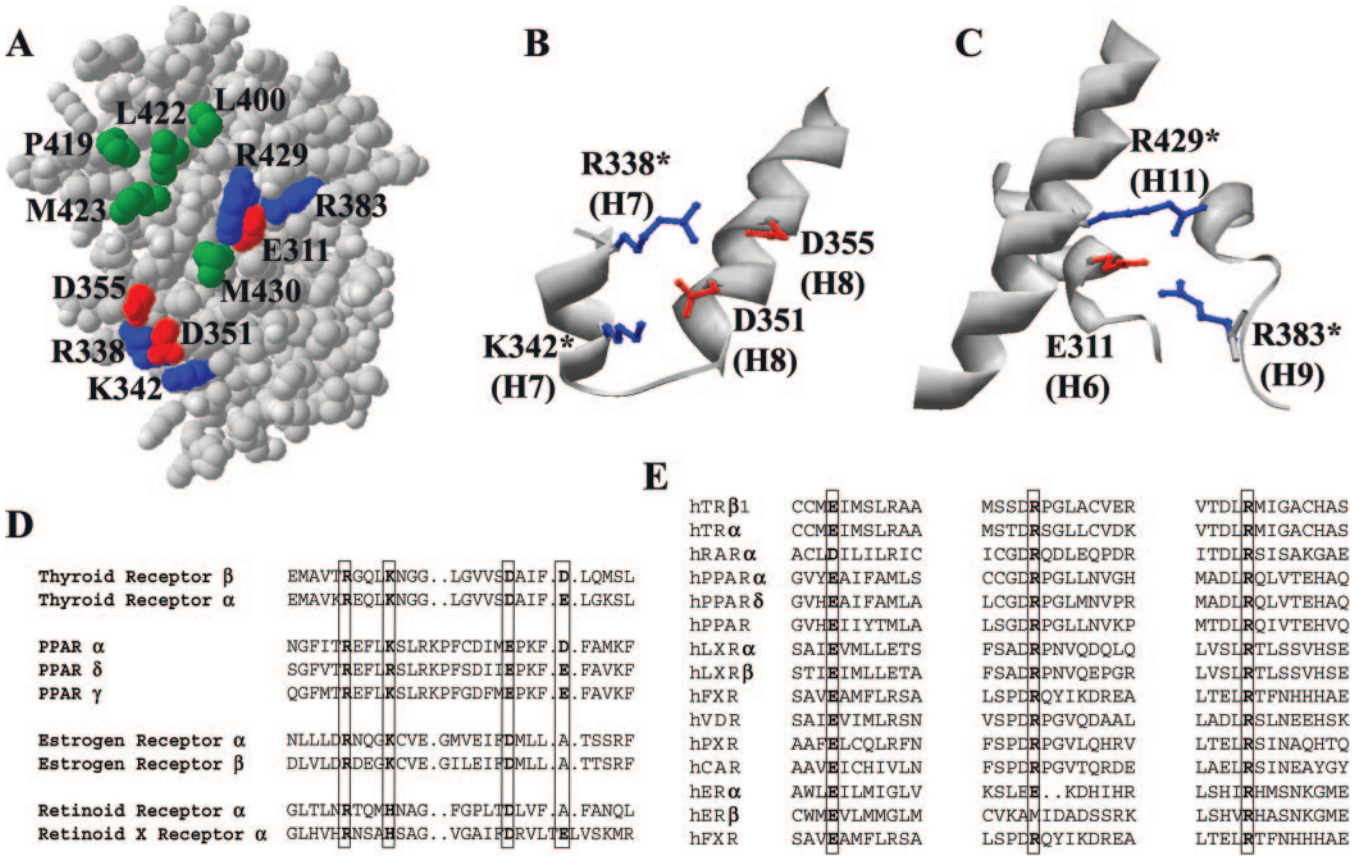

FIG. 1. **Location of RTH mutations in charge clusters that are adjacent to the dimer surface.** *A*, charge Clusters 1 and 2 are adjacent to the TRβ dimer surface. The figure shows a space-filling model of the TRβ LBD. Residues in the dimerization surface (Leu^{400}, Pro^{419}, Leu^{422}, Met^{423}, and Met^{430}) are shown in *green*. Residues in Cluster 1 (Arg^{338}, Lys^{342}, Asp^{351}, and Asp^{355}) and Cluster 2 (Glu^{311}, Arg^{383}, and Arg^{429}) are shown in *blue* (positively charged) and *red* (negatively charged). *B* and *C*, closer view interactions between the residues that comprise charge Clusters 1 (*B*) and 2 (*C*). Positively charged residues are shown in *blue*, and negatively charged residues are shown in *red*. *Asterisk* represents residues mutated in RTH. *D*, alignment of Cluster 1 residues in TR and other NRs. *E*, alignment of Cluster 2 residues in TR and other NRs. *hTR*, human TR; *hRAR*, human retinoic acid receptor; *hLXR*, human liver X receptor; *hER*, human estrogen receptor; *hPXR*, human pregnane X receptor; *hVDR*, human vitamin D receptor; *hCAR*, human constitutive androstane receptor.

vivo (25, 26). Thus, it is thought that T_3-dependent inhibition of homodimer formation relieves transcriptional repression by unliganded TRs. Nevertheless the mechanisms involved in coupling of T_3 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 T_3 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 elements are involved in coupling T_3 binding to inhibition of DNA binding. Whereas most TRβ mutations that arise in resistance to thyroid hormone syndrome (RTH) reduce the affinity of TRβ for T_3 (3, 28, 29), a small subset also inhibits binding of TRβ homodimers, but not heterodimers, to TREs (30, 31). Here we report that these RTH mutations affect clusters of charged amino acids in the LBD with potential for electrostatic stabilization of TR conformation but that distinct arrangements of charged residues are needed for stable T_3 binding and DNA binding by unliganded TRs. We propose that the charge clusters rearrange upon T_3 binding to block homodimer formation and create new ionic bonds that stabilize bound hormone.

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 (Elim 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 ml/4.5 × 10^7 cells) containing 0.1% dextrose and typically 4 μg of reporter, 1 μg of TR expression vector or empty vector control, and 2 μg of pCMV-β-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 T_3 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 (TNT, Promega). N-CoR (amino acids 1944–2453) and GRIP1 (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 (Coomassie Plus protein assay reagent, Pierce) with 1–2 μl of ^{35}S-labeled human TRβ in 150 μl of binding buffer (20 mM HEPES, 150 mM KCl, 25 mM $MgCl_2$, 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

TABLE I
Location and conservation of TR charge clusters
RTH mutants known to inhibit DNA binding activity are shown in bold.

Cluster	Residues	Location	RTH mutants	Conservation
1	Arg^{338}, Lys^{342}, Asp^{351}, Asp^{355}	Surface, links H7-H8	**R338W**, **R338L**, **K342I**, A337del	Charged residues at similar location in several NRs
2	Arg^{429}, Arg^{383}, Glu^{311}	Partially buried, links H11 and H10 to H6	R383H, **R429Q**	Arg^{429} conserved in 70% of NRs
3	Arg^{316}, Gln^{374}, Thr^{232}, His^{229}	Buried, links H6 and H9 to H1	**R316H**	Polar cluster observed in similar location in many NRs
4	Arg^{410}, His^{412}, Asp^{366}, Glu^{369}	Buried, links H9 and H10-H11 loop	None	Glu^{369} well conserved

were resuspended in SDS-PAGE loading buffer, and proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

T_3 Binding Assay—TRs were expressed using the TNT T7 quick coupled transcription translation system (Promega). The affinities of T_3 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 [^{125}I]T_3 (PerkinElmer Life Sciences) in 100 μl of E400 buffer (400 mM NaCl, 20 mM KPO_4, pH 8, 0.5 mM EDTA, 1.0 mM $MgCl_2$, 10% glycerol), 1 mM monothioglycerol, and 50 μg of calf thymus histones (Calbiochem). The bound [^{125}I]T_3 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 (k_{off}) was determined by adding a 1000-fold molar excess of unlabeled T_3 to a mixture containing TR and 1 nM [^{125}I]T_3 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 [^{125}I]T_3 was quantified using a γ-counter. As each T_3-TR complex dissociates at a random time, the amount of specific binding follows an exponential dissociation equation: $Y = \text{Span}\cdot e^{-kx} + \text{Plateau}$ where x is time (min), Y is total binding (cpm), Span is the difference between binding at time 0 and plateau (cpm), and k is the dissociation rate constant (k_{off}, expressed in min^{-1}). Binding curves were fit by nonlinear regression, and dissociation constant (K_d) and k_{off} 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 Shifts—Binding of TR to DNA was assayed by mixing 20 fmol of TRs produced in a reticulocyte lysate system, TNT T7 (Promega), with 300,000 cpm [γ-^{32}P]ATP-radiolabeled DR-4 and F2 oligonucleotides and 1 μg of poly(dI-dC) (Amersham Biosciences) in a 20-μl reaction (32). In cases in which TR ligand binding activity was severely affected by Cluster 1 mutations, the overall amount of translated TRs in the extracts was also verified independently by Western blot. The binding buffer contained 25 mM HEPES, 50 mM KCl, 1 mM dithiothreitol, 10 μM $ZnSO_4$, 0.1% Nonidet P-40, 5% glycerol. After 30 min at room temperature, 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 T_3 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. 1*A* 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

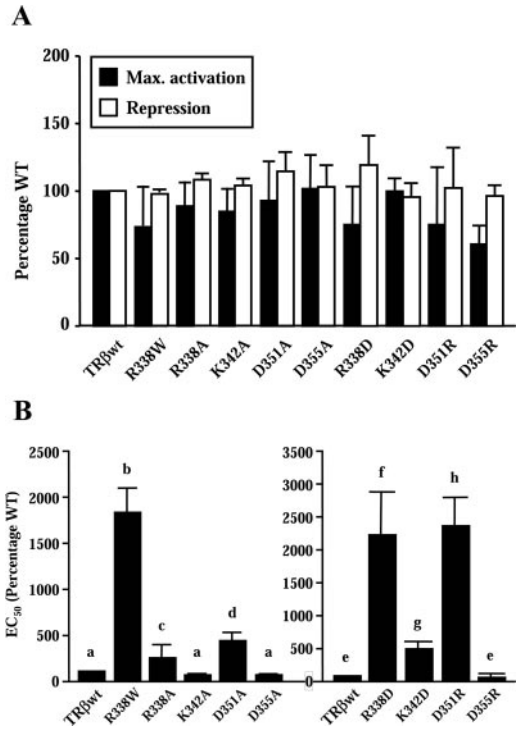

FIG. 2. **Charge Cluster 1 mutations inhibit T_3 activation.** Maximal (*Max.*) activation and repression (*A*) and dose of T_3 (*B*) required for half-maximal activation at a T_3-regulated reporter gene with an F2/IP-6 TRE. Activities obtained with TR mutants are compared with those obtained with wild type TRβ, which is set to 100%. The EC_{50} T_3 concentration for wild type TRβ was 15 nM for the F2 driven reporter. In *A*, no statistical difference was found among mutants and wild type ($p > 0.05$). In *B*, different *letters over bars* indicate statistical difference ($p < 0.05$) according to ANOVA and Tukey's test. *WT* or *wt*, wild type.

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. 1*A* and Table I). Unlike many residues that are affected by RTH mutations, none of the residues in the clusters directly contacts T_3 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 Arg^{338} and Lys^{342} on H7 and two negatively charged residues on H8, Asp^{351} and Asp^{355}, and is completely surface-exposed. We originally suggested that Arg^{338} and Lys^{342} engage in parallel ionic pairings with Asp^{355} and Asp^{351}, respectively, based on analysis of x-ray crystal structures of the TRα LBD (12). Reinvestigation of TRβ-LBD structures (13) suggested another arrangement: Arg^{338} and Lys^{342} both pair with Asp^{351}, and Asp^{355} is not directly engaged in the cluster (Fig. 1*B*). Cluster 2 includes Arg^{429} on H11 and Arg^{383} 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

TABLE II

Average EC_{50} for T_3 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.

TRE/TR	TR	R338A	K342A	D351A	D355A
F2 (IP-6)	$100^{a,1}$	$357 \pm 149.1^{b,1}$	$73 \pm 9.5^{a,1}$	$608 \pm 185.5^{c,1}$	$56 \pm 11.3^{a,1}$
DR-4	$100^{a,1}$	$112 \pm 14.6^{a,2}$	$68 \pm 11.8^{a,1,2}$	$389 \pm 98.6^{b,2}$	$102 \pm 27.7^{a,2}$
TREpal	$100^{a,b,1}$	$155 \pm 37.0^{b,1,2}$	$37 \pm 16.7^{a,2}$	$308 \pm 56.7^{c,2}$	$33 \pm 3.5^{a,1}$

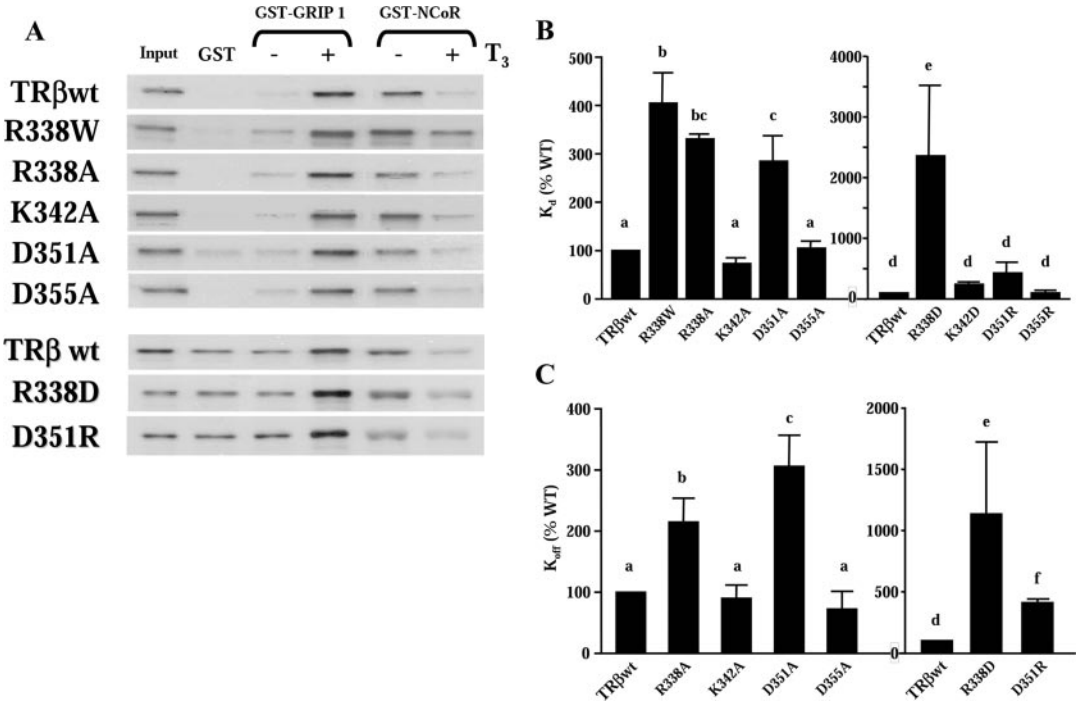

FIG. 3. **Effects of Cluster 1 mutations on activities of liganded TRs *in vitro*.** *A*, mutations in Cluster 1 do not affect coregulator binding. Shown are autoradiograms of SDS-polyacrylamide gels used to separate labeled TRs bound to bacterially expressed GRIP1 (amino acids 563–1121) and N-CoR (amino acids 1944–2453) in pull-down assays. The result is representative of three experiments. *B*, K_d, equilibrium dissociation constant. Mutants are compared with values obtained with wild type TR, which was 161.4×10^{-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, k_{off}. 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.

TRα and TRβ indicated that both Arg residues pair with Glu^{311} on H6 in the LBD core (Fig. 1*C*).

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. 1*D*). Residues in Cluster 2 show even better conservation (Fig. 1*E*). 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 T_3 binding and homodimer formation indicates that the clusters must play a role in activities associated with liganded and unliganded TRs.

Cluster 1 Is Required for Optimal T_3 Binding—We first examined effects of mutations in Cluster 1 on activities of liganded TRs. Because residues of this cluster are completely surface-exposed it appeared unlikely that these mutations would exert indirect effects on TR function by disrupting internal folding of the LBD. We introduced 1) Ala substitutions, which swap a residue with a small neutral side chain for a residue with a charged side chain and thereby eliminate the potential for electrostatic interactions, and 2) charge reversal mutations, which should disrupt ionic bonds between oppositely charged residues by juxtaposing residues with like charges.

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 reporter (F2) in the presence of saturating T_3 or repression of basal transcription in the absence of T_3 (Fig. 2*A*). Nevertheless several TRβ Cluster 1 mutants displayed altered T_3 concentration dependence (Fig. 2*B* and Table II) both in HeLa cells (shown here) and in other cells (U2-OS and CV-1, not shown). Mutations in two residues (Arg^{338} and Asp^{351}) led to reduced T_3 sensitivity. The TRβR338W RTH mutant required 17-fold more T_3 than wild type TRβ for half-maximal activation (EC_{50}). TRs bearing Ala substitutions at Arg^{338} and Asp^{351} (TRβR338A and TRβD351A) exhibited more modest reductions in T_3 sensitivity, and TRs with charge reversal mutations at Arg^{338} and Asp^{351} (TRβR338D and TRβD351R) displayed more marked reductions in T_3 sensitivity. In contrast, different mutations at Lys^{342} exhibited divergent effects. A relatively mild Ala substitution mutation (TRβK342A) had either no effect or slightly enhanced T_3 sensitivity (Table II). Nevertheless a more severe charge reversal mutation, TRβK342D, exhibited decreased T_3 sensitivity as did the TRβK342I RTH mutant (not shown). Finally mutations at Asp^{355} did not reduce T_3 sensitivity. TRβD355A and TRβD355R either exhibited T_3 sensitivity com-

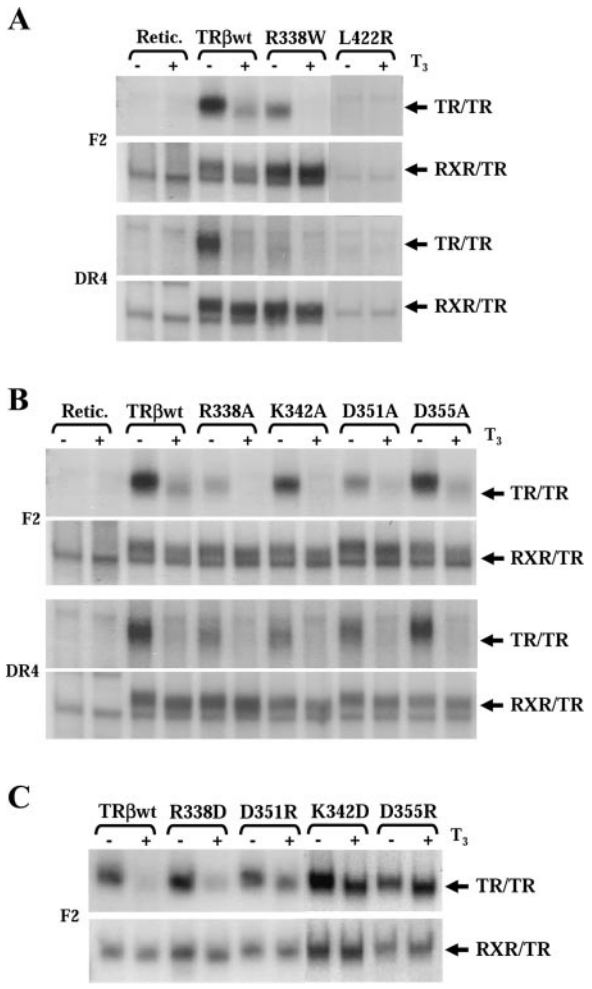

FIG. 4. **Mutations in charge Cluster 1 inhibit or enhance homodimerization on DNA.** *A–C*, autoradiograph of gel shift assays of labeled F2 (*A–C*) and DR-4 (*A* and *B*) element oligonucleotides with wild type TR (*TRβwt*) and various TR mutants in the presence or absence of both T_3 and RXR. *A*, 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.

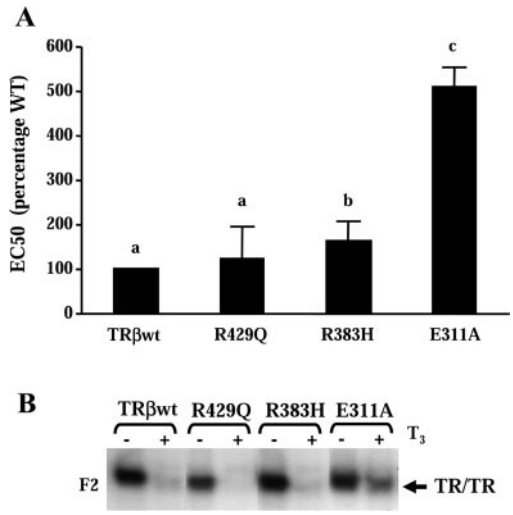

FIG. 5. **Mutations in Cluster 2 differentially affect T_3 response and DNA binding.** *A*, Glu^{311} is required for optimal T_3 response. Shown is a summary of relative EC_{50} values for T_3 response obtained in transfections assays performed in HeLa cells with an F2-driven reporter gene as in Fig. 3. *B*, Arg^{429} 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. In *A*, different *letters over bars* indicate statistical difference ($p < 0.05$) according to ANOVA and Tukey's test. *WT* or *wt*, wild type.

parable to wild type TRβ or enhanced T_3 sensitivity at some reporters (Fig. 2*B* and Table II).

None of the Cluster 1 mutations impaired binding to a coactivator (GRIP1, Fig. 3*A*) or to a corepressor (N-CoR, Fig. 3*A*) 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 reduced T_3 sensitivity *in vivo* also reduced the affinity of the TR for T_3 (Fig. 3*B*) and increased T_3 dissociation rates (Fig. 3*C*).

Together our results indicate that Arg^{338}, $Asp3^{51}$, and, to a lesser extent, Lys^{342} are required for optimal T_3 binding and response and that Asp^{355} is not. This is consistent with the apparent organization of Cluster 1 in TRβ crystal structures where Arg^{338}, Lys^{342}, and $Asp3^{51}$ side chains engage in electrostatic interactions with each other, and Asp^{355} does not (Fig. 1*B*).

Mutations in Cluster 1 Can Either Impair or Enhance Homodimer Binding to DNA, and Effects Do Not Correlate with T_3 Binding—Next we examined effects of mutations in Cluster 1 on TR homodimer formation on DNA. The data in Fig. 4*A* confirmed that the TRβR338W RTH mutant exhibits defective homodimer formation at F2 and DR-4 elements along with normal levels of heterodimer formation (30, 31). By contrast, an artificial mutation (TRβL422R) in the classical dimer interface abolished both homodimer and heterodimer formation. In parallel, TRs bearing Ala substitution mutants at Arg^{338} and Asp^{351} (both required for optimal T_3 binding) exhibited reduced homodimer but not heterodimer formation at F2 and DR-4 elements (Fig. 4*B*) just like the TRβR338W RTH mutant. However, TRβK342A displayed reduced homodimer formation even though it did not inhibit T_3 binding (compare Figs. 2*B* and 4*B*). More surprisingly, the charge reversal mutants exhibited enhanced DNA binding (Fig. 4*C*) even though most of these mutations inhibit T_3 binding (Fig. 2*B*). The precise effect of the charge reversal mutants varied; TRβR338D showed enhanced DNA binding in the absence of T_3, whereas TRβD351R and TRβK342D showed enhanced DNA binding in the presence or absence of T_3. TRβD355R exhibited enhanced DNA binding in the presence of T_3, reversing the usual effects of T_3 on TR DNA binding activity (Fig. 4*C*). Again these effects were largely homodimer-specific, although TRβK342D did exhibit somewhat enhanced heterodimer formation. Together our results confirm that Cluster 1 is required for TR homodimer but not heterodimer formation on DNA. Nevertheless the same data also revealed that different arrangements of charge are needed for optimal DNA and T_3 binding.

Charge Cluster 2 Residues Differentially Affect T_3 Activation and DNA Binding—Mutations in Cluster 2 (Arg^{383}, Arg^{429}, and Glu^{311}) also exhibited differential effects on activity of liganded TRs and DNA binding. Fig. 5*A* shows that TRβE311A exhibited a much larger reduction in T_3 sensitivity than TRs bearing mutations at Arg^{429} and Arg^{383} (TRβR429A and TRβR383H) (Fig. 5*A*). This finding is consistent with previous observations that RTH mutations in these Arg residues only affect T_3 sensitivity weakly (30) and is also consistent with the organization of Cluster 2 in TRβ structures (Fig. 1*C*); a mutation in Glu^{311} that breaks electrostatic interactions with both Arg residues exhibited a more severe defect than mutations at Arg^{429} and Arg^{383}, which only break one bond. By contrast, TRβE311A (and TRβR383H) bound to DNA as efficiently as wild type TRs in the absence of T_3, whereas TRβR429A exhibited decreased homodimer formation on DNA (Fig. 5*B*). Thus, Cluster 2 requires different charged residues for optimal T_3 response and DNA binding just like Cluster 1.

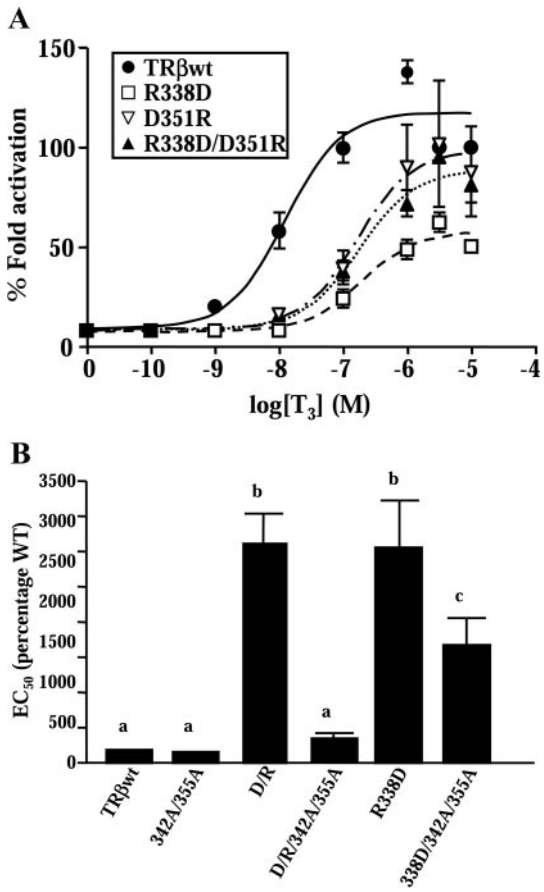

FIG. 6. **Reversibility of the putative Arg^{338}-Asp^{351} salt bridge.** *A*, Arg^{338} and Asp^{351} cannot be reversed without severe disruption of T_3 response. Shown is a comparison of activities of wild type TRβ and charge reversal mutants as a function of T_3 concentration. Transfections were performed in HeLa cells with an F2-driven reporter. *B*, Arg^{338} and Asp^{351} can be reversed in the absence of interfering charge at Lys^{342} and Asp^{355}. Ala substitution mutations at Lys^{342} and Asp^{355} restore activity of the TRβR338D,D351R charge reversal mutant (*D/R*). Data are presented as a comparison of EC_{50} values for different TRβ mutants as in Fig. 3*C*. In *B*, different *letters over bars* indicate statistical difference ($p < 0.05$) according to ANOVA and Tukey's test. *wt*, wild type.

An Arg^{338}-Asp^{351} Pair Stabilizes Bound T_3—To learn how individual residues within Cluster 1 interact, we examined effects of multiple mutations in the cluster upon TR function. First we reversed the positions of two residues that are most important for optimal T_3 binding, Arg^{338} and Asp^{351}. Fig. 6*A* shows that TRβR338D,D351R displayed markedly reduced T_3 sensitivity in transfections like the R338D and D351R single mutants. TRβR338D,D351R also displayed decreased affinity for T_3 and increased dissociation rates of bound T_3 with normal levels of coregulator binding and TR homodimer formation on DNA (not shown).

The phenotype of the TRβR338D,D351R double mutant was surprising; it is often possible to reverse the positions of residues in ionic pairs and regenerate wild type protein function. Nevertheless Fig. 6*B* shows that introduction of Ala substitutions at Lys^{342} and Asp^{355} restored the activity of the TRβR338D,D351R double mutant to near wild type levels but not that of a TRβ mutant with like charges at both positions (TRβR338D,K342A,D355A). Thus, Arg^{338} and Asp^{351} can be reversed without severe loss of TRβ function, suggesting that they can form a reversible ionic bond that stabilizes liganded TRβ. This effect can only be observed, however, when other charges are removed from the cluster.

Cluster 1 Is Dispensable for Ligand TR Activity—Because Lys^{342} and Asp^{355} interfere with TRβ activity and T_3 binding when the putative Arg^{338}-Asp^{351} ionic bond is reversed (Fig. 6), we asked whether Lys^{342} and Asp^{355} might also interfere with TRβ activity and T_3 binding in the context of wild type TRβ. To do this, we examined effects of multiple Ala substitutions in Cluster 1.

Mutations at Lys^{342} and Asp^{355} rescued effects of mutations at Arg^{338} and Asp^{351}. Fig. 7*A* shows that a TRβ double mutant bearing Ala substitutions at residues that are required for optimal T_3 binding (TRβR338A,D351A) displayed reduced T_3 sensitivity and T_3 binding and increased dissociation rates of bound T_3. Furthermore a TRβ double mutant bearing Ala substitutions at residues that are not required for optimal T_3 binding (TRβK342A,D355A) did not affect TRβ activity. These results confirm that Arg^{338} and Asp^{351} are needed for optimal hormone binding, and Lys^{342} and Asp^{355} are not. More surprisingly, a double mutant that eliminated both positive charges in Cluster 1 (TRβR338A,K342A) exhibited a phenotype that was similar to wild type TRβ. Furthermore a double mutant that removed both negative charges (TRβD351A,D355A) exhibited a phenotype that was intermediate between TRβD351A, reduced affinity for T_3, and TRβD355A, similar to wild type TRβ. Thus, Ala substitution mutations at Lys^{342} and Asp^{355} rescue effects of similar mutations at Arg^{338} and Asp^{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 (TRβ4A) failed to inhibit T_3 binding or liganded TRβ function. TRβ4A displayed enhanced T_3 sensitivity in transfections (Fig. 7*B*), slightly increased affinity for T_3 (Table III), and normal levels of coactivator and corepressor binding (not shown). Nevertheless TRβ4A exhibited strongly reduced homodimer formation on DNA (Fig. 7*C*). 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. 7*B*, *inset*).

Together our results show that, whereas two individual residues in the cluster (Arg^{338} and Asp^{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 TRβ 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

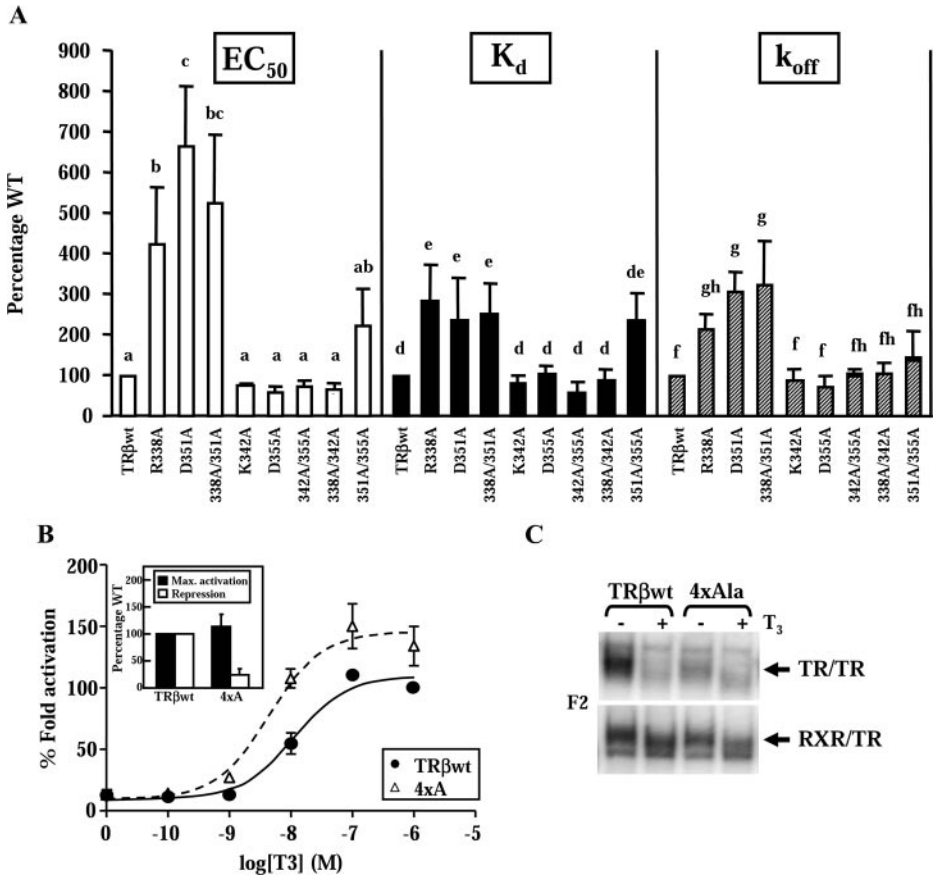

FIG. 7. **Cluster 1 is dispensable for liganded TR action.** *A*, mutations at Lys[342] and Asp[355] rescue effects of mutations at Arg[338] and Asp[351]. Shown is a comparison of EC_{50}, K_d, and k_{off} values obtained with TR mutants with single and double Ala substitutions in Cluster 1; data are 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 (*4xAla*) 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
Charge Cluster 1 is dispensable for T_3 binding

Means ± S.D. are the average of at least three experiments. The same letters in the same column indicate no statistical difference ($p > 0.05$) according to *t* test.

	K_d	k_{off}
	$\times 10^{-12}$ M	$\times 10^{3}$ min^{-1}
TRβ	134.5 ± 44.6[a]	2.32 ± 0.72[a]
TRβ4A	67.1 ± 23.6[a]	1.92 ± 0.56[a]

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[355] (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-

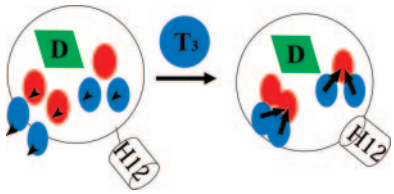

FIG. 8. **Model to explain coupling of T_3 binding to inhibition of TRβ DNA binding activity.** The *blue spheres* represent positively charged residues in Cluster 1, whereas *red spheres* represent negatively charged residues. The charged residues are in an unspecified organization required for homodimer formation in the absence of hormone and rearrange to form the ionic bond organization detected in our x-ray structures in the presence of hormone.

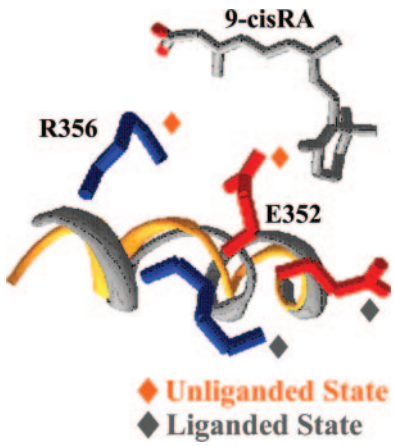

FIG. 9. **Ligand-dependent rearrangements in RXR H7 (equivalent to TRβ H8).** A schematic shows apoRXR H7 in *orange* and holo-RXR in *gray* with ligand (9-*cis*-retinoic acid (*RA*)) in *gray* and *red*. H7 changes backbone conformation: Lys^{356} and Glu^{352}, paired in apoRXR, move from inside to outside on ligand binding.

zation that is distinct from that observed in x-ray structures of liganded TRβ LBDs but required for optimal homodimer formation on DNA. Given the placement of these residues, we favor the notion that the clusters comprise homodimer-specific extensions of the dimer surface that engage in flexible contacts with oppositely charged residues in partner LBDs or possibly influence homodimer formation via distant stabilizing effects on the dimer surface. We further suggest that T_3 binding promotes structural rearrangements in the TR LBD that reposition the charged residues, simultaneously breaking the interactions that are needed for optimal homodimer formation on DNA and creating new ionic bonds that hold the walls of the hormone binding pocket in an appropriate configuration for stable T_3 interactions.

Our model suggests explanations for several apparently paradoxical results. 1) Different charge arrangements are required for T_3 binding and homodimer formation on TREs because the clusters adopt different organizations in the presence and absence of T_3. 2) Cluster 1 could be eliminated without obvious effect on TRβ even though mutations in Arg^{338} and Asp^{351} inhibit T_3 binding because the Arg^{338}-Asp^{351} ionic bond counteracts the tendency of the Cluster 1 to revert toward the organization in the unliganded state. If Cluster 1 is eliminated, the requirement for the stabilizing element is eliminated. 3) Lys^{342} and Asp^{355} inhibited T_3 binding because they stabilize the unliganded TRβ conformer that binds to DNA as a homodimer but only provide limited stability (Lys^{342}) or no additional stability (Asp^{355}) to the liganded TRβ conformer. Here mutation of Lys^{342} and Asp^{355} enhanced T_3 response and T_3 binding by counteracting the tendency of the cluster to revert to its organization in unliganded state.

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 Glu^{352} and Lys^{356} 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 T_3 binding are not restricted to H12 and that T_3 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. 7*B*). 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

1. Apriletti, J. W., Ribeiro, R. C. J., Wagner, R. L., Feng, W., Webb, P., Kushner, P. J., West, B. L., Nilsson, S., Scanlan, T. S., Fletterick, R. J., and Baxter, J. D. (1998) *Clin. Exp. Pharmacol. Physiol. Suppl.* **25,** S2–S11
2. Ribeiro, R. C. J., Apriletti, J. W., Wagner, R. L., West, B. L., Feng, W., Huber, R., Kushner, P. J., Nilsson, S., Scanlan, T. S., Fletterick, R. J., Schaufele, F., and Baxter, J. D. (1998) *Recent Prog. Horm. Res.* **53,** 351–394
3. Yen, P. M. (2001) *Physiol. Rev.* **81,** 1097–1142
4. Zhang, J., and Lazar, M. A. (2000) *Annu. Rev. Physiol.* **62,** 439–466
5. Glass, C. K., and Rosenfeld, M. G. (2000) *Genes Dev.* **14,** 121–141
6. Baxter, J. D., Dillmann, W. H., West, B. L., Huber, R., Furlow, J. D., Fletterick, R. J., Webb, P., Apriletti, J. W., and Scanlan, T. S. (2001) *J. Steroid Biochem. Mol. Biol.* **76,** 31–42
7. Webb, P., Nguyen, N. H., Chiellini, G., Yoshihara, H. A., Cunha Lima, S. T., Apriletti, J. W., Ribeiro, R. C., Marimuthu, A., West, B. L., Goede, P., Mellstrom, K., Nilsson, S., Kushner, P. J., Fletterick, R. J., Scanlan, T. S., and Baxter, J. D. (2002) *J. Steroid Biochem. Mol. Biol.* **83,** 59–73
8. Gothe, S., Wang, Z., Ng, L., Kindblom, J. M., Barros, A. C., Ohlsson, C., Vennstrom, B., and Forrest, D. (1999) *Genes Dev.* **13,** 1329–1341
9. Wondisford, F. E. (2003) *J. Investig. Med.* **51,** 215–220
10. Ribeiro, R. C., Apriletti, J. W., Wagner, R. L., Feng, W., Kushner, P. J., Nilsson, S., Scanlan, T. S., West, B. L., Fletterick, R. J., and Baxter, J. D. (1998) *J. Steroid Biochem. Mol. Biol.* **65,** 133–141
11. Weatherman, R. V., Fletterick, R. J., and Scanlan, T. S. (1999) *Annu. Rev. Biochem.* **68,** 559–581
12. Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fletterick, R. J. (1995) *Nature* **378,** 690–697
13. Wagner, R. L., Huber, B. R., Shiau, A. K., Kelly, A., Cunha Lima, S. T., Scanlan, T. S., Apriletti, J. W., Baxter, J. D., West, B. L., and Fletterick, R. J. (2001) *Mol. Endocrinol.* **15,** 398–410
14. Huber, B. R., Desclozeaux, M., West, B. L., Cunha-Lima, S. T., Nguyen, H. T., Baxter, J. D., Ingraham, H. A., and Fletterick, R. J. (2003) *Mol. Endocrinol.* **17,** 107–116
15. Huber, B. R., Sandler, B., West, B. L., Cunha Lima, S. T., Nguyen, H. T., Apriletti, J. W., Baxter, J. D., and Fletterick, R. J. (2003) *Mol. Endocrinol.* **17,** 643–652
16. Borngraeber, S., Budny, M. J., Chiellini, G., Cunha-Lima, S. T., Togashi, M., Webb, P., Baxter, J. D., Scanlan, T. S., and Fletterick, R. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15358–15363
17. Feng, W., Ribeiro, R. C. J., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998) *Science* **280,** 1747–1749
18. Marimuthu, A., Feng, W., Tagami, T., Nguyen, H., Jameson, J. L., Fletterick, R. J., Baxter, J. D., and West, B. L. (2002) *Mol. Endocrinol.* **16,** 271–286
19. Hu, X., and Lazar, M. A. (1999) *Nature* **402,** 93–96
20. Nagy, L., Kao, H. Y., Love, J. D., Li, C., Banayo, E., Gooch, J. T., Krishna, V., Chatterjee, K., Evans, R. M., and Schwabe, J. W. (1999) *Genes Dev.* **13,** 3209–3216
21. Perissi, V., Staszewski, L. M., McInerney, E. M., Kurokawa, R., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1999) *Genes Dev.* **13,** 3198–3208
22. Ribeiro, R. C., Kushner, P. J., Apriletti, J. W., West, B. L., and Baxter, J. D. (1992) *Mol. Endocrinol.* **6,** 1142–1152
23. Cohen, R. N., Brzostek, S., Kim, B., Chorev, M., Wondisford, F. E., and Hollenberg, A. N. (2001) *Mol. Endocrinol.* **15,** 1049–1061
24. Liang, F., Webb, P., Marimuthu, A., Zhang, S., and Gardner, D. G. (2003)

J. Biol. Chem. **278,** 15073–15083
25. Williams, G. R., Zavacki, A. M., Harney, J. W., and Brent, G. A. (1994) *Endocrinology* **134,** 1888–1896
26. Yoh, S. M., and Privalsky, M. L. (2001) *J. Biol. Chem.* **276,** 16857–16867
27. Ribeiro, R. C., Feng, W., Wagner, R. L., Costa, C. H., Pereira, A. C., Apriletti, J. W., Fletterick, R. J., and Baxter, J. D. (2001) *J. Biol. Chem.* **276,** 14987–14995
28. Chatterjee, V. K., and Beck-Peccoz, P. (1994) *Bailliere's Clin. Endocrinol. Metab.* **8,** 267–283
29. Kopp, P., Kitajima, K., and Jameson, J. L. (1996) *Proc. Soc. Exp. Biol. Med.* **211,** 49–61
30. Collingwood, T. N., Adams, M., Tone, Y., and Chatterjee, V. K. (1994) *Mol. Endocrinol.* **8,** 1262–1277
31. Kitajima, K., Nagaya, T., and Jameson, J. L. (1995) *Thyroid* **5,** 343–353
32. Ribeiro, R. C., Apriletti, J. W., Yen, P. M., Chin, W. W., and Baxter, J. D. (1994) *Endocrinology* **135,** 2076–2085
33. Ando, S., Nakamura, H., Sasaki, S., Nishiyama, K., Kitahara, A., Nagasawa, S., Mikami, T., Natsume, H., Genma, R., and Yoshimi, T. (1996) *J. Endocrinol.* **151,** 293–300
34. Takeda, T., Suzuki, S., Nagasawa, T., Liu, R. T., and DeGroot, L. J. (1999) *Biochimie* (*Paris*) **81,** 297–308
35. Takeda, T., Nagasawa, T., Miyamoto, T., Minemura, K., Hashizume, K., and Degroot, L. J. (2000) *Thyroid* **10,** 11–18
36. Clifton-Bligh, R. J., de Zegher, F., Wagner, R. L., Collingwood, T. N., Francois, I., Van Helvoirt, M., Fletterick, R. J., and Chatterjee, V. K. (1998) *Mol. Endocrinol.* **12,** 609–621
37. Perutz, M. F. (1978) *Science* **201,** 1187–1191
38. Perutz, M. F. (1989) *Trends Biochem. Sci.* **14,** 42–44
39. Serrano, L., Horovitz, A., Avron, B., Bycroft, M., and Fersht, A. R. (1990) *Biochemistry* **29,** 9343–9352
40. Strop, P., and Mayo, S. L. (2000) *Biochemistry* **39,** 1251–1255
41. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **375,** 377–382
42. Renaud, J. P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **378,** 681–689
43. Gampe, R. T., Jr., Montana, V. G., Lambert, M. H., Miller, A. B., Bledsoe, R. K., Milburn, M. V., Kliewer, S. A., Willson, T. M., and Xu, H. E. (2000) *Mol. Cell* **5,** 545–555
44. Bourguet, W., Vivat, V., Wurtz, J. M., Chambon, P., Gronemeyer, H., and Moras, D. (2000) *Mol. Cell* **5,** 289–298
45. Gampe, R. T., Jr., Montana, V. G., Lambert, M. H., Wisely, G. B., Milburn, M. V., and Xu, H. E. (2000) *Genes Dev.* **14,** 2229–2241