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Quantification of Ligand-Regulated Nuclear Receptor Corepressor and Coactivator Binding, Key Interactions Determining Ligand Potency and Efficacy for Thyroid Hormone Receptor

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Abstract

The potency and efficacy of ligands for nuclear receptors (NR) result both from the affinity of the ligand for the receptor and the affinity that various coregulatory proteins have for ligand-receptor complexes; the latter interaction, however, is rarely quantified. To understand the molecular basis for ligand potency and efficacy, we developed dual time-resolved fluorescence resonance energy transfer (*tr*-FRET) assays and quantified both ligand and coactivator/corepressor binding to the thyroid hormone receptor (TR). Promoter-bound TR exerts dual transcriptional regulatory functions, recruiting corepressor proteins and repressing transcription in absence of thyroid hormones (THs), and shedding corepressors in favor of coactivators upon binding agonists, activating transcription. Our *tr*-FRET assays involve a TRE sequence labeled with terbium (fluorescence donor), TRβ•RXRα heterodimer and fluorescein-labeled NR interaction domains of coactivator SRC3 or corepressor NCoR (fluorescence acceptors). Through coregulator titrations, we could determine the affinity of SRC3 or NCoR for TRE-bound TR•RXR heterodimers, unliganded or saturated with different THs. Alternatively, through ligand titrations, we could determine the relative potencies of different THs. TR agonist potencies were GC-1~T_3~TRIAC~T_4>>rT_3, for both coactivator recruitment and corepressor dissociation; the affinity of SRC3 binding to TR-ligand complexes followed a similar trend. This highlights that the low activity of rT_3 derives both from its low affinity for TR and the low affinity of SRC for the TR-rT_3 complex. The TR antagonist NH-3 failed to induce SRC3 recruitment but did effect NCoR dissociation. These assays provide quantitative information on the affinity of two key interactions that are determinants of NR ligand potency and efficacy.

Our evolving understanding of the processes by which nuclear receptors (NRs) regulate gene transcription has revealed an increasing number of protein partners with which a receptor interacts to alter chromatin architecture and nucleosome structure, and to regulate the activity of RNA polymerase II. The best characterized proximal protein partners of the NRs are the p160 coactivators, exemplified by the steroid receptor coactivator 3 (SRC3), and corepressors, exemplified by nuclear receptor corepressor (NCoR). Both of these proteins interact with the NRs through specific nuclear receptor interaction domains (NRID) in a ligand-dependent manner, with the corepressor binding to unliganded or antagonist-liganded NR states that

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repress transcription and the coactivator binding to agonist-liganded NR states that activate transcription (1,2).

While in general, the potency of a NR ligand is related to the affinity with which it binds to its cognate receptor, potency and affinity do not always track in parallel; in particular, as the structure of a ligand is changed, it is sometimes observed that potency decreases faster than binding affinity. This more rapid decrease in potency could arise at the level of the receptor-coactivator interaction if, for example, a coactivator bound more weakly to a receptor-*low affinity ligand complex* than to a receptor-*high affinity ligand complex*, even if the receptor was fully saturated with ligands in both cases. Thus, ligand potency would be determined by a combination of two interactions, (i) the affinity of the ligand for binding to the receptor and (ii) the affinity of the coactivator for binding to the ligand-receptor complex.

To study these two key interactions that are thought to underlie the potency of NR ligands directly, we have developed dual *in vitro, tr*-FRET (time resolved fluorescence resonance energy transfer)- based assays, using purified components, through which it is possible to examine in a convenient and quantitative manner both ligand-induced association of the SRC3 NRID with a NR, and ligand-induced displacement of the NCoR NRID from the same NR. We have used this assay to study these interactions in the context of the thyroid hormone receptor (TR) and its ligands, the thyroid hormones (THs).

The TR/TH system is a particularly good one for this purpose, because TR remains promoter bound both in the absence and presence of ligands, with unliganded TR recruiting corepressors and repressing gene transcription, and agonist-liganded TR recruiting coactivators and activating gene transcription (3). Furthermore, there are both high and low affinity/low potency TR ligands, both natural (T_3, T_4 vs. rT_3) and synthetic (TRIAC, GC-1), as well as a recently described TR antagonist (NH-3) (Figure 1). The THs play essential roles in mammalian development, growth and homeostasis (4), and the human thyroid gland produces T_4, the major TH, and relatively smaller amounts of T_3 and rT_3. T_3 binds to TR with the highest affinity and rT_3 the lowest (5–8). Thus, T_4 is the major thyroid hormone, while T_3 is the most potent one.

Our *tr*-FRET assays recapitulate many of the molecular elements involved in TH regulation of transcription in cells: a direct repeat (DR+4 thyroid hormone response element (TRE) sequence from a known TH-regulated gene (labeled with terbium-strepavidin, the fluorescence donor), TRβ with its usual heterodimer partner RXRα, (9–12) and nuclear receptor interaction domains of the coactivator SRC3 (13,14) or the corepressor NCoR (labeled with fluorescein as the fluorescence acceptor) (15,16). The *tr*-FRET assays can be set up to measure either the affinity with which the coactivator or the corepressor binds to TR•RXR while it is TRE bound (*coregulator titration mode*) or the potency and efficacy with which a ligand dissociates NCoR or recruits SRC3 to the TRE-bound receptors (*ligand titration mode*).

With these assays, we have found both expected and unexpected trends in the relationship between the potencies with which ligands bind to TR and the affinities with which coactivators bind to ligand-TR complexes. We have characterized the basis for the low potency of rT_3, and explored how T_4 differs from T_3 and TRIAC in these interactions, and we have characterized the distinctly different coregulator recruitment patterns of TR agonists and antagonists. This convenient and versatile assay provides quantitative information on the affinity of two key interactions that are determinants of ligand potency and efficacy and should prove useful in investigations of both ligand discovery and molecular pharmacology with other nuclear receptor systems.

EXPERIMENTAL PROCEDURES

Reagents

T_4 (3,5,3',5'-tetraiodo-L-thyronine), T_3 (3,5,3'-triiodo-L-thyronine), TRIAC (Tri iodo acetic acid), rT_3 (3,3',5' triiodo-L-thyronine), 2-thio-glycerol and Sephadex G-25 (medium) were purchased from Sigma (St. Louis, MO). Calf thymus DNA was from Calbiochem. ^{125}I-T_4 (116 Ci/mmol) and ^{125}I-T_3 (97 Ci/mmol) were obtained from PerkinElmer. GC-1 (3,5-Dimethyl-4-(4'-hydroxy-3'-isopropylbenzyl) phenoxyl acetic acid) and NH-3 ({4-[4-Hydroxy-3-isopropyl-5-(4-nitrophenylethynyl)-benzyl]-3,5-dimethylphenoxy} acetic acid have been described previously (17,18). The thiol reactive flurophore, 5-iodoacetamidofluorescein and terbium-labeled streptavidin, were obtained from Molecular Probes/Invitrogen (Eugene, CA) and Invitrogen (Carlsbad, CA), respectively. A 48-bp DNA sequence containing a single copy DR+4 TRE (5'/5Bio/GAACAGATCTCCTTGGCTCTGGAGGTCACAGGAGGTCAGCGGATCCAT: core TRE is underlined) was derived from the rat α-myosin heavy chain promoter and was synthesized by Integrated DNA Technologies (Coralville, IA) (19). Biotin was covalently attached to 5' end of the sense strand. The core DR+4 sequence, AGGTGACAGGAGGACA, from the natural promoter was changed to the consensus DR+4 sequence AGGTCACAGGAGGTCA.

Plasmids and protein expression

The bacterial expression plasmids encoding 6XHis fusion proteins of hTRβ (amino acids 82–456 containing both the DNA and ligand binding domains), hRXRα (full length) and a GST-tagged NCoRC' encoding the NRID fragment mNCoR (residues 2057–2453) were kindly provided by Dr. Milan K. Bagchi, and their functional properties have been described elsewhere (20–22). The NdeI-BamHI fragment of the NCoRC' from the GST-NCoRC' vector was excised and cloned into the NdeI and BamHI sites of pET15b vector and expressed as a 6XHis fusion protein. Expression of the NRID fragment of hSRC3 protein (residues 627–829) has been previously described (23). The NRID fragments of SRC3 and NCoR were fluorescently labeled with 5-iodoacetamidofluorescein according to our published procedure (23,24).

HPLC analysis of TR ligands

T_4, T_3 and rT_3 were tested for their purity in an analytical C18 reversed phase column (Waters-Symmetry, 5 μm, 4.6 ×150 mm) using a gradient of 20–45% acetonitrile in water. While T_3 was found to be pure, T_4 stock was found to contain 0.08% of T_3, and rT_3 was found to contain 0.09% T_4. Peak fractions of T_4 and rT_3 devoid of contaminants were collected, verified for purity on the same column and used in our experiments.

Coregulator (SRC3 or NCoR) titration assays

Fluorescein-labeled SRC3 or NCoR fragment was serially diluted at 1.5× of the required final concentration in 1.5 ml amber microfuge tubes in buffer A (50 mM Tris pH 8 containing 10% glycerol, 0.05% Nonidet P-40, 50mM KCl 2 mM β-mercaptoethanol and 0.3 mg/ml of ovalbumin) and 10 μl aliquots of each dilution were added to the wells of a 96-well dark microplate (Molecular Devices, Sunnyvale, CA). TR ligands were dissolved at a concentration of 20 mM in 0.1 M NaOH (T_3, TRIAC, T_4 and rT_3) or 5 mM in DMSO (T_3, GC-1 and NH-3). Ligands in NaOH or DMSO were further diluted in buffer B (20mM Tris pH 8 and 100 mM NaCl) or buffer B containing 6% DMSO. A 3× premixture of SA-Tb, TRE, TR, RXR and various TR ligands or equivalent volume of the solvents was prepared and then added to each well in a volume of 5 μl for a final assay concentration of 2.5 nM (SA-Tb), 10 nM (TRE), 15 nM (TR and RXR each) and 3 μM (ligands). The total assay volume was 15 μl.

Each assay was run with corresponding negative control wells that contained all the components plus appropriately diluted solvents, but without the biotinylated-TRE, which was used to correct for diffusion-enhanced FRET. The plates were gently mixed, protected from light and incubated for 1 h at room temperature before measurement for *tr*-FRET. Coregulator titrations performed in the presence of 10, 15 and 25 nM TR•RXR indicated that 15 nM of TR•RXR was the lowest receptor concentration to yield an optimal assay sensitivity and assay window. Incubation of assay plates for different times indicated that the reaction reached equilibrium by 1 h and the *tr*-FRET signal remained unchanged up to 24 h (data not shown).

Ligand titration assays

The following 3X reaction components were made:Ligand or solvent dilutions in buffer B or buffer B plus 6% DMSO, fluorescein-labeled coregulators in buffer A and a premixture of SA-Tb, TR•RXR with (test) or without (control) biotinylated TRE in buffer A. Aliquots (5 µl) of different ligand dilutions and SA-Tb-DNA-receptor heterodimer premixture were added first, followed with the addition of 5µl fluorescein-labeled SRC3 or NCoR. The final assay concentration for SA-Tb, TRE, TR•RXR and fluorescein-labeled SRC3 or NCoR in each well were 2.5, 10, 15 and 125 or 24 nM, respectively, in the presence of the indicated ligand concentrations. Diffusion-enhanced background fluorescence was measured as described in the previous experiment. The plates were mixed gently and measured for *tr*-FRET after 1 h incubation at room temperature in the dark.

Ligand dissociation kinetic assays

T_4 and T_3 dissociation experiments were carried out essentially as previously described, except that ligand dissociations were carried out at 25 °C instead of 4 °C (6). Briefly, 3.0 nM TRβ alone or in complex with equimolar RXR or RXR plus DR+4 TRE was allowed to bind 25 nM ^{125}I-T_4 or ^{125}I-T_3 in buffer (pH 8.0) containing 20 mM KHPO4, 150 mM NaCl, 0.5 mM EDTA, 1.0 mM $MgCl_2$, 1mM 2-thio-glycerol and 500 µg/ml calf thymus histones (700 µl reaction volume) until the reaction reached equilibrium (12 hrs at 4° C). In some assays we used a saturating concentration (100 nM) of unlabeled SRC3 protein along with the other combinations described above. Following incubation, tubes were warmed in 25° C water bath for 30 minutes, and a 50-µl aliquot was applied to a Sephadex G-25 column (2 ml) before and at different time points after the addition of excess (2.0 µM) of unlabeled T_4 or T_3. Receptor bound radioactivity was collected and counted in a γ-counter.

Inhibition of T_3 and GC-1-dependent SRC3 recruitment by NH-3

Five microliters of serially diluted NH-3 (as 4×) in buffer B containing 4% DMSO was individually mixed with 5 µl of 120 nM of either T_3 or GC-1 in buffer B containing 4% DMSO. Five microliters each of 500 nM of fluorescein-labeled SRC3 and 4× SA-Tb, TR•RXR with (test) or without (control) biotinylated TRE was then added to the ligand mixture. The final concentrations of T_3 or GC-1 and SRC3 were 30 nM and 125 nM, and of SA-Tb, TRE and TR•RXR were 2.5, 10, and 15 nM, respectively.

While the presence of 2% DMSO in assays had no significant effect on the EC_{50} values for SRC3 recruitment, we observed a modest decrease in the affinity of NCoR for unliganded TR, with a concomitant decrease in IC_{50} values in NCoR dissociation assays in the presence of DMSO (compare Fig. 5B & Fig. 6 with Fig. 8C & 8D). However, in NCoR dissociation assays performed in the presence and absence of DMSO (2%), the *relative* potencies of different TR ligands remained essentially unchanged (data not shown).

tr-FRET Measurements

tr-FRET was measured on a Wallac Victor II plate reading fluorometer (Molecular Devices, Sunnyvale, CA) as previously described (25).

Statistical Analysis

Assays were performed in replicates, and each point in the binding curves represents the mean ± SD of three independent experiments. The Z'-factor was calculated from 6 replicates of specific *tr*-FRET values corresponding to the maximal response values (saturation points) and their respective negative control values for coregulator saturation assays (at 0.5 and 1 μM of SRC3 in the presence (positive control) and absence (negative control) of 3 μM of different TR ligands in the coactivator titration assay, and at 0.125 and 0. 25 μM of NCoR in the absence (positive control) and presence (negative control) of 3μM of ligands in the corepressor titration assay), using a previously described method (26). The Z' factors were similarly determined for ligand titrations of both SRC3 recruitment and NCoR dissociation using the respective positive and negative control values. The Z' equation is

$$Z' = 1 - [(3\sigma_{c1} + 3\sigma_{c2})/\{(\mu_{c1} - \mu_{c2})\}],$$

where σ_{c1} and σ_{c2} are the standard deviations of the positive and negative control values, respectively, and μ_{c1} and μ_{c2} are mean values for the positive and negative controls, respectively. The Z' value of each ligand and of different ligands tested in a given experiment is provided as a range in the respective figure legends.

The dose-dependent NH-3 displacement curve of agonist-induced SRC3 binding was analyzed by the Cheng-Prusoff equation to determine the relative affinities of NH-3 to block SRC3-TR interaction in the presence of 30 nM minimal dose of T_3 or GC-1 (27).

RESULTS

Time-resolved fluorescence resonance energy transfer (*tr*-FRET) assays of TR-coregulator interactions

tr-FRET is a convenient, fluorescence-based assay technology for studying molecular interactions, adaptable to a microplate format, that we have used to quantify the ligand-regulated interactions of a corepressor and a coactivator with hTRβ. To determine both the binding affinity and the ligand-dependent displacement of fluorescein-labeled NCoR from, and the recruitment of fluorescein-labeled SRC3 (both as NRID fragments) to, TRE-bound hTRβ•hRXRα heterodimers, we used TR•RXR indirectly labeled with terbium via a biotinylated TRE that is linked to a streptavidin-terbium (SA-Tb) conjugate. Excitation of the Tb chelate at 340 nm results in emission at 495 nm. However, if both TRE-bound TR and coregulator are in close proximity, energy from the excited state of the Tb complex can be transferred to the fluorescein acceptor, which emits at 520 nm (Fig. 2A and 2B). By monitoring the degree of FRET as the ratio of acceptor emission intensity (A, at 520 nm) to donor emission intensity (D, at 495 nm), expressed as A/D*1000, we could follow quantitatively the ligand-dependent association and dissociation of coregulators with TRE-bound TR.

Determination of SRC3 binding affinity to various TR-ligand complexes: Coactivator titration assay

A *coactivator titration assay* is used to measure the *affinity* of *SRC3* for TRE-bound TR•RXR heterodimer. As shown in Fig. 3A, when increasing concentrations of fluorescein-labeled SRC3 NRID were incubated with a fixed amount of DNA-bound TR•RXR (15 nM) and a fixed, saturating concentration of different TR ligands (3 μM, or in the absence of ligand, apo TR), we observed a concentration-dependent and ligand-specific increase in *tr*-FRET signal,

reflecting the binding of SRC3 to the various TR-ligand complexes. To correct for diffusion-enhanced FRET, *background* control fluorecence (*tr*-FRET measured in the absence of biotinylated DNA) is subtracted from the *total tr*-FRET values. This shows that specific binding approaches full saturation at the highest SRC3 concentration for all the ligands tested, except for rT_3 (Fig. 3B). The baseline binding in Figure 3B indicated that SRC3 did not bind at all to the unliganded TR•RXR (Apo vs. control, Fig. 3A). In preliminary experiments, SRC3 titration was performed at 1.0, 2.0, 3.0, 10.0 and 100 μM of the different ligands. We found that saturable binding of SRC3 to TR was observed at 3 μM of T_3, TRIAC and T_4. With rT_3, SRC3 did not reach a clear saturation plateau even at a concentration of 100 μM, although the level of SRC3 recruitment increased to about 85% of that induced by 3μM of TRIAC or T_4 (data not shown). Since we were interested in comparing the coactivator recruitment profiles of TR exposed to the same concentration of different ligands, a 3 μM concentration was used for all the ligands.

The concentration of SRC3 that gives 50% binding (EC_{50}), a measure of the apparent affinity of SRC3 for these TR ligand complexes, was found to be 30.6 ± 2.8, 34.7 ± 2.1, 39.7 ± 2.0, and 538 ± 44 nM for T_3, TRIAC, T_4 and rT_3, respectively (Fig. 3B, Table).The comparable EC_{50} and maximal binding (B_{max}) values with T_3, TRIAC and T_4 indicate that these ligands induce conformations in TRβ that have similar affinities for SRC3. By contrast, the 18-fold larger EC_{50} value for rT_3 indicates that this ligand stabilizes a TR conformation with much lower affinity for SRC3, but nevertheless suggests that rT_3 has potential agonistic activity. Earlier results from *in vitro* GST-pull down assays showed that some TR agonists had the same coactivator *binding capacity* (6,20,28); by contrast, our *tr*-FRET assay provides a direct measurement of both the *binding capacity* and the *binding affinity* of SRC3 to these different TR-ligand complexes.

The specificity of these TR-coregulator interactions was verified by confirming a complete loss of *tr*-FRET signal when the TRE half site AGGTCA was mutated to TCTCTG. Gel-shift assays confirmed the sequence specificity of the TRE interaction and showed that, in the presence of RXR, TR interacted with the DR+4 response element predominantly (>95%) as TR•RXR heterodimers Ligands have no effect on this interaction (data not shown).

Determination of ligand potency for TR-SRC3 recruitment: Ligand titration

A *ligand titration assay* was used to assess the relative potencies of different TR ligands in recruiting SRC3. The concentration of fluorescein-SRC3 used in this ligand titration assay, 125 nM, was selected from the previous experiment (Fig. 3B) to provide a near maximum specific *tr*-FRET signal and a minimum non-specific signal for most ligands with the concentration of TRE-bound TR•RXR heterodimers required to obtain a good FRET signal (15 nM).

The diffusion-enhanced FRET-corrected binding curves for these ligand titrations (Fig. 4) show that all ligands induced concentration-dependent and saturable SRC3 recruitment profiles. The EC_{50} values for coactivator recruitment (ligand potency) were 16.2 ± 1.2, 17.2 ± 0.9, 21.8 ± 1.7 nM and ~151 ± 9 nM for T_3, TRIAC, T_4, and rT_3, respectively (Fig. 4, Table). The maximum signal from rT_3, however, was only about 40% that induced by the other three ligands. Because SRC3 binds more weakly to the rT_3-TR complex (Fig. 3B), the concentration of SRC3 used in this assay (125 nM) is sufficient to saturate only about 40% of this complex.

Determination of NCoR binding affinity to unliganded TR and effects of ligands: Corepressor titration assays

To examine NCoR binding to TR, we used an mNCoR NRID fragment (residues 2057–2453) that contains 3 cysteine residues, all of which are located at some distance from the critical CoRNR boxes required for interaction with TR. In gel-shift assays with the DR+4 TRE, this

NCoR NRID showed good binding activity for the TR•RXR heterodimers, whether fluorescein labeled or not (data not shown). Fluorescein-labeled NCoR showed saturable binding to unliganded TRE-bound-TR•RXR (Apo), with a measured EC_{50} of 6.9 ± 0.4 nM (Fig. 5; curves in panel B are after correction for diffusion-enhanced FRET). NCoR binding to TR complexes with the high potency agonists, T_3, TRIAC and T_4, was much more limited and of lower affinity, yet there still appeared to be substantial interaction with the TR-rT_3 complex. These baseline levels of binding with T_3, TRIAC or T_4 were not reduced when NCoR dissociation was monitored from TR treated with 10 or 100 μM of these ligands. Only at 100 μM, however, did the ligand rT_3 reach a similar level of NCoR dissociation to that observed with T_3, TRIAC or T_4 at 3 μM concentration (data not shown).

We were curious about the fluorescence activity that remained associated with the TR•RXR when saturated with the agonist ligands T_3, TRIAC, and T_4. These background binding signals are clearly above that attributable to diffusion-enhanced tr-FRET (control curve in Fig. 5A, which is subtracted from the data in Fig. 5B). This NCoR binding does not appear due to a small fraction of TR that is unliganded, because it was not significantly reduced in assays performed with higher concentration of ligands (10 or 100 μM). Thus, we wondered whether it might represent some weak residual binding of NCoR for TR-agonist complexes.

Because the binding surfaces of coactivators and corepressor on NRs are known to partially overlap (29,30), we thought that any residual but specific NCoR binding to agonist-liganded TR•RXR could be disrupted by inclusion of excess of *unlabeled* SRC3 in the assay mixture. There were, however, no differences in the level of binding when NCoR interaction assays were carried out both with and without a saturating concentration of unlabeled SRC3 protein (10 μM) in the presence of 100 μM T_3, TRIAC or T_4 (data not shown). Thus, the residual interaction of NCoR for agonist-liganded TR is likely due to non-specific binding.

Determination of ligand potency for TR-NCoR dissociation: Ligand titration

To determine potencies of different TR ligands in the NCoR dissociation assay, we chose a concentration of NCoR (24 nM, determined from the previous experiment) that gave good specific but minimal background *tr*-FRET signals with 15 nM TRE-TR•RXR. All of the ligands, including rT_3, effected a concentration-dependent dissociation of NCoR from the receptor heterodimers, with IC_{50} values of 56.1 ± 3.2, 38.6 ± 2.8, 60.5 ± 4.4 and 1720 ± 52 nM for T_3, TRIAC, T_4 and rT_3, respectively (Fig. 6). As we found with ligand titration in the *coactivator recruitment assays* (Fig. 4), the potencies for T_3, TRIAC and T_4 obtained in this assay were not dramatically different (Fig. 6, Table), although TRIAC was consistently more potent than T_3 (1.5 fold) in this *corepressor dissociation* assay. Thus, TR ligand potency in terms of NCoR displacement follows the order: TRIAC>T_3~T_4>>rT_3.

As we saw in the SRC3 recruitment assay, rT_3 behaves as an agonist in effecting dissociation of NCoR from TR•RXR, but its low potency (Fig. 6) provides an explanation for the substantial affinity of NCoR for TRE-TR•RXR-rT_3 (Fig. 5B): The concentration of rT_3 used in the NCoR titration (3 μM) is sufficient to dissociate only about 65% of TR-bound NCoR, so unliganded TR in this assay is still available for NCoR binding.

Stability of TR-T_4 and TR-T_3 complexes: Ligand dissociation assays

By radiometric assays, T_4 binds to TR with 20 to 30-fold lower affinity than T_3 (5–8), yet T_4 had activity profiles that are only ~1.5 fold lower in potency compared to T_3 in both of our coregulator interaction assays. Ligand affinity is usually determined by equilibration of a radiolabeled hormone with the receptor protein of interest, in the absence of other interacting protein or DNA partners. The coregulator interaction assays reported here, however, monitor

SRC3 recruitment or NCoR dissociation from TR that is heterodimerized with RXR and bound to a TRE.

Previously, we demonstrated that T_4 dissociates more rapidly from TR than T_3, and we suggested that T_4 forms a less stable complex with TR than does T_3 (6). We now considered whether the additional components in our more complete assay format (RXR, DR+4 TRE, or SRC3) might have a stabilizing effect on T_4 interaction with TR. Therefore, we examined the kinetic stability of T_4-TR and T_3-TR alone (TR), or as complexes with the other components, TR•RXR, TRE-TR•RXR, or TRE-TR•RXR-SRC3 in ligand dissociation assays. In this assay, a preformed ^{125}I-T_4- or ^{125}I-T_3-TR complex is challenged with ~100-fold excess of unlabeled hormone, and the time course of radiolabled hormone dissociation is measured (6). A rapid dissociation rate is indicative of a less stable hormone-receptor complex.

T_4 dissociation from TR and TR•RXR at 25 °C is very rapid (Fig. 7A), having overall half lives of only 1.3–1.8 min; (Fig. 7 table) notably, however, DNA binding markedly slows T_4 dissociation, so that in the context of TRE-TR•RXR, T_4 dissociation half life increases 5.3 min., which is more comparable to the dissociation rate of T_3 (13–14 min. in all three receptor contexts; Fig. 7C). As in our earlier study (6), dissociation of both T_4 and T_3 is not a single exponential; nevertheless, the effect of TRE is clearly evident both in slowing the overall dissociation rate of T_4 and in reducing the magnitude of the initial, more rapid dissociation phase, whereas the T_3 dissociation profile is unaffected by the inclusion of the TRE.

In the estrogen receptor, we have shown that the binding of coactivators can markedly reduce the rate of agonist ligand dissociation (31). Here, we find that addition of SRC3 slows T_4 dissociation from TR and TR•RXR, but less so for the TRE-TR•RXR complex (Fig 7B), resulting in comparable half lives in all three complexes ($t_{1/2}$ = 12–16 min). These SRC3-induced half lives of the T_4 complexes are equivalent to the T_3 dissociation times, in all three complexes, without SRC3. Addition of SRC3 to the T_3 complexes, however, provides additional kinetic stabilization, giving them half lives of ca. 130 min (Fig. 7D), which are ca 8-times greater than those of the T_4-TR complexes in the presence of SRC3 (Fig. 7B). In the presence of SRC3, all T_3 and T_4, dissociation profiles are more cleanly single exponential (as analyzed by the equation for a one-phase exponential decay by GraphPad Prism).

Our results indicate that when TR is bound to a DR+4 TRE as a heterodimer with RXR, the T_4-TR interaction is stabilized compared to the T_4 interaction with TR alone. This effect could be one of the contributing factors for the enhanced T_4 activity in our assays. Nevertheless, once SRC3 is recruited to the liganded TR, TR•RXR or TRE bound TR•RXR heterodimer, the T_3-TR interactions are stabilized much more than the T_4-TR interactions, a factor that might account for the higher potency of T_3.

Evaluation of the synthetic TR ligands, GC-1 and NH-3, in the dual corepressor/coactivator interaction assay

There has been interest in developing agonist compounds that elicit desirable tissue-selective but not unwanted actions of thyroid hormone and also antagonists that block TH action for treatment of thyroid excess state. The ligand GC-1 (Fig. 1) displays selectivity in both its tissue uptake properties and for the β vs. α-isoforms of the TR; it promotes weight loss and lowers cholesterol without eliciting deleterious cardiac effects and is being evaluated as a potential pharmaceutical (17,32). The TR antagonist NH-3 (Fig. 1) can block tadpole metamorphosis (33).

We measured potencies and coregulator binding characteristics of these novel TR ligands in our four assay formats, together with T_3 as a reference. In SRC3 titrations with TRE-bound TR•RXR incubated with these three ligands (each 3 μM), SRC3 binding to T_3- and GC-1- TR

complexes showed saturation curves with EC_{50} values: 24.6 ± 2.7 and 30.1 ± 3.5 nM, respectively (Fig. 8A). By contrast, SRC3 shows no binding affinity for TR liganded with the antagonist NH-3, consistent with its antagonistic activity *in vivo* (18). Increasing NH-3 to 25 or 100 μM had no effect on SRC3 recruitment to TR (data not shown). In ligand titration assays, EC_{50} values for T_3 and GC-1 were 19.0 ± 1.8 and 13.2 ± 1.2, nM, respectively, indicating that GC-1 is slightly more potent that T_3 in recruiting SRC3 to the DNA-bound TR•RXR (Fig. 8B). These findings are consistent with the similar affinities of T_3 and GC-1 for TRβ and their nearly identical efficacies in mammalian cell transactivation assays (17).

In TR-corepressor dissociation assays (Fig. 8C), NCoR showed, as before, essentially no affinity for TRE-bound TR•RXR liganded with the agonists GC-1 and T_3, but saturable binding to unliganded TR. NCoR did show binding to the TRE-bound TR•RXR heterodimers in the presence of 3 μM NH-3, although it did so with very low potency (EC_{50} value of 2800 ± 82 nM). We believe this low affinity interaction reflects the fact that the concentration of NH-3 used in this assay (3 μM) is not sufficient to fully saturate TR, leaving some unliganded TR with which NCoR can interact. Consistently, NCoR interaction with TR performed with different NH-3 concentrations revealed that maximal inhibition of NCoR binding was observed only at 25 μM NH-3 (data not shown). In ligand titration assays, both GC-1 and NH-3 effected NCoR dissociation from apo-TR•RXR (Fig. 8D), with GC-1 being slightly more potent than T_3. NH-3 also disrupted TR-NCoR binding, although with much lower potency. The IC_{50} values were: 16.2 ± 1.8, 11.1 ± 1.1 and 874 ± 23 nM for T_3, GC-1 and NH-3, respectively, and are consistent with the binding affinities of these ligands (17,18).

NH-3 blocks T_3 and GC-1 mediated SRC3 recruitment

To determine whether the antagonist NH-3 could reverse the recruitment of SRC3 to TRE-bound TR•RXR induced by T_3 or GC-1, we titrated increasing concentrations of NH-3 into an assay in which SRC3 had been recruited to TR with 30 nM of T_3 or GC-1. Good competition curves were seen in both cases (Fig. 9), giving IC_{50} values of 2720 ± 180 and 2620 ± 230 nM, respectively. The apparent K_i values, obtained by correcting the IC_{50} values for the concentrations of T_3 or GC-1 used in this experiment (30 nM), were 447 ± 36 and 508 ± 47 nM, for NH-3 in competition with T_3 and GC-1, respectively, values that are consistent with the K_i values from inhibition curves of NH-3 suppression of T_3 and GC-1-induced transactivation in reporter gene assays (18).

DISCUSSION

Determinants of nuclear receptor agonist ligand potency: Ligand-receptor interaction and coregulator-receptor interaction measured *in vitro* in a biologically relevant context

Elucidation of the molecular components involved in NR regulation of target gene transcription suggests that the potency of a NR agonist in a cellular context will reflect both its affinity for binding to the receptor, as well as the affinity with which coactivator proteins are able to interact with the resulting agonist-NR complex. It is straightforward to measure *ligand-receptor binding affinities* directly by titrations in two-component (ligand and receptor) systems, assayed by radiometric or fluorometric methods, or to estimate them by *ligand titrations* in coactivator-recruitment assays. Neither of these methods, however, reveals the *affinity with which coregulators bind to different ligand-receptor complexes*. The favorable attributes of our *tr*-FRET assay system enabled us to measure these protein-protein binding affinities directly.

A convenient and quantifiable *tr*-FRET assay for ligand regulation of TR-coregulator interactions

The different protein components used in our *tr*-FRET assays of TR/THs have been well characterized in terms of mediating protein-protein interactions with NRs (NCoR and SRC3 NRIDs) and for their near normal ligand binding, DNA binding and *in vitro* transcriptional regulatory properties (TR and RXR) (21–23,34). We selected a natural DR+4 sequence from the rat myosin heavy chain promoter as the DNA component because, unlike palindromic or inverted palindromic sequences, both liganded and unliganded TR•RXR heterodimers bind DR+4 TRE with high affinity, ensuring that the changes we observe in *tr*-FRET are due to changes in ligand-specific coregulator binding and not alterations in DNA binding (4,35). To the best of our knowledge, this is the first report assessing quantitatively the affinity of both a coactivator and a corepressor for a response element-bound NR ligand complex, and thus it represents a significant step towards mimicry of the cellular context in which a NR normally works to regulate transcription.

The terbium donor fluorophore used in these *tr*-FRET assays provides some particularly favorable properties: a pronounced Stokes shift, a large R_0 value, and a long fluorescence lifetime (around 1 msec). The last of these enables pulsed excitation and gated emission measurement, which greatly minimizes signals due to background or direct excitation of the acceptor fluorophore (36–39), and makes feasible titration experiments with acceptor fluorophore (fluorescein)-labeled coregulators; such titrations are more difficult to do with conventional FRET assays. Thus, with *tr*-FRET we can measure two distinct parameters: (i) The affinity of coregulators for the receptor (either apo-TR or the various TR-ligand complexes) is measured in a *coregulator titration experiment*, and (ii) the potency of ligands in regulating receptor interaction with coactivator or corepressor is measured in a *ligand titration experiment*.

Potencies and efficacies of thyroid hormones

The potency and agonistic/antagonistic activities of THs are usually determined by standard reporter gene assays using mammalian cell lines in culture (7,8,17,18,40). A comprehensive evaluation of how both natural and synthetic THs affect coactivator and corepressor interactions in terms of their binding efficiency and affinity with TRE-bound TR•RXR heterodimer *in vitro* is typically not undertaken, however, because suitable, quantitative assay systems are not available. In addition, it is not even known whether the potency of a given TR ligand in mediating the distinct process of corepressor dissociation vs. coactivator association is the same or different, or to what extent the potency of a TH agonist depends on its affinity for TR vs. the affinity with which coactivators bind to the ligand-receptor complex it forms with TR.

The rank order of potencies we obtain for the THs by the ligand-titration *coactivator recruitment* assay, T_3~TRIAC~T_4>>rT_3, is very similar to the rank order of relative affinities of SRC3 for the respective ligand–TR complexes, with the first three (T_3, TRIAC and T_4) being the same within a factor of 1.5, and the fourth (rT_3) being 10 to 20-fold lower affinity. The concordance between the affinity of a ligand for its receptor and the affinity of a ligand-receptor complex for a coactivator, however, is not necessarily expected, and it is worth considering what these results mean for a poor TR ligand, such as rT_3. The low potency of this ligand appears due to two factors, (i) its low affinity for binding to TR, and (ii) the weak affinity that SRC3 has for the resulting rT_3-TR complex. Thus, while increasing rT_3 concentration could compensate for the low affinity that rT_3 has for TR, no increase in ligand concentration could compensate for its second “deficiency”, the fact that the complex that rT_3 makes with TR has itself a lower affinity for SRC3. Our findings with rT_3 could have broad pharmacological implications: They suggest that while high concentrations of a low affinity ligand may saturate

a receptor, the biological output from this complex might not be equivalent to that of a receptor complex with a high affinity ligand, because it would be unable to interact effectively with the downstream signaling components required mediate further biological effects.

It is notable that the crystal structure of TRβ complexed with T_4 shows that the receptor can undergo subtle structural alterations relative to the crystal structure of TRβ-T_3 complex to accommodate the bulky 5' iodine group of T_4 (6). Despite these changes, the TR-T_4 complex maintains its affinity for SRC3. While there is no crystal structure for the TR-rT_3 complex, the reduced affinity of SRC3 for this complex suggests that its conformation is significantly different from those TR complexes with T_3 and T_4.

Ligand potency in terms of *corepressor dissociation* follows the order TRIAC≥T_3~T_4>rT_3, with rT_3 being some 30-fold weaker than the other three (Fig. 6 Table), which is similar to that found in the coactivator recruitment assays. Interestingly, however, we repeatedly found TRIAC to be the most potent TR ligand (1.5 fold more potent than T_3) in the ligand titrations of NCoR dissociation, hinting at the possibility that ligands like TRIAC might be more effective in transcriptional derepression than transcriptional activation functions. While the ligand rT_3 was the least potent, it still promoted both NCoR dissociation and SRC3 recruitment, which are attributes of a TH agonist.

tr-FRET and other types of fluorescence-based assays have been used to study the interaction of coactivators with other NRs (39,41–43), and while they have provided some information on the binding affinity and the efficacy of various ligands, they have been conducted in less "intact" systems (i.e., lacking hormone response elements and using NR ligand-binding domains and short peptide sequences from coregulators). The studies of androgen receptors also revealed the effect of mutations on the agonist/antagonist balance of various ligands (38). The role of coactivator binding affinity as a co-determinant of ligand potency, however, has thus far not been studied in a coherent fashion. Other workers have used fingerprinting methods to characterize NR interaction with panels of peptides from both known coregulators or phage display-derived methods (44,45) While these approaches provide a broader view of NR-coregulator interactions, they do not provide quantitative affinities.

Reciprocal interactions of DNA and coactivator on TR-ligand complex stability and conformation

We were curious that in our assays T_4 appeared comparable to T_3 and TRIAC in terms of potency in SRC3 recruitment and NCoR dissociation and in terms of SRC3 binding affinity for the three TR-ligand complexes. In other assays, however, T_4 has been found to be of lower potency than T_3 and TRIAC (5,7,8). Because various NRs, including estrogen and glucocorticoid receptors and TRs, undergo specific conformational changes upon binding to different response elements, resulting in selective recruitment of coactivator/corepressor proteins and ultimately altering the transcriptional response to a particular ligand (25,35,46–49), we wondered whether the higher relative potency of T_4 in our hands might be due to a selective stabilizing effect of the heterodimer partner RXR, the TRE, or the coactivator components on the TR-T_4 complex relative to the TR-T_3 complex.

From ligand dissociation rate measurements, we found that the rapid T_4 dissociation from TR was moderated to some extent by the addition of TRE, but T_3 dissociation was unaffected. Given the fact that circulating levels of free T_4 is ~7-times higher than that of T_3 in humans (50), this suggests that T_4 might have greater effectiveness *in vivo*, despite its lower affinity for TR. The dissociation rate of T_3 from TR, TR•RXR, or TRE-TR•RXR, however, is still slower than that of T_4. Furthermore, similar to the effect that SRC1 has on slowing the dissociation rate of estrogen agonists in estrogen receptor-α (31), we found that the addition of SRC3 afforded kinetic stabilization of the TR-ligand complexes, with stabilization, in fact,

being greater for T_3 and for T_4 (from 1.3–5.3 to 11.8–15.6 min for T_4 and from 13.4–14.0 to 126–132 min for T_3, respectively), with a similar degree in shift of the half life between T_4 and T_3 with the addition of SRC3 (3–10 fold shift in T_4 vs. ~10 fold shift in T_3) This indicates that the coactivator stabilization process is ligand-structure dependent and is another factor contributing to overall ligand potency. Thus, we ascribe the more comparable potency of T_4 relative to T_3 and TRIAC we observe to a potency-leveling effect that results from the relatively high concentration of TR (15 nM) required to get adequate FRET signal levels in our assays.

Characterization of the bioactivity of the novel TR ligands, CG-1 and NH-3

Our results with GC-1, a synthetic T_3 agonist, and NH-3, a synthetic T_3 antagonist, are in good agreement with the TRβ binding affinities, potencies, and efficacies of these compounds measured in cell-based assays. GC-1 has approximately the same affinity as T_3 for TRβ and profiles as a potent agonist (17). Likewise, NH-3 lacks agonist activity and completely blocks T_3-driven gene activity in transfection assays, yet, in mammalian two-hybrid assays, it dissociates corepressors from TR, albeit weakly (18). We too found that NH-3 failed to recruit SRC3 to TR and displayed relatively low potency for corepressor dissociation; we were able to quantify the relative NCoR dissociation potency by NH-3 to be ca. 50-times less than that of GC-1 and T_3, which is similar to the NH-3 to T_3 potency ratio of 30 obtained in cell-based assays (18)..

CONCLUSION

We have described a dual, *in vitro tr*-FRET-based assay through which the interaction of DNA-bound-TR•RXR heterodimers with a coactivator (SRC3) and a corepressor (NCoR), both bona fide coregulators for TRs, can be measured in response to the binding of various thyroid hormones and their analogs. This assay is convenient (mix and measure format), miniaturizable (15–20 μl), readily quantifiable, and reproducible (Z' factor in the range of 0.72–0.84) (26). We provide several lines of evidence showing that these interactions are specific and that the potency and efficacy values we measure in this assay are predictive of the inherent potencies and the agonistic or antagonistic nature of various TR ligands. We can also clearly distinguish the corepressor dissociation and coactivator recruitment characteristics of TR agonists vs. TR antagonists. Furthermore, we can determine the affinity with which SRC3 interacts with TR complexes with different agonist ligands, an interaction that can modulate overall ligand potency in a cellular context, but has thus far been difficult to quantify. The assay system developed here can be easily extended to determine how other TREs, such as palindromic and inverted palindromic response elements, influence TR ligand potencies and how they compare to that obtained with DR+4 TRE. Thus, our results provide new insight into the molecular interactions that underlie agonist ligand potency and the nature of antagonism of various TR ligands. It is notable, as well, that the type of *tr*-FRET assay we employ can be modified using two acceptor fluorophores, so that coactivator recruitment and corepressor dissociation can be measured *simultaneously* in a single assay, as we have done with TRE-bound TR•RXR and SRC3 and NCoR (Jeyakumar and Katzenellenbogen, unpublished), and as has recently been reported in a different system (51). It is likely that assays of this type, with yet further refinement in components and context, will play an increasing role in identifying and characterizing novel ligands for members of the NR family.

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Abbreviations

DMSO, dimethyl sulfoxide
DR+4, direct repeat spaced by 4 nucleotides
Fl, fluorescein
NCoR, nuclear receptor corepressor
NR, nuclear receptor

NRID, nuclear receptor interaction domain
RXR, retinoid X receptor
SA, streptavidin
SRC3, steroid receptor coactivator 3
Tb, terbium
TH, thyroid hormone
tr-FRET, time resolved fluorescence resonance energy transfer
TR, thyroid hormone receptor
TRE, thyroid hormone response element

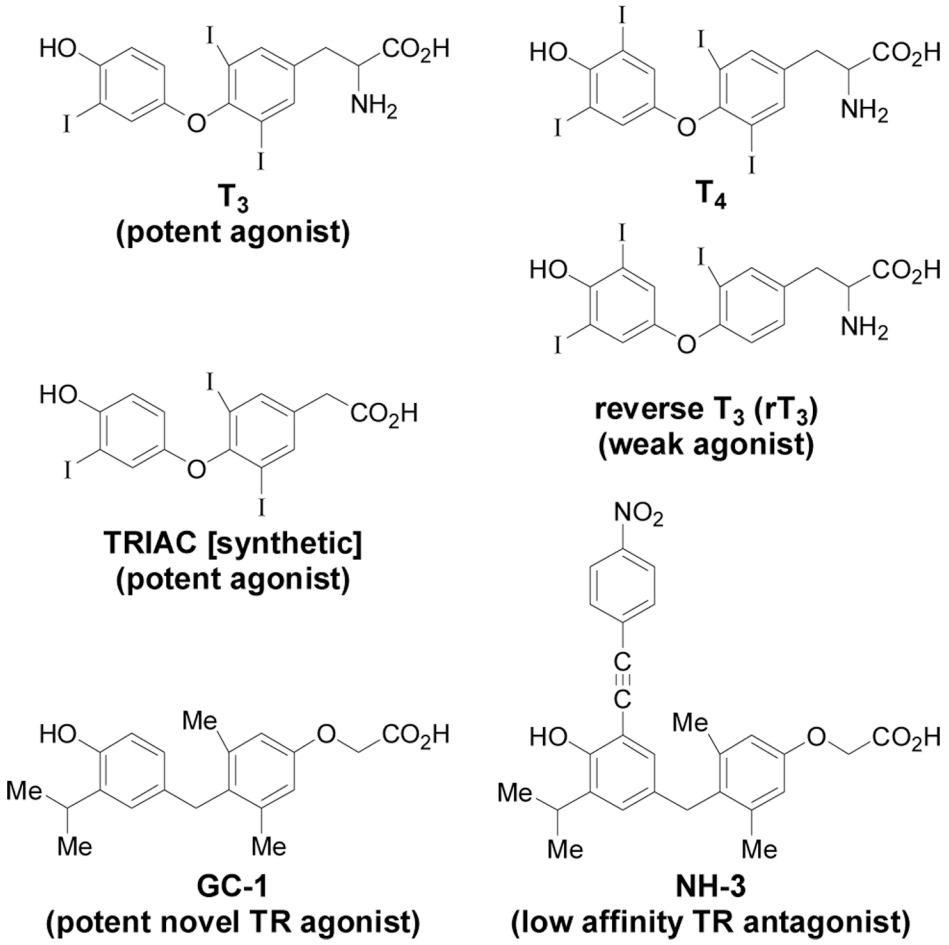


FIGURE 1.

The structures of the different ligands used: endogenous thyroid hormones, T_3, T_4 and rT_3, and synthetic TRβ-selective agonists, TRIAC and GC-1, and antagonist, NH-3.

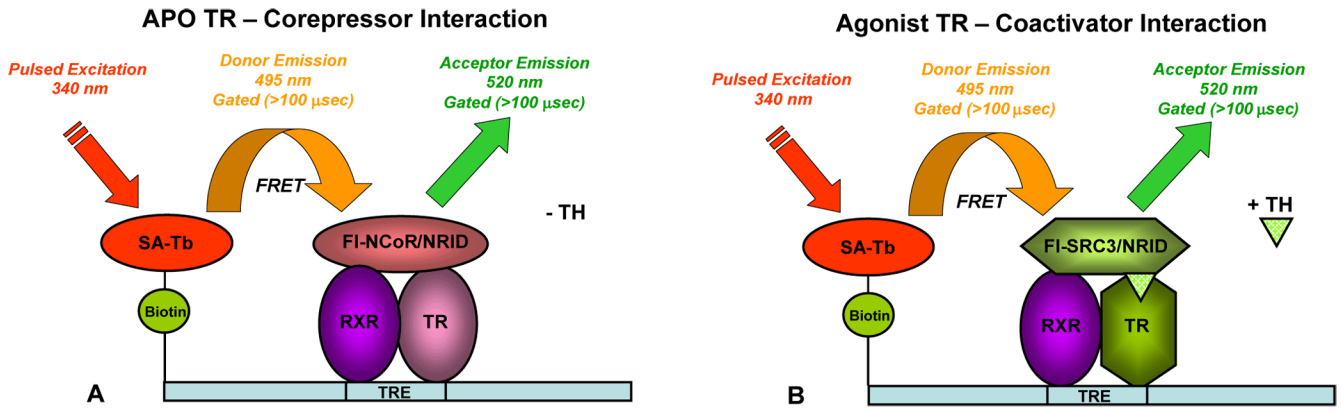


FIGURE 2. Principle of the dual *tr*-FRET assays

A, NCoR interacts with unliganded TR•RXR heterodimer bound to a TRE. hTRβ (residues 82–456)•hRXRα (full length) heterodimer was assembled onto a terbium-labeled streptavidin-bound biotinylated DR+4 sequence (49 bp derived from the rat myosin heavy chain promoter) and incubated with the fluorescein labeled-NRID fragment of mNCoR (residues 2057–2453). The terbium (donor) was excited at 340 nm, and tr-FRET was measured after 100-μsec delay at 495 nm for terbium (donor) and 520 nm for fluorescein (acceptor). *B*, SRC3 interacts with liganded TR•RXR heterodimer bound to a TRE. The assay format is essentially the same as that described for Fig. 2A, except that fluorescein-labeled NRID fragment of hSRC3 (residues 627–829) recruitment to TRE-bound TR•RXR was measured in the presence of different TR ligands.

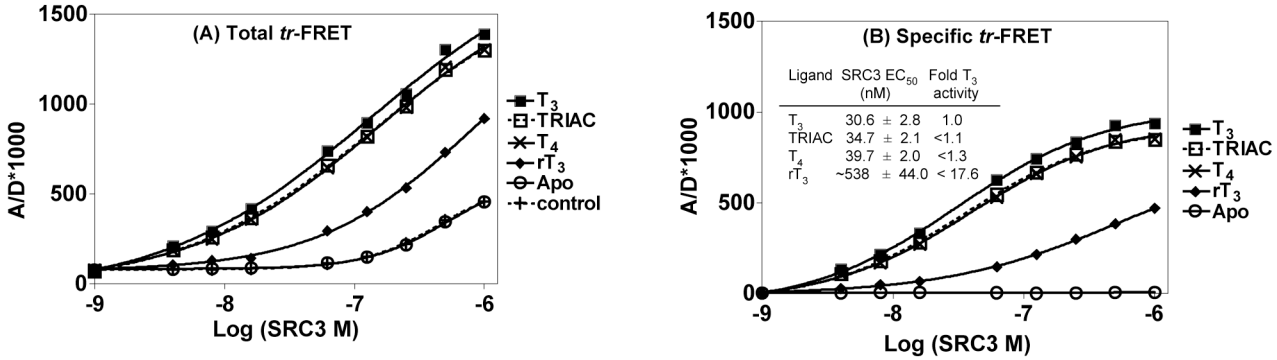


FIGURE 3. Ligands specify affinity of SRC3 to TRE-bound TR•RXR heterodimers (coactivator titration)

A, A fixed amount of TR•RXR heterodimer (15 nM) bound to a biotinylated TRE was incubated with increasing concentrations of fluorescein-labeled SRC3 (NRID fragment) in the absence (Apo) and presence of 3 μM of T_3, TRIAC, T_4 and rT_3, as described under "Experimental Procedure". Control assays containing all the components minus the biotinylated DNA were used to correct for diffusion-enhanced FRET. After incubation at room temperature for 1 h, *tr*-FRET was measured and plotted as the ratio of acceptor to donor × 1000 (A/D*1000) against log of fluorescein-labeled SRC3 concentration. The binding curves obtained for different ligands and the control are shown. *B*, The *tr*-FRET values presented in each of the binding curves in Fig. 3A were subtracted from the corresponding diffusion-enhanced FRET values, and the resulting specific FRET units are plotted against the log SRC3 concentrations. Three independent sets of experiments were performed in replicate, and each assay point in the binding curves represents mean ± SD of six measurements. Data in Fig. 3B were analyzed by non-linear regression with an equation for sigmoidal dose response (variable slope) in GraphPad Prism, and the concentrations of fluorescein-labeled SRC3 at 50% (EC_{50}) of maximal binding in the presence of indicated TR ligands were obtained and listed in the table as mean EC_{50} ± SD of three different experiments. Fold T_3 activity was calculated as a ratio of respective ligand EC_{50} value to that of T_3. The Z' factor was calculated by using the six replicates of maximally responsive specific *tr*-FRET values obtained with each ligand and the corresponding value obtained in the absence of ligand (Apo) as described in the Experimental Procedure section. The Z' factor for SRC3 recruitment by each ligand ranged 0.80–0.84.

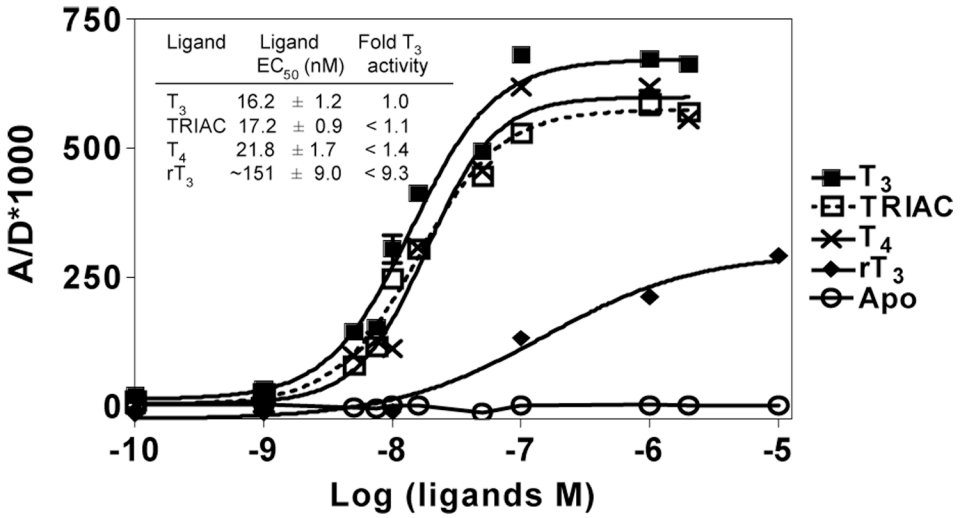


FIGURE 4. Measurement of ligand potency for SRC3 recruitment to TRE-bound TR•RXR heterodimers (ligand titration)

Increasing amounts of different TR ligands were tested for their ability to recruit a submaximal concentration of fluorescein-labeled SRC3 (125 nM) to a fixed amount of TRE-bound TR•RXR (15 nM). *tr*-FRET values obtained in the presence of each ligand were subtracted from the respective diffusion-enhanced control FRET values and plotted against the log of ligand concentration. Apo represents the SRC3 binding pattern to the vehicle-treated TR•RXR (unliganded). Each point in the binding curves represents mean ± SD of six measurements from three different experiments performed in replicate. Data were analyzed as described in the legend to Fig. 3B, and the ligand concentration to induce 50% of maximal SRC3 recruitment was determined and listed in the table as mean EC_{50} ± SD of three independent experiments. Fold T_3 activity was calculated as described in the previous experiment. The Z' factor for T_3, TRIAC, T_4 and rT_3 induced SRC3 recruitment was in the range of 0.76–0.82.

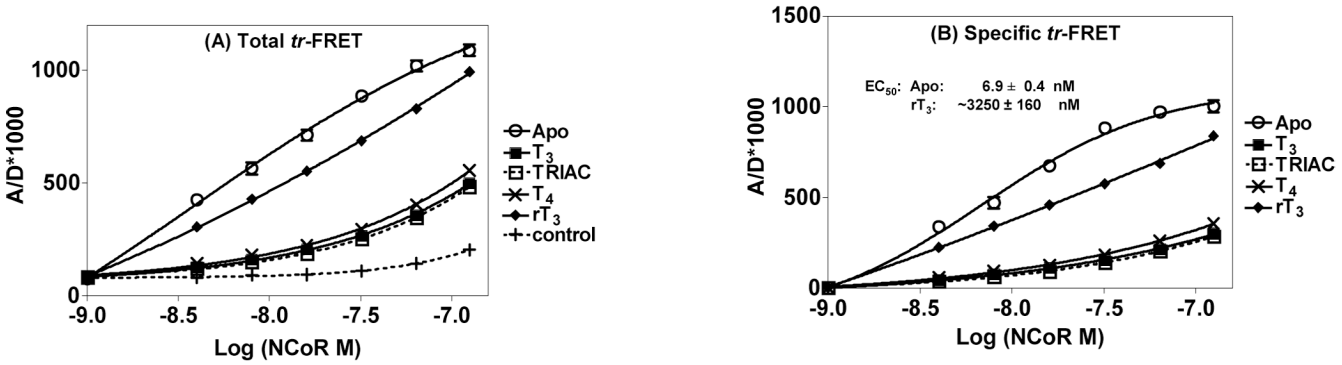
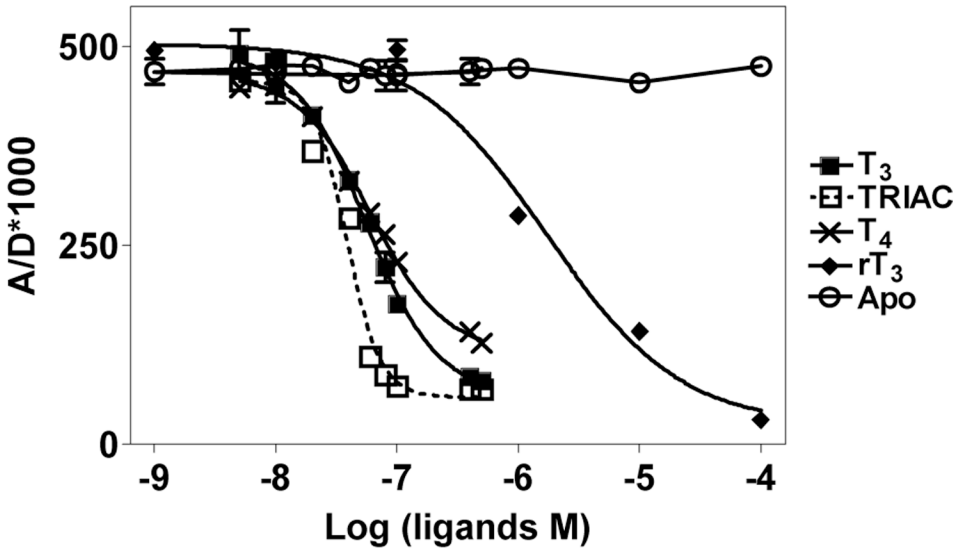


FIGURE 5. Ligand specify affinity of NCoR to TRE-bound TR•RXR heterodimers (corepressor titration)

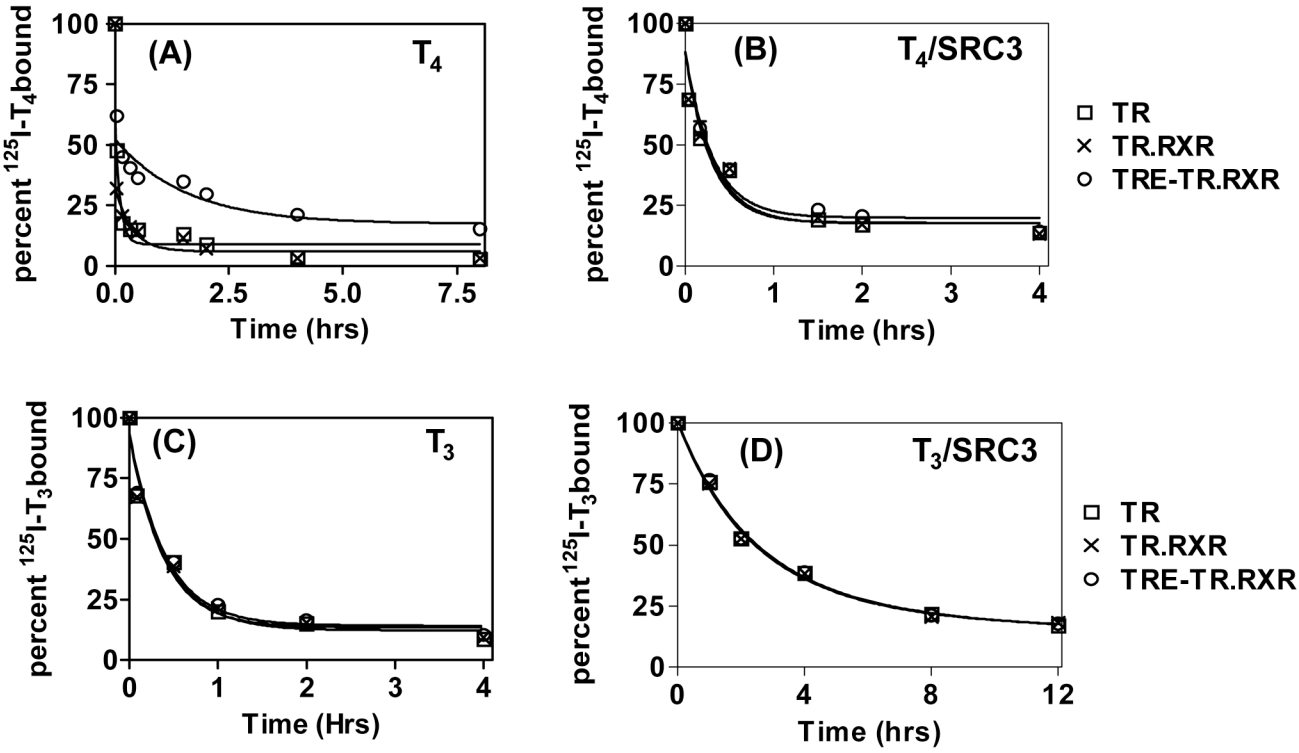
A, Serially diluted fluorescein-labeled NCoR was incubated with a fixed amount of DNA-bound TR•RXR (15 nM) in the presence and absence (Apo) of the indicated TR ligands (3 μM each), and the resulting *tr*-FRET signal was measured as described under "Experimental Procedure". *B*, Binding curves represent results in Fig. 5A after correction for diffusion-enhanced *tr*-FRET. The EC_{50} ± SD of NCoR recruitment to unliganded TR•RXR from three experiments is shown. In this assay NCoR recruitment for un-liganded TR (Apo) had a Z' factor of 0.74.



Ligand	Ligand IC_{50} (nM)	Fold T_3 activity
T_3	56.1 ± 3.2	1.0
TRIAC	38.6 ± 2.8	> 1.5
T_4	60.5 ± 4.4	< 1.1
rT_3	1720 ± 52.0	< 31

FIGURE 6. Measurement of ligand potency for NCoR dissociation from TRE-bound TR•RXR heterodimers (ligand titration)

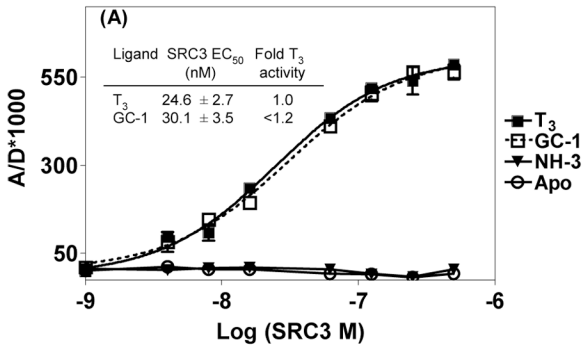
A submaximal concentration of fluorescein-NCoR (24 nM) was incubated with a fixed amount of TR•RXR (15 nM) bound to a TRE in the presence and absence (Apo) of indicated concentrations of different TR ligands. The *tr*-FRET values corrected for diffusion-enhanced *tr*-FRET values for each of the ligands are shown. Three sets of experiments in replicate were performed, and each point in the curves represents mean ± SD of six measurements. Data in Fig. 6 were analyzed by non-linear regression with an equation for sigmoidal dose response (variable slope) in GraphPad Prism, and the concentrations of each of the TR ligands to effect 50% dissociation (IC_{50s}) of maximal NCoR binding were obtained and listed in the table as mean IC_{50} ± SD of three different experiments. Fold T_3 activity was calculated as a ratio of respective ligand value IC_{50} to that of T_3. NCoR remained maximally bound to the corresponding unliganded (solvent-treated) TR•RXR sample wells (Apo). The Z' factor for each ligand induced NCoR disociation was measured to be 0.73–0.85.

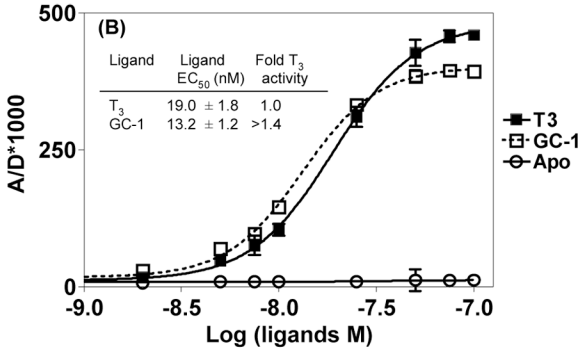


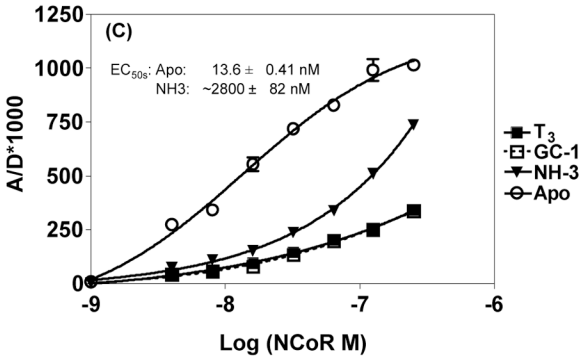
	T_4 $t_{1/2}$		T_3 $t_{1/2}$	
	- SRC3 (mins)	+ SRC3 (mins)	-SRC3 (mins)	+ SRC3 (mins)
TR	1.8 ± 0.09	11.8 ± 1.2	13.4 ± 1.1	132 ± 7.2
TR.RXR	1.3 ± 0.09	13.8 ± 0.6	13.6 ± 0.9	126 ± 5.4
TRE-TR.RXR	5.3 ± 0.30	15.6 ± 0.6	14.0 ± 1.2	126 ± 6.0

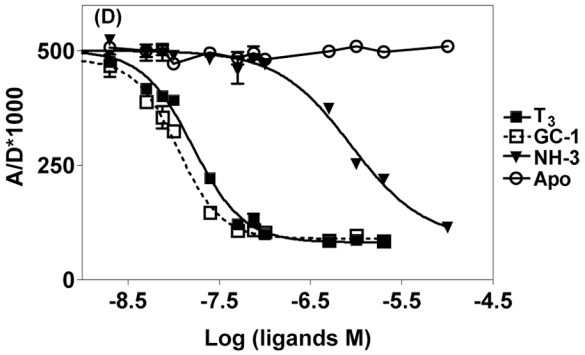
FIGURE 7. Ligand dissociation assays

TR alone or in a complex with RXR or RXR plus DR+4 TRE (3. nM each) was incubated with 25 nM ^{125}I-T_4 (Fig. 7A) or ^{125}I-T_3 (Fig. 7C) in the absence (total binding) and in the presence of 2.5 μM of the respective unlabeled-hormone (non-specific binding) until the reaction reached equilibrium (12 hrs at 4 °C). Experiments in Fig. 7B and Fig.7D are similar to those in Fig. 7A and Fig. 7C, respectively, but contained a saturating concentration of unlabeled SRC3 NRID fragment (100 nM). 50 μl aliquots from the reactions set for total binding were applied onto a sephadex G-25 column (2 ml) before and at the indicated times, after the addition of corresponding unlabeled hormones (2 μM), and the hormone-bound fraction was collected and counted in a γ-counter. Non specific binding was determined similarly by column fractionation and subtracted from the total binding of corresponding experiment to obtain the specific binding. The specific binding from the fraction collected before the addition of unlabeled hormone was set to 100%. Dissociation curves were generated by plotting the percent bound radioactivity *vs* time. Data were analyzed by non-linear regression with an equation for three phase exponential decay. The time taken for 50% dissociation ($t_{1/2}$) is measured from the curve. Each point in the dissociation curves represents mean ± SD of three independent experiments, and the respective $t_{1/2}$ values are provided in the accompanying table.









Ligand	Ligand IC_{50} (nM)	Fold T_3 activity
T_3	16.2 ± 1.8	1.0
GC-1	11.1 ± 1.1	> 1.5
NH-3	874 ± 23	< 54

FIGURE 8. Evaluations of GC-1 and NH-3 in the dual coregulator interaction assays

A, T_3, GC-1 and NH-3 specified affinity of SRC3 to TRE bound TR•RXR heterodimer. Serially diluted fluorescein SRC3 was incubated with DNA-bound TR•RXR (15 nM) in the absence (Apo) and presence of T_3, GC-1 and NH-3 (3 μM), and the resulting *tr*-FRET values were measured. Binding curves after correction for diffusion-enhanced control values are shown. *B*, Determination of the potency of T_3 and GC-1 to recruit SRC3. Different dilutions of T_3 or GC-1 were incubated with TRE-TR•RXR (15 nM) and SRC3 (125 nM), and the resulting *tr*-FRET was measured. The specific *tr*-FRET values are shown. *C*, NCoR binding in the presence of T_3, GC-1 and NH-3. TRE-bound TR•RXR was incubated with increasing amounts of fluorescein-labeled NCoR in the absence (Apo) and the presence of T_3, GC-1 and NH-3 (3 μM), and resulting binding curves after correction for diffusion-enhanced FRET control are shown. *D*, Determination of ligand potency of T_3, GC-1 and NH-3 in NCoR dissociation assay. TRE-bound TR•RXR heterodimer was incubated with fluorescein-labeled NCoR (24 nM) in the presence of the indicated levels of T_3, GC-1 and NH-3. The specific *tr*-FRET values are shown. Data from Fig. 7A, B, C, or D was analyzed by non-linear regression with an equation

for sigmoidal dose response (variable slope) in GraphPad Prism, and the respective EC_{50} and IC_{50} values listed in the corresponding tables represent the mean ± SD of three different experiments performed in replicates. The Z' factor for all the four formats described here was determined to be in the range of 0.72–.0.84.

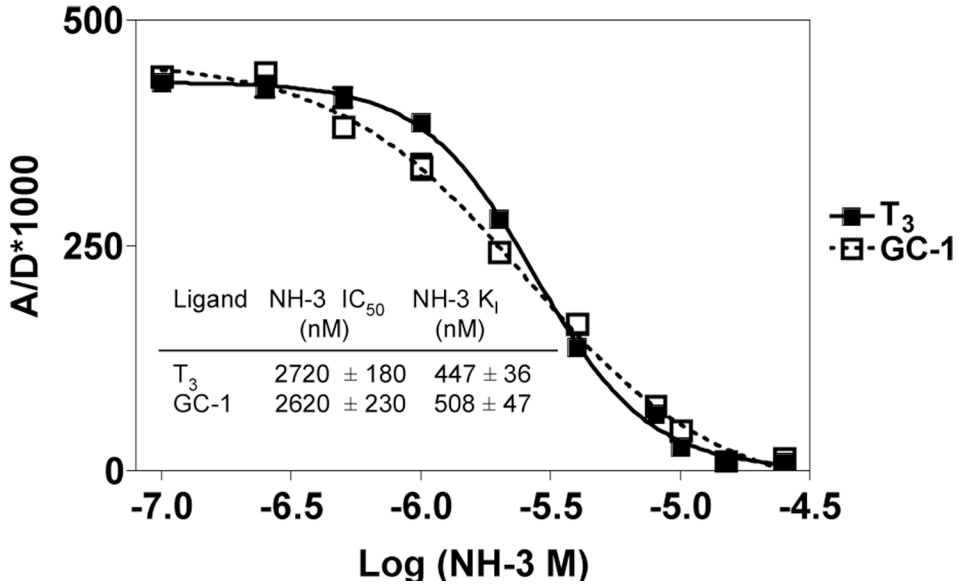


FIGURE 9. NH-3 blocks T3 and GC-1 induced SRC3 recruitment to TRE-bound TR•RXR heterodimer

Increasing amounts of NH-3 were tested for their ability to block fluorescein-labeled SRC3 recruitment to TR•RXR by a submaximal dose of T_3 or GC-1 (30 nM). NH-3 displacement curves that were corrected for diffusion-enhanced FRET were analyzed by non-linear regression with an equation for sigmoidal dose response (variable slope) in GraphPad Prism, and the concentration of NH-3 to displace 50% of T_3- or GC-1-induced SRC3 recruitment was determined (Fig. 9, Table). These IC_{50} values and the EC_{50} values from Fig. 8A (which approximate the apparent affinity of SRC3 to TRE-bound TR•RXR in the presence of T_3 and GC-1) were used with the Cheng-Prusoff equation to estimate the relative affinity of NH-3 for displacement of SRC3 recruited to TR by T_3 or GC-1.

$$\text{Cheng–Prusoff equation;} K_I = IC_{50}/(1+T_0/K_D^*)$$

Where IC_{50} is the concentration of NH-3 to give 50% inhibition of T_3- or GC-1-induced SRC3 recruitment (IC_{50} from Fig. 9 and Table); K_I is dissociation constant of NH-3 that is to be determined; T_0 is the concentration of SRC3 used in the experiment (125 nM); K_D^* is the EC_{50} values obtained from the SRC3 saturation binding curves in the presence of T_3 and GC-1, 24.6 nM and 30.1 nM, respectively (Fig. 8A table). The determined apparent K_I values are shown in Fig. 9 Table. Three independent experiments in replicates were performed, and the NH-3 IC_{50} values in the Fig. 9 Table represent the mean ± SD from three different experiments.