Thyroid hormone (TH) actions are mediated by nuclear receptors (TRs α and β) that bind triiodothyronine (T₃, 3,5,3'-triiodo-l-thyronine) with high affinity, and its precursor thyroxine (T₄, 3,5,3',5'-tetraiodo-l-thyronine) with lower affinity. T₄ contains a bulky 5' iodine group absent from T₃. Both TRs are intracellular receptors. In the context of the ligand binding domain (LBD), we have predicted that TH analogues with 5' substituents should fit poorly into the ligand binding pocket and perhaps behave as antagonists. We therefore examined how T₄ affects TR activity and conformation. We obtained several lines of evidence (ligand dissociation kinetics, migration on hydrophobic interaction columns, and non-denaturing gels) that TR-T₄ complexes adopt a conformation that differs from TR-T₃ complexes in solution. Nonetheless, T₄ behaves as an agonist in vitro (in effects on coregulator and DNA binding) and in cells, when conversion to T₃ does not contribute to agonist activity. We determined x-ray crystal structures of the TRβ LBD in complex with T₃ and T₄ at 2.5-Å and 3.1-Å resolution. Comparison of the structures reveals that TRβ accommodates T₄ through subtle alterations in the loop connecting helices 11 and 12 and amino acid side chains in the pocket, which, together, enlarge a niche that permits helix 12 to pack over the 5' iodine and complete the coactivator binding surface. While T₄ is the major active TH, our results suggest that T₄ could activate nuclear TRs at appropriate concentrations. The ability of TR to adapt to the 5' extension should be considered in TR ligand design.

Thyroid hormone (TH) plays important regulatory roles in metabolism, homeostasis, and development by binding and altering the transcriptional regulatory properties of two related nuclear receptors (NRs), the thyroid hormone receptors (TRs) α and β (1, 2). Most TH produced in the thyroid gland is secreted in the form of thyroxine (T₄; 3,5,3',5'-tetraiodo-l-thyronine) (2, 3). The thyroid gland also produces smaller amounts of triiodothyronine (T₃; 3,5,3'-triiodo-l-thyronine) and reverse T₃ (rT₃; 3',3',5'-triiodo-l-thyronine), and 80% of T₄ is converted to T₃ and rT₃ in peripheral tissues by two selenoprotein deiodinases, which are tissue-specific (4). Current beliefs are that T₃ is the dominant active form of TH; T₄ binds the TRs with an affinity about 20–30 times higher than that of T₃ (5–9), and some studies suggest that T₄ is present at higher concentrations in the nucleus than T₃ (10, 11). Nonetheless, the question of whether T₄ is simply a prohormone or an active TH species is not completely resolved. T₄ exerts rapid nongenomic effects at several loci distinct from TRs (12). Moreover, saturating levels of T₄ activate transcription of TH-responsive genes in cell culture (see for example Ref. 5). Whereas it is possible that at least some of this activity is due to T₃ generated from T₄ in the cell, these results suggest that T₄ may act as a TR agonist. Normal concentrations of plasma-free T₄ are about 4–6-fold higher than those of T₃ (19 pmol/liter of T₃ versus 4.3 pmol/liter T₄) and intracellular T₄ and T₃ levels can differ because of variations in uptake and T₄ to T₃ conversion (3); thus, it is conceivable that intracellular T₄ in some context could occupy a significant fraction of nuclear TRs.

If T₄ does behave as an agonist, then it should bind to TR in a similar way to T₃ and induce conformational changes in the TR similar to those induced by T₃ (13, 14). T₃ interacts with the TR ligand binding domain (LBD), located in the receptor C terminus. The x-ray crystal structure of TRα or TRβ complexed with T₃ reveals that hormone is completely enclosed in a ligand binding pocket within the core of the LBD. It is thought that the enclosure is due to ligand-induced packing of the LBD C-terminal helix 12 (H12) against the LBD; a rearrangement that also disrupts the corepressor binding surface and completes the coactivator binding surface, leading to the recruitment of coregulators and influence on gene expression in vivo (15).

Unlike T₃, T₄ possesses a bulky iodine substituent at the 5'-position of the first thyronine ring. X-ray crystal structures have been determined for TR-LBDs complexed with several different high affinity agonists, including T₃, Dimit (3, 5-di methyl-3'-isopropyl-l-thyronine), and the TRβ-specific ligands GC-1 (3, 5 dimethyl-4-(4-hydroxy-3'-isopropylbenzyl)-phenoxy acetic acid), and KB141 (3,5-dichloro-4-[(4-hydroxy-3-isopropyl phenoxy)phenyl] acetic acid) (16–18). In each of these cases,

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The atomic coordinates and structure factors (codes IYOX and 1ZXZ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† Has proprietary interests in, and serves as a consultant and deputy director to Karo Bio AB, which has commercial interests in this area of research. To whom correspondence should be addressed: Metabolic Research Unit and Diabetes Center, University of California School of Medicine, HSW1210, 513, Parnassus Ave., San Francisco, CA 94122-0540. Tel.: 415-476-3166; Fax: 415-564-5813; E-mail: Jbaxter918@aol.com.

‡ The abbreviations used are: TH, thyroid hormone; LBD, ligand binding domain; TR, thyroid receptor; T₃, triiodothyronine (3,5,3'-triiodo-l-thyronine); T₄, thyroxine (3,5,3',5'-tetraiodo-l-thyronine); R.m.s., root mean square; MIBRT, 3,5-dibromo-4-(3's-isopropyl-4'-hydroxyphenoxy)benzoic acid; DIBRT, 3,5-dibromo-4-(3',5'-disopropyl-4'-hydroxyphenoxy)benzoic acid; HIC, hydrophobic interaction columns; NR, nuclear receptor.

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the agonist contains a 5' hydrogroup that lies close to the inner surface of H12. We therefore predicted that compounds with bulky side groups would perturb the folding of H12 against the body of the LBD and exploit this feature to create TR antagonists based on the notion that 5' extensions would preclude appropriate H12 packing and coactivator binding (19–24). For example, addition of a 5’ isopropyl group, similar in size to an iodine group, to the agonist MIBRT (3,5-dibromo-4-(3’isopropyl-4’-hydroxyphenoxy)benzoic acid) creates the TR antagonist DIBRT (3,5-dibromo-4-(3’,5’-diisopropyl-4’-hydroxyphenoxy)benzoic acid) (22). Thus, it is conceivable that T4, with a 5’ iodine extension, could even behave as an antagonist in some settings. Improved understanding of the way that the TRs adapt to the 5’ iodine group will be therefore important for understanding T4 action and key principles of NR antagonist design.

In this study, we examine T4 interactions with TR, the way that T4 influences TR activity in vitro and in cells in culture and determined the x-ray crystal structure of TR in complex with T4. We find that the TR-T4 complex is less stable than the TR-T3 complex, and that T4-ligated TRs exhibit properties that are similar to unliganded TRs in solution. Nonetheless, T4 behaves as an agonist in cell-free assays and transfected cells. The x-ray structure of the TR LBD-T4 complex reveals that a previously undetected niche in the ligand binding pocket widens, relative to the size of the pocket observed in the TR-B-T3 complex, to accommodate the 5’ iodine, permitting H12 to pack against the LBD surface in the presence of the larger ligand. Thus, the enclosed TR hormone binding pocket accommodates T4 without complete disruption of overall TR-LBD structure. These results suggest that T4 will act largely as a TR agonist if present at high enough concentrations in the nucleus.

MATERIALS AND METHODS

Thyroid Hormone Binding Assay—Thyroid hormone binding and analog competition assays were performed as previously described (9). Kd values were calculated by fitting saturation curves and competition data to the equations of Swillens (25) using the GraphPad Prism program (GraphPad Software, San Diego, CA).

Hydrophobic Interaction Chromatography of TR—TR-LBDs were expressed in E. coli and partially purified on phenyl-Tyopearl, TSK-DEAE, TSK-heparin, and TSK-phenyl columns without TH as described previously (9). For each analog tested, TR was incubated for 1 h with a 5-fold molar excess of the analog relative to the final TR concentration, and where appropriate, radiolabeled T4 in complex with the TR was prepared in reticulocyte lysates, TNT T7 Quick (Promega), and 20 fmols of translated receptor were incubated with 300,000 cpm of [32P]ATP-radiolabeled F2 oligonucleotides and 1 µg of poly(dI-dC) (Amersham Biosciences) in a 20-µl volume (29–31). The binding mixture was loaded with 55 nm HEPES, 50 mM KCl, 1 mM diithiothreitol, 10 µM ZnSO4, 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 separate TR-DNA complexes, the gel was run at 4 °C for 120–180 min at 200 V, using a running buffer containing 45 mM Tris borate (pH 8.0), and 1 mM EDTA.

Reporter Cells—The assay procedure, described previously (22), utilized Chinese hamster ovary cells stably expressing TRα1 and TRβ1 containing a stably integrated reporter gene with a single TRE (DR-4) cloned into the position of the mouse mammary tumor virus promoter hormone response element, driving expression of alkaline phosphatase coding sequences.

Crystalization and Data Collection—The TRβ-LBD was purified for crystallization trials using cobalt affinity and hydrophobic interaction chromatography first without and then with hormone as previously described (18). Crystals of the TRβ complex were obtained by the hanging drop method, with a 10.5 mg/ml protein stock solution and mother liquor consisting of 100 mM sodium cacodylate and 900 mM sodium acetate, pH 7.2. Crystals were cryoprotected by immersion in sequential baths of 100 mM sodium cacodylate and 1.1 µl sodium acetate, pH 7.2, with 3, 8, and 15% glycerol. Crystals were subjected to a final swipe through a bath with 25% glycerol before flash-freezing in liquid nitrogen.

Crystals of the TRα complex were obtained similarly, with a crystallization mother liquor of 100 mM sodium cacodylate and 700 mM sodium acetate, pH 7.4. Use of extremely fresh protein and microseeding with TRα-T3 LBD crystals (< 0.1% of the final crystal) were found to be essential to obtaining diffraction quality crystals. Crystals were then cryoprotected using sequential glycerol baths as for the TRβ complex, but with a mother liquor of 100 mM sodium cacodylate and 900 mM sodium acetate, pH 7.4. Crystals were analyzed at the Advanced Light Source synchrotron facility, beamline 5.02. For the TRβ complex, 15° of data were collected with 1.5° oscillations, for the TRα complex, 75° of data were collected with 0.5° oscillations. Reflections were indexed in DENZO and scaled in SCALEPACK.

Structural Refinement of the TRβ Complex—A molecular replacement solution was found using EPMR, employing the wild-type TRβ/TRiac complex with hormone omitted as a probe. The structure was then subjected to multiple rounds of simulated annealing, followed by positional and B-factor refinement in CNS. Occupancies were refined in CNS for aromatic atoms only. Refinement was then continued using REFMAC of the CCP4 suite. In the final stages of refinement, water molecules were added to the structure both manually and in ARP/WARP. Refinement steps alternated with manual rebuilding steps in Quanta98 and O, guided by wARP. Refinement steps alternated with manual rebuilding steps in Quanta98 and O, guided by wARP. Refinement steps alternated with manual rebuilding steps in Quanta98 and O, guided by wARP.

Structural Refinement of the TRα Complex—The TRα data set was subjected to molecular replacement in EPMR and simulated annealing and positional, B-factor, and occupancy refinement in CNS as for the TRβ complex. In light of the markedly higher resolution of the data for the TRα complex, the refined TRα structure was then least squares fitted in O to the model of the TRβ complex. A composite model consisting of protein from the fitted TRβ complex and ligand from the TRα complex was created and used for subsequent refinement against the TRα complex data.

To guard against model bias, this composite model was subjected to simulated annealing and positional refinement in CNS. However, this treatment raised R resid markedly, as compared with R resid of the composite after positional refinement only. This indicates that the true structure of the TRα complex is close to the TRβ complex model used to create the composite. Subsequent refinement and model rebuilding were carried out as described above for the TRβ complex.

RESULTS

The TR-T3 Complex Is Less Stable Than the TR-T3 Complex and Adopts a Different Conformation in Solution—We first investigated interactions of TRs with T3 and T4 (Fig. 1A). We previously determined that T3 has an affinity for TRs (Kd = 0.06 nM) about 30-fold higher than T4 (Kd = 2 nM) (6). T4 also dissociates from TRs more rapidly than T3 (Fig. 1, B and C). Whereas it took 8.4 and 6.2 h for half of the T3 to dissociate from TRα and TRβ, respectively, TRs form a complex with T4 that is significantly less stable than the TR-T3 complex.

We next examined elution of T4-liganded TRs from TSK-
phenyl hydrophobic interaction columns (HIC), an assay that detects ligand-dependent conformational alterations in TR-LBDs and provides a crude index of hydrophobicity (9). In accordance with previous results (9), liganded TR/H9251-LBDs eluted ahead of unliganded TRs (Fig. 2A). Moreover, TR preparations in complex with several agonists T3, Triac (3,3',5'-triiodothyroacetic acid), 3'-IPT2 (3'-isopropyl-3,5-diido-o-thyronine), and Dimit eluted nearly together, with elution order paralleling relative affinities of ligand for TR (T3 = Triac > IPT2 > Dimit). By contrast, the TRα-T4 complex eluted closer to unliganded TR. Similar results were also obtained with the TRβ-LBD; the TRβ-T4 complex eluted from the column between the TRβ-T3 complex and unliganded TR (Fig. 2B).

It is unlikely that the unusual elution profile of the TR-T4 complex is related to the low affinity of T4 for TRs; the TRα-Dimit complex eluted at a similar position to other TR agonist complexes even though Dimit binds TR/H9251 with an affinity five times lower than T3 (Kd = 9 nM for Dimit versus 2 nM for T3). It is also unlikely that the unusual elution profile is related to rapid T4 release during passage over HIC. Continuous dissociation of T4 would lead to a broad curve and not a discrete symmetric peak as observed here, although the “shoulder” observed with the TRβ-T4 complex may reflect T4 dissociation (Fig. 2B). Nonetheless, we directly examined migration of [125I]T4 prebound to TR α on HIC (Fig. 2C). Radiolabeled T4 migrated at the same position as the TRα-T4 complex, whereas free T4 did not elute from the column in these timescales (not shown), confirming that TR remains bound to T4 as it passes over HIC. Thus, the unique elution profile of the TR-T4 complex reflects an unusual conformation that exposes more hydrophobic surface than TRs in complex with T3 or other analogues.

Modulator Binding Properties of TR-T4 Complexes—We next determined whether T4 behaved as an agonist under cell-free conditions. Fig. 3A shows that T4 and T3 promoted equivalent levels of TR binding to bacterially expressed nuclear receptor interacting regions of the coactivators GRIP1 and TRAP220. T4 and T3 also showed comparable activity in promoting binding of radiolabeled full-length GRIP1 and TRAP220 to bacterially expressed TRβ-LBD (not shown). Finally, both ligands promoted TR release from bacterially expressed preparations of the receptor-interacting region (C terminus) of the corepressor, N-CoR (Fig. 3B). Thus, T4 and T3 behave as agonists in cell-free conditions.

TR agonists promote near complete dissociation of TR dimers, but not RXR-TR heterodimers, from DNA response...
elements (TREs) containing half-sites aligned as inverted palindrome (F2/IP-6) or direct repeats (DR-4) (29, 30). T₄ and T₃ both promoted TRα homodimer release from an F2/IP-6 element and enhanced interactions of TR monomers with the same element (Fig. 4). Both forms of TH only modestly reduced RXR-TRα heterodimer binding in the same conditions. Similar results were also obtained using DR-4, and with TRβ and both TREs (not shown). Nonetheless, TR migration was slower in the presence of T₄ than T₃ (this was most evident for the monomer). Thus, T₄ resembles T₃ in terms of regulation of DNA binding activity, but TR-T₄ and TR-T₃ complexes exhibit different mobilities, underscoring the idea that TR-T₄ complexes adopt a different structure from TR-T₃ complexes in solution (see Fig. 2).

### T₄ Behaves as a TR Agonist in Cell Culture

We next examined the behavior of T₄ in cell culture. T₄ elicited a similar maximal response to T₃ in cultured Chinese hamster ovary cells that were stably transfected with a TH-regulated reporter gene and a vector that expresses either TRα (TRAFα cells, Fig. 5A) or TRβ (TRAFβ cells, Fig. 5B) (22, 32). In both cases T₄ exhibited a potency that is about 10% that of T₃. It is unlikely that T₄ to T₃ conversion accounts for the activity of administered T₄ in these conditions for several reasons. Treatment of TRAF cells with optimal doses of the deiodinase inhibitors iopanoic acid or propylthiouracil (PTU) did not alter the T₄ or T₃ dose response, and we did not detect significant T₄ to T₃ conversion assayed by high performance liquid chromatography of TRAF cell extracts (not shown). Moreover, spiking T₄ preparations with T₃ (to account for 10% of total TH on a molar basis) yielded a leftward shift in EC₅₀ relative to T₃ alone (Fig. 5C), suggesting that T₄ to T₃ conversion of 10% (and probably lower) can be detected in this system. Thus, T₄ behaves as a full agonist in cells in conditions in which conversion to T₃ is unlikely to contribute significantly to agonist activity.

### The X-ray Crystal Structure of the TRβ-T₄ Complex Reveals That the LBD Adapts to the 5'-Iodine of T₄ and Completes the Coactivator Binding Surface

To determine how TRs accommodate T₄ within the ligand binding pocket and perceive the compound as an agonist despite the presence of the bulky 5'-iodine group, we obtained structures of the TRβ-LBD in complex with T₄ and T₃. Data collection and refinement statistics are presented in Table I. A ribbon diagram of the T₄-ligated TRβ structure (pink), superimposed over the T₃-ligated TRβ structure (cyan) is shown in Fig. 6. The complexes are similar in overall fold, both to each other and to previously determined structures of TR LBDs bound to agonists. Moreover, T₄ is completely buried within the core of the LBD, just like other agonists. The TRβ-T₃ structure has a pocket volume of 572 Å³, whereas the TRβ-T₄ structure exhibits a pocket volume of 607 Å³, as determined by GRASP (33). The TRβ-T₃ and TRβ-T₄ structures share the same space group and crystal contacts. Thus, differences in pocket volume between these two structures are probably attributable to the difference in the size of the ligand.

Direct comparisons of the TR-T₄ and TR-T₃ complexes (using “blinking” between aligned LBD structures in Insight II (Accelrys) revealed concerted backbone shifts in four distinct regions. The first comprises H12, the H11-H12 loop and the wall of the ligand binding pocket and lies close to the 5’ iodine group (Fig. 6). The other regions include: N-terminal residues 199–212 (part of the DNA binding domain C-terminal helix (H0), which is included in this structure); H2 residues 234–243, portions of the underlying β-sheet (residues 318–321 and 325–339) and the loop between H2 and H3; and the N terminus of H3 (residues 248–267). Each of these regions of TR usually exhibits poor electron density in crystals, suggesting that they correspond to mobile regions of the protein (16–18). Thus, alterations in these regions are less likely to be significant for understanding ligand discrimination than those of H11-H12 region.

The H11-H12 loop (residues 445–453) is shifted by about 1 Å in the TR-T₃ structure relative to the TR-T₄ structure (Fig. 7), the C terminus of H11 (residues 437–444) is pulled inwards toward the pocket, accentuating a kink also present in the T₃ structure and other agonist-bound TR LBD structures, and the C-terminal end of H12 (residue 460) is pushed outwards in the presence of T₄. Despite these alterations, residues that comprise the coactivator binding surface (on H12 and the upper part of H3 and H5) adopt a structure with backbone positions identical to those seen in the TR-T₃ structure, and side chain positions nearly identical. This is consistent with the finding that T₄ promotes coactivator binding in vitro, and displays agonist activity in vivo. Nonetheless, direct comparisons in Rasmo indicate that H12 (residues 452–460) has closer contacts with the main body of the LBD in the T₄ complex than in the T₃ complex. This suggests that H12 packs less tightly against the LBD. Moreover, the average B-factor for protein atoms was higher for TR-T₄ (54.05) than for TR-T₃ (49.61), and the TR-T₄ structure had lower resolution (3.1 Å) than the TR-T₃ structure (2.5 Å). Thus, the TRβ-T₄ complex exhibits a greater degree of disorder than the TR-T₃ structure.

The conformational alterations that occur within the hormone binding pocket near the T₄ 5’ iodine group are shown in detail in Figs. 8 and 9. Strikingly, the 5’ iodine fits neatly into a small “niche” in the wall of the pocket (Fig. 8A). This feature is analogous to similar niches that accommodate the other iodine groups in the T₄ and T₃ structures and is comprised of two distinct parts: an upper region that consists of residues

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**Fig. 3.** T₄ acts as an agonist in vitro. A, autoradiograms of SDS-polyacrylamide gels, showing the amount of radiolabeled TRβ retained on columns containing bacterially expressed nuclear receptor-interacting fragments of GRIP1 and TRAP220. B, as in A, except that the NR-interacting region of the corepressor N-CoR is used as bait for TR.
from several static helices that line the pocket of the LBD (Ile276 on H3, and Met310, Met313 on H6), and a lower region comprised of His435 on H11, and Phe455 and Phe459 on H12. This precisely positioned niche permits TR to accommodate T4 completely within the enclosed pocket despite the presence of the 5'-iodine group. The niche is also present within the TRβ-T4 structure, but it is smaller (Fig. 8B). Superimposition of the TR-T4 and TR-T3 complexes reveals this region of the pocket expands slightly in the presence of T4 (Fig. 9, compare mesh surface, T4 with solid surface, T3). This expansion is a result of a number of amino acid side chain shifts. The largest involves Met310 (on H6), which lies above the 5'-iodine in the TR-T4 complex. If one considers the receptor in the orientation seen in Fig. 9, a steric clash between Met310 and the 5'-iodine shifts the entire ligand toward the “left” of the receptor relative to the position of ligand in the TRβ-T3 complex (detailed in Table II). This repositioning accentuates further steric clashes between the 5'-iodine group and side chains of two residues (Phe455, Phe459) on H12 itself. In addition, the kink in H11 probably results from a steric clash between the 5'-iodine and His435 (see Table II for distances). These alterations enlarge the niche that accommodates the 5'-iodine substituent and permit H12 to pack against the LBD and complete the coactivator binding surface.

**FIG. 5.** T4 acts as an agonist in mammalian cell cultures. *A*, graph shows alkaline phosphatase activity (the alkaline phosphatase gene is under control of a TH-regulated promoter) as a function of TH concentration in Chinese hamster ovary cells stably transfected with human TRα (TRAF-α cells). *B*, as in A, except that the experiment was performed in CHO cells stably transfected with TRβ (TRAF-β cells). *C*, as in A, except that an additional treatment (T4 preparations spiked with 10% T3) was employed.

**FIG. 6.** Structure of the TRβ-T3 complex. Superimposed ribbon diagrams of TRβ-T3 complex (cyan) and the TRβ-T4 complex (pink) show that the overall fold is nearly identical. The coactivator binding surface H3, 4, 5, and 12 is labeled.

**FIG. 7.** Superimposed views of the backbone trace of the TRβ H11-H12 region derived from TRβ-T4 and TRβ-T3 structures. Backbone displacements in TR-T4 (red) relative to TR-T3 (blue). Helix 12 is highlighted. Displacements near the ligand binding pocket are confined to three areas: (a) C terminus of helix 12, (b) loop connecting helices 11 and 12, (c) disordered loop consisting of residues 250–265 linking nearby helices 2 and 3. Despite these structural alterations, the center of helix 12 shows no backbone displacement and completes the coactivator binding surface.

**TABLE I**

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TR-\textsubscript{T\textsubscript{4}} Structure

**DISCUSSION**

The current studies address \textsubscript{T\textsubscript{4}} interactions with TRs. As developed in the Introduction, \textsubscript{T\textsubscript{4}} is thought to be the major active form of TH and binds TRs with \textasciitilde30-fold the affinity of \textsubscript{T\textsubscript{3}} (6). Free circulating \textsubscript{T\textsubscript{4}} levels are, however, about four to six times that of \textsubscript{T\textsubscript{3}} and intracellular \textsubscript{T\textsubscript{4}}/\textsubscript{T\textsubscript{3}} ratios can vary, so it is conceivable that \textsubscript{T\textsubscript{4}} could occupy a significant fraction of nuclear TRs in some contexts. The lower affinity of \textsubscript{T\textsubscript{4}} for TRs is probably related to the bulky 5\textsuperscript{'}/iodine moiety that, based on our previous structures of TR-LBDs in complex with \textsubscript{T\textsubscript{3}} and related agonists, should not fit readily into the hormone binding pocket (14, 16–18). Indeed, placement of some bulky 5\textsuperscript{'} extensions on high affinity TR agonists can even create antagonists (21). Thus, we asked how \textsubscript{T\textsubscript{4}} interacts with TR, whether it behaves as an agonist or antagonist, and how it can fit into the TR ligand binding pocket.

We initially examined properties of TR-\textsubscript{T\textsubscript{4}} complexes. We confirmed that \textsubscript{T\textsubscript{4}} bound to TR more weakly than \textsubscript{T\textsubscript{3}}, and further demonstrated that \textsubscript{T\textsubscript{4}} dissociates from TRs faster than \textsubscript{T\textsubscript{3}} (Fig. 1). Moreover, the TR complex with \textsubscript{T\textsubscript{4}} is less compact than that with \textsubscript{T\textsubscript{3}}, as suggested by migration of TR-\textsubscript{T\textsubscript{4}} complexes closer to unliganded TRs than to TR-agonist complexes on HIC (Fig. 2, A and B) and in gel shift assays with DNA (Fig. 4). The unusual HIC elution profile is not a reflection of lower affinity of TR for \textsubscript{T\textsubscript{4}}, because TR complexes with Dimit (which lacks a 5\textsuperscript{'}/iodine substituent yet only exhibits 20\% of the affinity of \textsubscript{T\textsubscript{4}} for TR) elute at a similar position to TR-\textsubscript{T\textsubscript{3}} complexes (Fig. 2). It is also unlikely to reflect rapid dissociation of \textsubscript{T\textsubscript{4}} while on the column, because TRs in complex with radiolabeled \textsubscript{T\textsubscript{4}} also elute at a similar position to TRs in complex with unlabeled \textsubscript{T\textsubscript{4}} (Fig. 2C). Despite the less compact nature of the TR-\textsubscript{T\textsubscript{4}} complex, maximally effective doses of \textsubscript{T\textsubscript{4}} were as effective as those of \textsubscript{T\textsubscript{3}} in stimulating association of coactivators (GRIP1 and TRAP220; Fig. 3), release of corepressors (N-CoR and SMRT; Fig. 3) and dissociation of TR homodimers from DNA (Fig. 4). Moreover, maximally effective doses of \textsubscript{T\textsubscript{4}} were as effective as those of \textsubscript{T\textsubscript{3}} in stimulating activity of a TRE-regulated reporter (21). Thus, it is likely that \textsubscript{T\textsubscript{4}} and \textsubscript{T\textsubscript{3}} promote similar overall conformational rearrangements within the TR-LBD in these conditions and that H12 must fold into the active conformation in the presence of \textsubscript{T\textsubscript{4}}.

The crystal structure of the TR\textbeta-\textsubscript{T\textsubscript{4}} complex supports the notion that \textsubscript{T\textsubscript{4}} induces a TR conformation similar to that observed with higher affinity agonists (Fig. 6). TR adopts this fold because, overall, \textsubscript{T\textsubscript{4}} fits tightly into the ligand binding pocket despite the presence of the 5\textsuperscript{'}/iodine group. The pocket accommodates the bulky 5\textsuperscript{'}/iodine via shifts in the position of several amino acid side chains in the pocket relative to their positions in the TR-\textsubscript{T\textsubscript{3}} complex. These changes enlarge a niche that lies close to the 5\textsuperscript{'}/position of the first thyronine ring and closely matches the size and shape of the 5\textsuperscript{'}/iodine (Figs. 8 and 9). The requirements for these structural alterations for fitting of \textsubscript{T\textsubscript{4}} relative to \textsubscript{T\textsubscript{3}} likely explain the reduced affinity of the TRs for \textsubscript{T\textsubscript{4}} relative to \textsubscript{T\textsubscript{3}}. However, the niche permits H12 to fold over the bulky iodine group and complete the coactivator binding surface. Thus, the presence of an adaptable niche in the TR ligand binding pocket allows \textsubscript{T\textsubscript{4}} to behave as agonist, despite the 5\textsuperscript{'}-extension.

**Table II**

*Table of neighbor atoms*

Distances between the 5\textsuperscript{'}/iodine of \textsubscript{T\textsubscript{4}} and neighbor atoms, defined as atoms within 4 Å. Hypothetical distances are measured between the \textsubscript{T\textsubscript{4}} iodine and side chain atoms of the TR\beta-\textsubscript{T\textsubscript{4}} structure, least-squares fitted to the TR\beta-\textsubscript{T\textsubscript{3}} structure in Insight II.

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<th>Hypothetical</th>
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**Fig. 8.** Comparison of the architecture of the TR ligand binding pocket in the presence of different ligands. A and B, close-up view of the ligand binding pocket with side chains of the residues lining the niche, which accommodates the 5\textsuperscript{'}/iodine group. Inner surfaces of the pockets are shown as *solid contours*, whereas surfaces of the ligands are shown as *mesh contours*, whereas surfaces of the ligands are shown as *solid contours*. A, pocket of the TR-\textsubscript{T\textsubscript{4}} complex. B, pocket of the TR-\textsubscript{T\textsubscript{3}} complex. The figures were created in PyMol (pymol.sourceforge.net).

**Fig. 9.** Structural alterations in the ligand binding pocket that permit TR\beta to accommodate \textsubscript{T\textsubscript{4}}. Superimposed images of the side chains and pockets of \textsubscript{T\textsubscript{3}} (pink bonds, mesh surface) and \textsubscript{T\textsubscript{4}} (cyan bonds, solid surface) liganded TRs.
Although H12 adopts the typical active conformation in the presence of T₄, our crystal structures indicate that the H11-H12 loop is more mobile and more loosely packed against the LBD in the presence of T₃ than in the presence of T₄ (Figs. 6 and 7). These features suggest explanations for the observed differences between the behavior of TR-T₄ and TR-T₃ complexes. A tendency of H12 to oscillate between conformations would make the LBD of the TR-T₄ complex in HIC and gel shifts. While T₄ more readily. Loose packing of H12 would also expose more of the hydrophobic interior of the protein, explaining the significant differences between the behavior of TR-T₄ and TR-T₃ complexes.

We previously proposed that TR ligands with bulky 5′ iodine group, but with a 3′ phenyl extension and a 5′ hydrogen (39), by opening up a hydrophobic patch on the inner surfaces of H3 and H11 that is not normally part of the pocket. It will be interesting to determine how TR accommodates ligands with even bulkier 5′ extensions (22–24), or why ligands with particular 5′ extensions, such as the DIBR isopropyl group and the NH₃ phenyl group, act as antagonists.

Our studies do not address the question of whether T₄ is a relevant species of TH in physiological settings. As indicated in the Introduction, many factors regulate relative T₄/T₃ concentrations in the nucleus, making it difficult to gauge the extent to which intracellular T₄ participates in TR binding. Nonetheless, the observation that TR can reorganize to create a niche that precisely accommodates the T₄ 5′ iodine, coupled with the fact that the potency of T₄ is about 10% that of T₃ in cell culture and that free circulating T₄ concentrations are 4–6-fold those of T₃, raises the distinct possibility that T₄ could exhibit significant agonist activity in humans.

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