Triiodothyronine ($T_3$) Differentially Affects $T_3$-Receptor/Retinoic Acid Receptor and $T_3$-Receptor/Retinoid X Receptor Heterodimer Binding to DNA*

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Thyroid hormone receptor (TR) heterodimerizes with retinoic acid receptor (RAR), retinoid X receptor (RXR), and triiodothyronine receptor auxiliary protein (TRAP) on natural and synthetic hormone response elements. Recently we showed that triiodothyronine ($T_3$) decreased TR homodimer, but not TR/TRAP heterodimer, binding to several thyroid hormone response elements (TREs). The effect of ligand on TR/RAR and TR/RXR heterodimer binding to DNA is not known. In this study, we showed that TR formed heterodimers with RAR and RXR on a retinoic acid (RA) response element and two TREs. Surprisingly, $T_3$, but not RA, decreased TR/RXR heterodimer binding to DNA. In contrast, $T_3$, all-trans-RA, or 9-cis-RA did not affect TR/RXR binding to DNA. This finding suggests that TR/RXR heterodimer is a stable receptor complex that remains bound to response elements in the presence of ligand and therefore may be a receptor complex involved in $T_3$-regulated transcription.

There are two major thyroid hormone receptor (TR) isoforms, TRα and TRβ, which are encoded on separate genes (1–3). These TRs bind to thyroid hormone response elements (TREs) in the regulatory regions of target genes, and mediate thyroid hormone ($T_3$)-regulated gene transcription (4, 5). TR binding to TREs is a complex process which has been only partially characterized. TRs bind to TREs, which have considerable degeneracy in their nucleotide sequences as well as variable number, orientation, and spacing of their half-sites (6–9). TRs also can bind to TREs as monomers, dimers, and TRα/TRβ dimers (10–14). Additionally, TRs heterodimerize with nuclear proteins such as TRα receptor auxiliary protein (TRAP), retinoic acid receptor (RAR), and retinoid X receptor (RXR) (14–23). These heterodimers appear to bind better to TREs than TR homodimers, and therefore may play a role in $T_3$-regulated transcription (20–22). In support of this possibility, we recently showed that $T_3$ caused dissociation of TR homodimers, but not TR/TRAP heterodimers, from several TREs (14). This finding suggests that TR/TRAP heterodimer is a stable receptor complex occupying TREs in the presence of $T_3$. In this paper we studied the formation of TR/RAR and TR/RXR heterodimers on a retinoic acid response element (RARE), DR5 (8, 9), and two TREs, DR4 and F2 (8, 9, 14, 24), using an electrophoretic mobility shift assay (EMSA). Since both RARs and RXRs are expressed in a wide variety of tissues (25, 26), it is possible that TR heterodimers containing these proteins may exist in many cells. Additionally, we examined the effect of $T_3$ and retinoic acid (RA) on these heterodimers. Surprisingly, $T_3$, but not RA, caused dissociation of TR/RAR heterodimers from these response elements. However, neither $T_3$, all-trans-RA, or 9-cis-RA decreased TR/RXR heterodimers binding to DNA, suggesting that TR/RXR heterodimers, like TR/TRAP heterodimers, are stable receptor complexes in the presence of $T_3$. As such, they may play an important role in $T_3$-regulated gene transcription.

MATERIALS AND METHODS
Preparation of in Vitro Translated Receptors—Previously described cDNA clones of rat TRα-1 (RAR-1), human TRβ-1 (TRβ-1), mouse retinoid X receptor β (RXRβ or H-2RIBP) in pBS (kindly provided by Dr. K. Ozato, National Institutes of Health), and human RARα in pGEM 1 (kindly provided by Dr. R. M. Evans, Salk Institute, La Jolla, CA) were used in these experiments (3, 27–29). Each cDNA was linearized with the appropriate restriction endonuclease and used as a template for RNA synthesis with T7 RNA polymerase. Human TRβ-1 truncation mutants missing the last 15, 35, and 121 carboxyl-terminal amino acid residues (–15, –35, and –121) were generated from the TRβ-1 cDNA plasmid with BsmI, BgelII, and PoulI restriction endonucleases, respectively, before RNA synthesis. Unlabeled [35S]methionine-labeled receptors then were produced from rabbit reticulocyte lysates according to the manufacturer’s instructions (Bethesda Research Laboratories). Unprogrammed reticulocyte lysate also was incubated under the same conditions. The amount of translated protein was quantitated by trichloroacetic acid precipitation (15, 30) and by SDS-PAGE analysis of [35S]methionine-labeled receptors which showed proteins of the expected molecular weights.

Preparation of Nuclear Extracts—Nuclear extract from the rat pituitary lactotrophic cell line, 231-5, was prepared and stored as previously described (15). These cells do not bind $T_3$, so presumably possess at most a minimal amount of endogenous TRs. Extracts then were dialyzed against 20 mM HEPES (pH 7.3), 5 mM 2-mercaptoethanol, 50 mM NaCl, 2 mM EGTA, 10% (v/v) glycerol, and 0.1 mM phenylmethylsulfonyl fluoride and centrifuged 10,000 g for 15 min. Aliquots were stored frozen at –70°C until used in DNA-binding assays.

Design and Preparation of Labeled DNA Probes—Double-stranded oligonucleotides containing TREs either from the chicken lysozyme gene (F2) [–2324 to –2326 (24)] or that are direct repeats of two half-sites (AGGTCA) separated by a gap of four (DR4) or five (DR5) nucleotides (8, 9) in the context of the F2 flanking sequences were used in our experiments. The oligonucleotides were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase. The labeled probes then were purified on a 5% polyacrylamide gel under nondenaturing con-
**RESULTS**

We used EMSA to examine formation of TR/RAR heterodimers on the synthetic RARE, DR5, which previously was shown to mediate RA-regulated transcription (8, 9). TRα bound as a monomer, and TRβ bound as a homodimer, on this response element (Fig. 1, lanes 1 and 7). Retinoic acid receptor β (RAR) also bound as a homodimer on DR5 (Fig. 1, lane 6, and Fig. 2A, lanes 5–10). When increasing amounts of TRα or TRβ were added to RAR, complexes with intermediate mobility were formed representing TRα/RAR or TRβ/RAR heterodimers (Fig. 1). Formation of these heterodimers was favored over homodimers, and enhanced overall TR binding to DNA. Although not seen on this gel, we previously showed that T3 decreases TRα/RAR heterodimer dissociation from several TRES at an EC50 of 10^{-9} M (14), and the Kd of RA for RAR is <10^{-8} M (31), so that essentially all of the RARs should be ligand-bound at 10^{-6} M RA. Surprisingly, T3, but not RA, decreased TRα/RAR heterodimers from DR5 (Fig. 2A, lanes 7–10). RA had no effect on RAR homodimer binding to DR5 (Fig. 2A, lane 6). Addition of T3 increased slightly the mobility of the TRα monomer band, which is similar to previous observations for TRα binding to several TRES (14).

We next examined TRα, RAR, and TRα/RAR heterodimer binding to the synthetic TRE, DR4 (8, 9). TRα bound mostly as a monomer to DR4, whereas RAR was unable to bind DR4 (Fig. 2B). As in the case of DR5, T3, but not RA, decreased heterodimer binding to DNA. Although not seen on this exposure, a faint homodimer band in TRα-containing samples (Fig. 2B, lane 1) disappeared after T3 addition similar to what we reported for several other natural and synthetic TRES (14). TRβ also formed heterodimers with RAR on DR4 and DR5. Similar effects of T3 and RA on TRα/RAR heterodimer binding to these response elements were observed (data not shown).

We then studied TR/RAR heterodimer formation on the chicken lysozome TRE, F2 (24). TRα, but not TRβ, hetero-
dimerized with RAR on this TRE, suggesting that the two TR isoforms can have different abilities to dimerize with RAR depending on the nucleotide sequence of the TRE (Fig. 3, lane 5, and Fig. 5, lane 3). Additionally, TRα formed relatively less heterodimer with RAR on F2 than on either DR4 or DR5, suggesting that different nucleotide sequences and/or orientation of half-sites in TRES determine the amount of TR/RAR isoforms can have different abilities to dimerize with RAR that one particular member of the TR/RAR heterodimer (TR) prebound to DNA. We observed loss of TR homodimer binding depending on the nucleotide sequence of the TRE (Fig. 5). DNA binding of TR/RAR heterodimers was due to dissociation of TR/RAR heterodimer from DNA, we examined the effect of T3 on TR homodimer and TR/RAR heterodimer prebound to DNA. We observed loss of TR homodimer binding to F2 when T3 was added either simultaneously with receptor and probe or after TR homodimer was prebound to probe (Fig. 4, lanes 2–4). We also observed similar results when we examined the effect of T3 on TR/RAR heterodimer prebound to DR4 (Fig. 4, lanes 6–8). These results suggest that T3 can induce dissociation of TR homodimers and TR/RAR heterodimers from DNA. However, the rates of dissociation from DNA by these two dimers may be different since T3 caused complete dissociation of TR homodimer from F2 by 5 min (data not shown), whereas 14.3% of TR/RAR heterodimer remained bound after 15 min.

We next examined TR/RXRα (TR/RXR) heterodimer formation on DR4, DR5, and F2. XRα (XRα) alone did not bind to these response elements (data not shown); in contrast, TRα/RXR heterodimers bound to these DNA sequences (Fig. 5). XR enhanced TRα binding to DR4 and DR5 more than an equivalent amount of RA (Fig. 5). When nuclear extract was added to the DNA-binding reactions, TRα/TRAP heterodimer as well as TRα homodimer bound to each of the DNA sequences. It was not possible to compare directly the relative amount of TRα/RXR and TRα/TRAP heterodimer formation since we could not quantify the amount of TRAP in 235-1 nuclear extract. The TR/RXR heterodimer band migrated slower than the TR/RAR heterodimer but faster than the TR/TRAP heterodimer bands. Neither 10^{-7} M T3 nor 10^{-6} M RA affected the overall binding of TR/RXR to these response elements, although the bands containing T3-bound TR/XR migrated slightly faster than bands containing TR/RXR in the presence or absence of RA (Fig. 6). We also observed no effect of the putative RXR ligand 9-cis-retinoic acid on TR/RAR heterodimer binding to DNA. In order to determine whether the T3-mediated decrease in DNA binding of TR/RAR heterodimers was due to dissociation of TR/RAR heterodimer from DNA, we examined the effect of T3 on TR homodimer and TR/RAR heterodimer prebound to DNA. We observed loss of TR homodimer binding to F2 when T3 was added either simultaneously with receptor and probe or after TR homodimer was prebound to probe (Fig. 4, lanes 2–4). We also observed similar results when we examined the effect of T3 on TR/RAR heterodimer prebound to DR4 (Fig. 4, lanes 6–8). These results suggest that T3 can induce dissociation of TR homodimers and TR/RAR heterodimers from DNA. However, the rates of dissociation from DNA by these two dimers may be different since T3 caused complete dissociation of TR homodimer from F2 by 5 min (data not shown), whereas 14.3% of TR/RAR heterodimer remained bound after 15 min.

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Fig. 5. TRα heterodimerization with RAR, RXR, and TRAP on F2, DR4, and DR5. Equal amounts of in vitro translated RAR or RXR (based on trichloroacetic acid precipitation and SDS-PAGE of 35S-labeled protein) or 235-1 cell nuclear extract (1 µg), were mixed with TRα (0.5 µl) and labeled probes and analyzed by EMSA. In lanes 5, 11, and 17, the small arrows denote TRα/TRAP heterodimers. NE, nuclear extract.

Fig. 6. Effect of T3 and RA on TRα/RXR heterodimer binding to F2, DR4, and DR5. In vitro translated XRα (4 µl) was mixed with TRα (2 µl) and labeled probe, in the presence or absence of T3 (10^{-7} M) or RA (10^{-6} M), and analyzed by EMSA.
RXR heterodimer binding to DNA (data not shown). These findings suggest that TR/RXR, like TR/TRAP (14), is a stable complex bound to some TREs and RAREs in the presence of T₃.

Last, we examined TR subregions that may be important for homo- and heterodimerization. We made carboxyl-terminal truncation mutants of TRβ in which the last 15, 35, and 121 amino acids were deleted. Deletion of the last 15 and 35 amino acids markedly decreased homo- and heterodimerization, suggesting that the distal carboxyl-terminal region of TRβ may be important for dimerization (Fig. 7, A and B). T₃ did not affect mutant TR homodimer binding to F2 since the truncation mutants do not bind T₃ (Fig. 7A, lanes 2–4 and 6–8). Mutant -121 probably weakly heterodimerized with RAR but not RXR (Fig. 7B, lanes 4 and 8).

**DISCUSSION**

We have demonstrated the formation of TR/RAR heterodimers on two TREs (DR4 and F2) and a RARE (DR5). These heterodimers enhanced overall TR binding to these response elements in the absence of ligand. Interestingly, these heterodimers dissociated from DNA in the presence of T₃ but not RA. In this regard, TR/RAR heterodimers behave similarly to TRα and TRβ homodimers as well as TRα/β dimers which all dissociate from DNA in the presence of T₃ (14). Recently, we observed that TRα and TRβ form dimers with Mf-1 (32, 33), a mutant human TRβ derived from a patient with generalized resistance to thyroid hormone in which glycine is substituted by arginine at amino acid position 345, and only minimally binds T₃. These dimers dissociated from TREs at T₃ concentrations similar to those that cause TR homodimer dissociation from DNA (33). As in the case of TR/RAR heterodimers, these findings would suggest that, for a certain subset of TR heterodimers, only one molecule of T₃ needs to bind to the dimer before it dissociates from DNA. RA did not affect RAR homodimer binding to DR5 or TR/ RAR heterodimer binding to these response elements. The effects of T₃ and RA on homodimer and TR/RAR heterodimer binding to response elements, then, seem to be fundamentally different.

Cotransfection studies have shown that both RARs and TRs mediate ligand-regulated transcription with TREsα as a response element (34, 35). However, unliganded RA blocked RA-regulated transcription with TREsα as response element (34, 35). This repression was unaffected by RA addition, but T₃ addition by itself, or in combination with RA, caused full transcriptional activation. Our results showed that T₃ decreases TR/RAR heterodimers binding to two TREs and a RARE. Inasmuch as unliganded TRs repress basal transcription of T₃α-regulated genes (34, 36), it is possible that unliganded TR/RAR heterodimers also may play a role in basal repression of transcription since they bind some TRES better than TR homodimers (18). We speculate that addition of T₃ would relieve this repression by causing dissociation of TR/ RAR heterodimer. Liganded TR complexes that can bind to DNA, such as TR/RXR or TR/TRAP heterodimers, could then bind to the response element and mediate transcriptional activation. Similar repression of RAREs such as DR5 or TREsα by TR/RAR heterodimers may also occur. In support of these possibilities, recent data suggest there is a transcriptional inhibitor that binds to the carboxyl-terminal region of TRs which is released after T₃ addition (37). Perhaps RAR, when heterodimerized with unliganded TR, could be such an inhibitor.

Recently, RXRα and RXRβ have been shown to heterodimerize with TRs and enhance overall binding to TREsα. We now have observed similar heterodimerization with TR and enhancement of overall TR binding by RXRβ using hormone response elements containing half-sites arranged as direct repeats (DR4 and DR5) and an inverted palindrome (F2). Additionally, when T₃ or RA was added, the heterodimers remained bound to DNA, suggesting that TR/RXR was a stable complex bound to response elements in the presence of ligand. These findings are similar to what we observed for TR/TRAP heterodimers (14). The issue of whether TRAP may be one of the known RXRs or a member of its family remains unresolved. While RXRα and RXRβ display the properties of TRAP by enhancing TR binding to TREs (19–23), it is not clear that all TRAPs are RXRs. Cross-linking experiments suggest that TRAP in 235-1 rat pituitary cells is approximately 10 kD larger than the predicted molecular of the known murine, rat, and human RXRs (19, 20, 23, 29, 38). We recently compared the tissue distribution of TRAP by performing EMSA of different tissue nuclear extracts and found that it had a different tissue distribution than reported.

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Classes of TR heterodimers that bind to response elements. We currently are investigating this possibility. The decrease in TR/RAR heterodimer binding supports this possibility. Additionally, several point mutants were observed, it is possible that TR may contact addition to the previously described "heptad repeat" region, differences in RAR and RXR interactions with truncation...