Thyroid Hormone Receptor Functions as Monomeric Ligand-induced Transcription Factor on Octameric Half-sites

CONSEQUENCES ALSO FOR DIMERIZATION*

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The thyroid hormone (3,5,3’-triiodothyronine) receptor (T3R) belongs to the nuclear receptor superfamily of ligand-inducible transcription factors. T3Rs are known to bind as homodimers and heterodimers with retinoid X receptors (RXRs) to two hexameric half-sites in directly repeated, palindromic, and inverted palindromic orientations. The binding of T3R monomers to individual half-sites was often reported, but no clear ligand-induced transactivation activity has been shown. Here, we analyzed interactions of T3R monomers with individual half-sites of the sequence NNGGTCA. We found that the two nucleotides 5’ of the AGGTCA core half-site strongly influence T3R binding and transactivation activity: octameric half-sites of the consensus sequence (T/C)AAGGTCA were bound by T3Rs with the highest affinity. This suggests T3R functioning also as a monomeric transcription factor like the orphan nuclear receptors NGFI-B and FTZ-F1. Moreover, we observed that the function of T3R-RXR heterodimers on response elements composed of two half-sites in a directly repeated orientation spaced by 4 nucleotides is determined in major parts by the 5’-flanking sequence of the upstream half-site. Consequently, we noted that the affinity of T3R homodimers is influenced by both 5'-flanking sequences. Our findings suggest that the binding of dimerizing receptors like T3R and other nuclear receptors to their cognate response elements is determined not only by the half-site core sequence, orientation, and number of spacing nucleotides, but also by the nucleotide sequence preceding the half-sites.

Thyroid hormone (3,5,3’-triiodothyronine, T3) regulates diverse aspects of cellular development and homeostasis by serving as a biological signal to control cell growth and differentiation in vertebrates (1, 2). Its effects are primarily mediated by the nuclear hormone receptor superfamily (3–5).

T3Rs activate transcription through DNA sequences in the promoter of target genes, referred to as response elements.

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1 The abbreviations used are: T3, 3,5,3’-triiodothyronine (thyroid hormone); T3R, T3 receptor; CAT, chloramphenicol acetyltransferase; TRE, T3 response element; RXR, retinoid X receptor; RA, retinoic acid; RAR, RA receptor; VDR, vitamin D receptor; PPAR, peroxisome proliferator-activated receptor.

These T3 response elements (TREs) contain degenerated pairs of the consensus half-site AGGTCA, which can be arranged as direct repeats, palindromes, or inverted palindromes (6–9). It is generally accepted that TREs consist of hexameric core motifs, whereas it is less widely recognized that also the nucleotides flanking this core motif can have a major influence on the affinity of T3R for its response elements.

T3Rs regulate transcription in two ways. In the presence of T3, the receptor activates transcription from genes bearing TREs. In the absence of T3, the receptor still binds DNA but acts as a transcriptional repressor of genes bearing TREs. The binding of T3Rs to their response elements is enhanced by auxiliary proteins, of which major forms are identical to RXRs (10–15). These receptors are abundantly expressed in adults (16) and are activated by their specific ligand 9-cis-retinoic acid (RA) (17, 18). RXRs can heterodimerize with T3Rs, thus influencing the selection of target sequences and modulating the binding of T3Rs to their cognate response elements (10–15).

The enhanced DNA binding and functional activity of these heterodimers seems to require two distinct hormones, suggesting a direct cross-talk between 9-cis-RA and T3 (19, 20). Recent reports suggested that nuclear signalling by T3 is also transmitted through RXR-independent pathways (9, 21, 22) most likely mediated by homodimers. T3R homodimers exhibit DNA binding specificities that are different only on inverted palindromes from those of the respective T3R-RXR heterodimers (9).

Classified by its interactions with response elements, which are determined essentially by the sequence of the DNA binding domain (P-box), T3R belongs to the same subgroup of the nuclear receptor family as RA receptors (RARs), RXRs, vitamin D receptor (VDR), and peroxisome proliferator-activated receptors (PPARs) (23). All these receptors are known to function only in dimeric complexes, whereas for T3R in vitro binding of monomers to DNA has been reported (24–27). These T3R monomers may function as precursors of dimeric complexes (21).

The studies described here were undertaken to characterize T3R as a transcription factor that is able to function as a monomer. We found that two 5’-flanking nucleotides preceding the AGGTCA core motif are important for efficient monomer binding. We show that on these octamer binding motifs T3Rs can transactivate in a ligand-induced manner. We also show that the selectivity to 5’-flanking sequences influences the binding affinity of dimeric receptor complexes.

MATERIALS AND METHODS

DNA Constructs—For 16 monomer response element with the general core sequence NNGGTCA and 5 dimer response elements with

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FIG. 1. Discrimination of the 5'-flanking sequence by T3R and RXR monomers. The binding of in vitro translated T3R (A) and RXR (B), respectively, on 16 monomeric response motifs of the structure NNAGGTCA is shown; the two varied nucleotides are indicated above the lanes. M indicates the respective monomer complex, whereas NS represents a nonspecific complex.

the core sequences,

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\begin{align*}
\text{DR}_{4,17/7C} & : 5'\text{CAGGGTCATTTCAGGTCC} 3' \\
\text{DR}_{4,14/14A} & : 5'\text{CAGGGTCATTTCAGGTCC} 3' \\
\text{DR}_{4,14/7D} & : 5'\text{CAGGGTCATTTCAGGTCC} 3' \\
\text{DR}_{4,14/14M} & : 5'\text{CAGGGTCATTTCAGGTCC} 3' \\
\text{DR}_{4,14/14S} & : 5'\text{CAGGGTCATTTCAGGTCC} 3'
\end{align*}
\]

for each element a pair of oligonucleotides was synthesized, purified, phosphorylated, and annealed to yield double-stranded DNA fragments. These fragments were fused to the tk promoter to drive the expression of the CAT reporter gene by subcloning into the XbaI site of pBLCAT2 (28). Chicken T3R and human RXR cDNAs were subcloned into pSG5 (Stratagene).

Cell Culture, Transfection, and CAT Assays—The Drosophila cell line, SL-3 (29), was grown at room temperature in Schneider's medium (Life Technologies, Inc.) supplemented with 15% fetal calf serum. For transfection, 5 \times 10^6 cells/well on a six-well plate were grown overnight in Schneider's medium supplemented with 5% charcoal-treated fetal calf serum. Liposomes were formed by incubating 1 \mu g of reporter plasmid, 1 \mu g of the reference plasmid pCH110 (Pharmacia LKB Biotechnology Inc.), and 0.25 \mu g of T3R or RXR expression vectors (or combinations of them) with 15 \mu g of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim) for 15 min at room temperature in a total volume of 20 \mu l. After dilution with 1 ml of Schneider's medium the liposomes were added to the cells and 0.5 ml of Schneider's medium supplemented with 30% charcoal-treated fetal calf serum was added 8 h after transfection. At this time T3R, 9-cis RA, combinations of them (final concentrations 100 nM), or solvent were also added. The cells were harvested 16 h after onset of the treatment and

CAT assays were performed as described (30). The CAT activities were normalized to \beta-galactosidase activity, and induction factors were calculated as the ratio of CAT activity of ligand-stimulated cells to that of mock-induced control. Each condition was analyzed in triplicates and data are shown as mean with standard deviation.

In Vitro Translation of Nuclear Receptors and DNA Binding Assays—Linearized cDNAs for T3R and RXR were used for in vitro transcription as recommended by the supplier (Promega). Five pg of each RNA was mixed with 175 \mu l of rabbit reticulocyte lysate, 100 units of RNasin, and 20 \mu M complete amino acid mixture (all from Promega) in a total volume of 250 \mu l and incubated at 30 °C for 180 min. The response element probes were prepared by double digestion of the respective plasmid DNA with HindIII and BamHI and purified and quantified by gel electrophoresis. All DNA templates were labeled by a fill-in reaction using [\alpha^-32P]dCTP and T7 DNA polymerase (Pharmacia). Five pg of in vitro translated receptors were preincubated for 10 min at room temperature in a total volume of 20 \mu l of binding buffer (10 mM Hepes (pH 7.9), 80 mM KCl, 1 mM diethiothreitol, 0.2 \mu g/\mu l poly(dI-dC) and 5% glycerol). About 1 ng of labeled probe (25,000 cpm) was added and the incubation was continued for 20 min. For Scatchard analysis 5 pg of in vitro translated T3R was incubated with different concentrations of probe ranging from 0.1 to 10 ng as described (26). Protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel (at room temperature) in a total volume of 250 \mu l of binding buffer (10 mM Hepes, 80 mM KCl, 1 mM dithiothreitol, 0.2 \mu g/\mu l poly(dI-dC) and 5% glycerol). After exposing to a film to localize the free probe and protein-probe complexes, the respective bands were excised and counted directly in a scintillation counter. Extracts from SL-3 cells were prepared as described (31).

RESULTS

Orphan receptors that bind to DNA as monomers like, e.g., NGFI-B and FTZ-F1, show a preference for specific sequences.
transactivation by T3R monomers. The transactivation in Drosophila SL-3 cells by T3Ra (black columns) and RXRa (white columns), stimulated with T3 or 9-cis-RA, respectively, on 16 monomeric response motifs of the structure NNAGGTCA is shown. The two varied nucleotides are indicated below the columns. The CAT activities were normalized, compared with the activity of endogenous nuclear receptors and expressed as -fold induction of the heterologous tk promoter. The columns indicate mean values and s represent physiologically relevant affinities not standard deviations.

We wished to test whether T3R monomers show a similar preference. Therefore, we examined the ability of in vitro translated T3Ra to bind double-stranded oligonucleotide probes carrying the AGGTCA half-site preceded by either of 16 different dinucleotide pairs (NNAGGTCA) (Fig. 1A). The response elements containing the 5'-flanking dinucleotide pairs CA, CG, TA, and TG were more efficiently bound than any of the other sequences. The elements with the pairs CC, CT, TC, and TT were bound less efficiently, whereas the remaining elements displayed only very faint or no in vitro binding affinity to T3R monomers. Thus, whereas a purine in the -1 position is preferred, a pyrimidine at the -2 position of the AGGTCA core motif is critical. As a control the same 16-monomer elements were incubated with in vitro translated RXRa (Fig. 1B). We observed only very little RXR monomer-DNA complexes on the elements preceded by the pairs AA, CA, and TA, suggesting that RXRs prefer an A residue in the -1 position of the AGGTCA half-site.

To quantify the binding affinity of T3R monomers, we performed Scatchard binding analysis on the complexes of the electrophoretic mobility shift assay. We plotted the ratio between T3R-bound and free DNA with respect to T3R-bound DNA. T3R monomers have dissociation constants ($K_d$) of 2.9 and 2.1 nM to the probes CG and TG, respectively (Fig. 2). These $K_d$ values represent physiologically relevant affinities not much higher than those of FTZ-F1 or NGFI-B for their cognate DNA elements (32, 33). In contrast, the probes AG and GG gave greater $K_d$ values of 10.3 and 15.3 nM, respectively.

To measure the individual transcriptional activity of T3R and RXR on these response elements, we fused each of the 16 monomer response elements to the tk promoter driving the CAT gene. Since Drosophila cells are devoid of mammalian nuclear receptors by definition, we carried out our studies on T3R- and RXR-mediated transactivation in the Drosophila cell line SL-3. However, we observed basal CAT activity on some of the response elements, even if only an empty expression vector was co-transfected with the reporter vector. We hypothesize that endogenous Drosophila nuclear receptors bind as monomers and activate the reporter gene. Therefore, we subtracted these basal values from the CAT activities measured for T3R or RXR-driven expression. Based on these corrected CAT activities the -fold induction of ligand versus mock stimulation was determined for each response element (Fig. 3). The transactivation data closely parallel in vitro DNA binding affinities of T3R for each of the 16 monomer response elements, suggesting a direct correlation between efficiency of monomer binding and transactivation activity. Accordingly, in SL-3 cells expressing RXRs transiently none of the 16 monomeric response elements provided substantial induction of CAT activity by 9-cis-RA.

We wished to exclude the possibility that the transactivating activity of T3R monomers that we observe in SL-3 cells is due to heterodimerization with an endogenous Drosophila receptor like, e.g. the RXR homologue ultraspiracle (usp) (34), which has been reported to form heterodimers with T3Rs (35). Therefore, binding of in vitro translated T3R to the monomeric half-site that provides strongest interaction, TAGGGTCA, was analyzed in the presence and absence of in vitro translated RXRa, usp, or SL-3 cell extract (Fig. 4). We did not observe any enhanced T3R monomer-DNA complex formation nor could we detect any
change in migration of the T3R-DNA complex brought about by higher order aggregations with auxiliary proteins like RXR, usp, or other Drosophila proteins. Neither RXR nor any Drosophila proteins bound to the TG element. Interestingly, in the presence of T3 the mobility of the T3R-DNA complex was significantly increased, suggesting a ligand-induced conformational change of T3R monomers. Taken together, these data demonstrate that T3R monomers are able to bind, and therefore also to transactivate, in the absence of RXR or Drosophila "accessory factors."

Having observed that T3Rs, and even RXRs, show preferences for selected 5'-flanking sequences when they bind to DNA as monomers (Fig. 1), we next investigated whether this might be also true for T3R homodimers and T3R-RXR heterodimers.
To this end, we have chosen the TRE of Moloney murine leukemia virus (36), which constitutes a direct repeat of tws (G/AGGTCA/A/C) half-sites spaced by four nucleotides (DR4). Its 5'-flanking nucleotide pairs are CA and TC, respectively, which we have shown to direct some T3R monomer binding affinity. On CAAGGTCA we also saw faint RXR monomer binding (Fig. 1). To gain more T3R and RXR specificity, we designed mutant Moloney murine leukemia virus TREs having the four possible combinations of TG and AA as 5'-flanking nucleotide pairs (see "Materials and Methods"). Analyzing the binding of T3R homodimers and T3R-RXR heterodimers to the five different DR4s (Fig. 5A), we observed the highest affinity of T3R homodimers to DR4CATG, marked binding to the natural TRE (DR4CATG) and to DR4TAAA, but only low affinity to DR4AAATG and to DR4AA/AA. In contrast, we observed highest affinity of T3R-RXR heterodimers to DR4AAATG, marked affinity to the Moloney murine leukemia virus TRE (DR4CATG), and lower affinities to the other DR4s. This analysis shows that although response elements, like DR4AAATG, have a high affinity to heterodimers and only low affinity to homodimers, other response elements like DR4TAAA exhibit inverse specificity. We also performed CAT assays in SL-3 cells to monitor functional activity of ligand-induced T3R homodimers and T3R-RXR heterodimers on all 5 DR4s (Fig. 5B). The data supported the in vitro DNA binding analysis: highest -fold stimulation of T3R homodimers by T3 was observed on DR4TAAA, whereas T3- and 9-cis-RA-induced T3R-RXR heterodimers were found to be most active on the natural TRE and on DR4AAATG.

DISCUSSION

Biological responses to T3 are mediated by its intracellular receptor proteins. One of the advances in understanding the molecular mechanism by which nuclear receptors operate was provided by establishing the hexameric consensus half-site AGGTCA as the core binding motif of response elements for T3Rs, RXRs, RARs, and PPARs (7, 8). However, it has not been generally noted that 5'-flanking nucleotides can determine the affinity of T3Rs for their TRE. Here, we demonstrated that the octameric half-site TGAGGTCA is bound most strongly by T3R monomers. Comparing original and mutated half-sites of the rat growth hormone gene promoter, Kim et al. (37) reported that the TRE half-site sequence CTGGAGGTAACG is bound by T3Rs with highest affinity. The sequence fits the consensus motif (T/C/CA/GAGGTCA) we have identified. Moreover, we observed on this response element up to 10-fold induction of reporter gene activity in response to T3 stimulation. We showed that this transactivational activity is mediated by T3R monomers, apparently acting independent of any auxiliary protein. Our finding suggests T3R as the first ligand-inducible monomeric transactivation factor known. Its half-site specificity is modulated in major parts by the nucleotides preceding the AGGTCA half-site motif reminiscent of some orphan nuclear receptors like NGFI-B, FTZ-F1, and Rev-ErbA. Most classical nuclear receptors do not bind to DNA sequences that contain a single half-site, because the interaction of one monomer with one half-site does not provide sufficient free energy to stabilize the protein-DNA complex. In contrast, some orphan receptors are known to bind to their response elements exclusively as monomers. In this sense, T3Rs are an exception, as they bind to their response elements both as monomers and as dimers. We recently identified an orphan nuclear receptor, called RZR (38), which binds to certain response elements both as monomer and dimer.3 Classified by this property, T3R and RZR may define a new subgroup of the nuclear receptor superfamily.

The finding that T3R monomers as well as T3R homodimers bind efficiently to DNA suggest a two-step mechanism for the recognition of response elements. Accordingly, T3R monomers screen the DNA for appropriate octameric half-sites. After having identified such a site a second receptor molecule, either T3R or RXR, binds in appropriate vicinity to facilitate dimerization. In this sense, T3R monomers may be interpreted as active precursors of dimeric complexes.

Following the 3-4-5 rule (8), all DR4s, including the five we studied, should be equally good TREs. However, we observed that the affinity of homo- and heterodimeric T3R complexes to appropriate response elements composed of two hexameric consensus half-sites was modulated by 5'-flanking nucleotide pairs preceding the two half-sites. Highest affinity of T3R-RXR heterodimers was observed only, if the 5'-flanking sequence of the upstream half-site was optimal for RXRs, suggesting that in the heterodimeric complex RXR binds to the upstream half-site motif. This interpretation is supported by two recent reports (39, 40) that showed that in heterodimeric complexes with T3Rs, RXR always binds to the upstream half-site Moreover, our observations indicate that the binding of T3R homodimers is even preferred over heterodimer binding, if both half-sites are preceded by nucleotide pairs, like TC, that are favored by T3R monomers. The discrimination of 5'-flanking sequences as described here suggests a mechanism to distinguish T3R-determining pathways mediated by either T3R homodimers or T3R-RXR heterodimers. For VDRs we recently reported a different mechanism (31): VDR homodimers and VDR-RXR heterodimers discriminate their respective response elements by the number of spacing nucleotides between directly repeated half-site motifs. Taken together, not only the number of spacing nucleotides, but also the sequence of the spacer is important for response element recognition. Future investigations following the indications for the existence of RAR homodimers in vivo (9, 20, 41) will show whether the homo- and heterodimeric pathways of retinoid action are also defined at the level of one of these two mechanisms.

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