Single thyroid hormone receptor monomers are competent for co-activator-mediated transactivation

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Thyroid hormone receptor (T₃R) belongs to the superfamily of nuclear receptors containing highly related transcription factors that transform an incoming signal in the form of a lipophilic hormone into an activation of the basal transcriptional machinery. Like many other nuclear receptors, T₃R acts preferentially as a heterodimer with retinoid X receptor (RXR) but it also has the unique property of binding as a monomer to DNA. This study demonstrates that T₃R monomers bind preferentially to AGGTCA binding motifs and are able to co-exist with T₃R–RXR heterodimers in the presence of limiting amounts of RXR. DNA-bound T₃R monomers efficiently contact all three members of the p160 co-activator family, which in turn boost T₃R monomer-mediated transactivation. In solution T₃R monomers take only

one agonistic conformation (c2_{L_{PD}}), whereas bound to DNA they also stabilize, like T₃R–RXR heterodimers, a second agonistic conformation (c1_{L_{PD}}). Conformation c2_{L_{PD}} seems to be of lower ligand sensitivity (10 nM), whereas, both in T₃R–RXR heterodimers and in DNA-bound T₃R monomers, c1_{L_{PD}} is already activated at a ligand concentration of 1 nM. Taken together, these results suggest that single T₃R monomers are fully competent for ligand-induced transactivation and that their role in gene regulation by thyroid hormone might have been underestimated.

Key words: co-activator protein, gene regulation, receptor conformations.

INTRODUCTION

Nuclear receptors form the largest known family of transcription factors and have a crucial role in nearly all aspects of vertebrate development and adult physiology by transducing the effects of small, lipophilic hormones into transcriptional responses [1]. The family is defined by two domains: (1) the central, highly conserved, DNA-binding domain (DBD) of approx. 66 amino acids, and (2) the C-terminal, structurally conserved, ligand-binding domain (LBD) of approx. 250 amino acids [2,3]. The LBD serves as a molecular switch that interacts in its agonistic conformation with co-activator proteins such as p160 family members TIF2, SRC-1 and RAC3 or TRAP220/DRIP205, and thus activates target gene transcription [4]. Classical nuclear receptors, which are the receptors for the steroids oestrogen, progesterone, testosterone, cortisol and aldosterone and also those for 1 α ,25-dihydroxyvitamin D₃ (vitamin D receptor or NR1H1), for the thyroid hormone 3,3',5-tri-iodothyronine (T₃) [thyroid hormone receptor (T₃R) or NR1A1-2] and for the vitamin A derivative all-*trans* retinoic acid [retinoic acid receptor (RAR) or NR1B1-3], act as ligand-inducible transcription factors [5]. However, the vast majority of the superfamily members were cloned before their specific ligands were known; these nuclear receptors are referred to as orphans [6]. For some of these orphan nuclear receptors interesting ligands have been identified, such as 9-*cis*-retinoic acid for the retinoid X receptor (RXR or NR2B1-3) [7] and oxysterols, bile acids, fatty acids, pregnanes and androstanol for receptors named LXR, FXR, PPAR, PXR and CAR [8]. However, some orphan nuclear receptors show constitutive activity and use ligand-independent pathways to mediate their function.

'True' orphans bind to DNA as homodimers, such as the five classical steroid receptors, or monomers, whereas the orphans with ligands all seem to form heterodimers with RXR like the three other classical receptors, vitamin D receptor, T₃R and RAR [9]. This seems to suggest that monomeric nuclear receptors cannot act as ligand-inducible transcription factors. Interestingly, of all the dimer-forming nuclear receptors only T₃R is known to have sufficient DNA binding affinity to bind a monomer too [2]. Hormone-responsive genes are defined through the presence and specific recognition of nuclear receptor binding sites, referred to as response elements (REs), in their promoter regions [2,5]. Most REs are formed by two hexameric binding motifs of the consensus sequence RGKTCA [9], which are mainly in a 'direct repeat' (DR) arrangement with a critical number of spacing nucleotides [10]. Nuclear receptors such as NGFI-B and RZR/ROR, which bind to DNA preferentially as monomers, have a region within the less conserved C-terminal extension of the DBD, referred to as the GRIP box [11], which provides receptor-specific interfaces for an interaction with 5'-flanking nucleotides of the hexameric motif. The GRIP box also seems to have a role for T₃R, because the DNA binding affinity of T₃R monomers has been shown to be strongly modulated by two nucleotides that directly flank the hexameric binding motif [12]. The crystal structure of DNA-bound T₃R–RXR heterodimers [13] confirmed the contact of amino acids of the C-terminal extension of the T₃R DBD with two 5'-flanking nucleotides.

In the present study the functional profile of T₃R monomers was compared with that of T₃R–RXR heterodimers. T₃R monomers were shown to co-exist with T₃R–RXR heterodimers, to contact co-activator proteins TIF2, SRC-1 and RAC3 efficiently and to stabilize the agonist conformations of the LBD

Abbreviations used: DBD, DNA-binding domain; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate; DR, direct repeat; FBS, fetal bovine serum; GST, glutathione S-transferase; LBD, ligand-binding domain; RAR, retinoic acid receptor; RE, response element; RXR, retinoid X receptor; T₃, 3,3',5-tri-iodothyronine; T₃R, thyroid hormone receptor.

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Figure 1 Complex formation of T₃R monomers and T₃R–RXR heterodimers on DNA

Gel shift experiments were performed with T₃R protein, both alone (monomer) and in combination with RXR protein translated *in vitro* (heterodimer) on ³²P-labelled REs formed from either one or two copies of an AGGTCA consensus motif (filled columns in bottom panel, AGGTCA; open bars, AGTTCA). Protein–DNA complexes were separated from free probes on 8% (w/v) non-denaturing polyacrylamide gels. Representative experiments are shown. The amounts of specific protein complexes were quantified on a bioimager in relation to their respective free probes. The relative amounts of complex formation of T₃R monomers and T₃R–RXR heterodimers on their respective RE types were calculated. Columns represent means from triplicate determinations; bars indicate S.D. Arrows represent the hexameric consensus motif and the letters below the arrows indicate the nucleotides at the third position. The domain structures of T₃R monomers and T₃R–RXR heterodimers are depicted schematically.

as sensitively as T₃R–RXR heterodimers. This suggests that T₃R is also competent as a monomer for ligand-induced transactivation.

MATERIALS AND METHODS

DNA constructs

Full-length cDNA species of chicken T₃Rα [14], human RXRα [7] and human TIF2 [15] were subcloned into the simian virus 40 promoter-driven pSG5 expression vector (Stratagene, Heidelberg, Germany), whereas full-length cDNA species of human SRC-1 [16] and human RAC3 [17] were subcloned into the cytomegalovirus promoter-driven CMX expression vector. The cDNA of the DBD of the yeast transcription factor Gal4 (residues 1–147) was fused with the cDNA of the LBD of human T₃Rβ1 (residues 165–461). The luciferase reporter gene was

Figure 2 T₃R monomers are stable when RXR concentration is limiting

Gel shift experiments were performed with T₃R translated *in vitro* and different amounts of RXR translated *in vitro* in the presence of two ³²P-labelled REs in the same experiment (a monomeric RE with one AGGTCA motif and a DR4-type RE with two AGTTCA motifs). Protein–DNA complexes were separated from free probes on 13% (w/v) non-denaturing polyacrylamide gels. A representative experiment is shown. The amounts of specific protein complexes were quantified on a bioimager in relation to their respective free probes. The amounts of complex formation of T₃R monomers (filled columns) and T₃R–RXR heterodimers (open columns) at increasing T₃R-to-RXR ratios were calculated in relation to their respective maximal values. Columns represent means from triplicate determinations; bars indicate S.D. Arrows represent the hexameric consensus motif and the letters below the arrows indicate the nucleotides at the third position. The domain structures of T₃R monomers and T₃R–RXR heterodimers are depicted schematically.

driven by three copies of a Gal4-binding site or one copy of the octameric sequence TGAGGTCA, each fused to the *tk* promoter [18]. The nuclear receptor interaction region of human TIF2 (spanning residues 646–926) [15], human SRC-1 (spanning residues 596–790) [16] and human RAC3 (spanning residues 673–1106) [17] were subcloned into the pGEX glutathione S-transferase (GST) fusion vector (Amersham-Pharmacia, Freiburg, Germany).

Gel shift and supershift assays

Linearized DNA species of the pSG5-based constructs of T₃R and RXR were transcribed with T₇ RNA polymerase and the respective RNA species were translated with rabbit reticulocyte lysate as recommended by the supplier (Promega, Mannheim, Germany). T₃R protein (2.5 μl) translated *in vitro*, both alone (monomer) and together with 2.5 μl of RXR protein translated

Figure 3 T₃R monomers and T₃R–RXR heterodimers form complexes with co-activator proteins

Gel shift experiments were performed with T₃R translated *in vitro*, both alone (monomer) and in combination with RXR translated *in vitro* (heterodimer), which had been preincubated with bacterially expressed GST–TIF2_{646–926} (black columns), GST–SRC-1_{596–790} (hatched columns) or GST–RAC3_{673–1106} (white columns) at saturating T₃ concentration (1 μ M) or with solvent as a control, on a monomeric RE with one AGGTCA motif and on a DR4-type RE with two AGGTCA motifs respectively (both ³²P-labelled; see Figure 2). Protein–DNA complexes were separated from free probes on 8% (w/v) non-denaturing polyacrylamide gels. Representative experiments are shown. The amounts of specific protein complexes were quantified on a bioimager in relation to their respective free probes. The relative amounts of supershifted T₃R–co-activator or T₃R–RXR–co-activator complexes were calculated in relation to their respective controls (gel shift without co-activator; left lane). Columns represent means from triplicate determinations; bars indicate S.D. Arrows represent the hexameric consensus motif and the letters below the arrows indicate the nucleotides at the third position. The domain structures of T₃R monomers and T₃R–RXR heterodimers are depicted schematically. Abbreviation: CoA, co-activator protein.

in vitro (heterodimer), was incubated with saturating concentrations of T₃ (Sigma, Deisenhofen, Germany) or with solvent (DMSO) for 15 min at 20 °C in a total volume of 20 μ l of binding buffer [10 mM Hepes (pH 7.9)/1 mM dithiothreitol/0.2 μ g/ μ l poly(dI-dC)/5% (v/v) glycerol]. The buffer had been adjusted to 150 mM univalent cations by the addition of KCl. For supershift assays, approx. 3 μ g of bacterially expressed GST–TIF2_{646–926}, GST–SRC-1_{596–790} or GST–RAC3_{673–1106} fusion proteins were included in the incubation. In both cases, approx. 1 ng of indicated ³²P-labelled monomeric or dimeric REs (50 000 c.p.m.) was added to the protein/ligand mixture and incubation was continued for 20 min. Protein–DNA complexes were resolved through 8% or 13% non-denaturing polyacrylamide gels in 0.5 $\times$ TBE [45 mM Tris/45 mM boric acid/1 mM EDTA (pH 8.3)]. Gels were then dried and exposed to a Fuji MP2040S imager screen. The ratio of free probe to protein–probe complexes

was quantified on an FLA2000 bioimager (Fuji, Tokyo, Japan) with Image Gauge software.

Transfection and reporter gene assays

For mammalian one-hybrid assays, HeLa human cervix carcinoma cells were seeded into six-well plates (10⁵ cells/ml) and grown overnight in Phenol-Red-free DMEM supplemented with 5% (v/v) charcoal-treated fetal bovine serum (FBS). Liposomes were formed by incubating 1 μ g of a Gal4-binding-site-driven luciferase reporter gene construct, 1 μ g of an expression vector for the Gal4_{DBD}–T₃R_{LBD} fusion protein (or, as a control, a vector for Gal4_{DBD}), 1 μ g of expression vectors for TIF2, SRC-1 or RAC3 (as indicated) and 1 μ g of the reference plasmid pCH110 (Amersham-Pharmacia) with 15 μ g of *N*-[1-(2,3-dioleoyloxy)-propyl]-*N,N,N*-trimethylammonium methylsulphate (DOTAP;

Roth, Karlsruhe, Germany) for 15 min at 20 °C in a total volume of 100 μ l. After dilution with 900 μ l of Phenol-Red-free DMEM, liposomes were added to the cells. Transfections for regular reporter gene assays were performed very similarly in Cos-7 simian-virus-40-transformed African Green monkey kidney cells that had been grown overnight in Phenol-Red-free DMEM supplemented with 5 % (v/v) FBS. In this case, liposomes were formed by incubating 1 μ g of an octameric-sequence-driven luciferase reporter gene construct, 1 μ g of an expression vector for T₃R α , 1 μ g of expression vectors for TIF2, SRC-1 or RAC3 (as indicated) and 1 μ g of the reference plasmid pCH110 with 15 μ g of DOTAP. In both cases, Phenol-Red-free DMEM supplemented with 15 % (v/v) charcoal-treated FBS (500 μ l) was added 4 h after transfection. At this time, T₃ or DMSO (0.1 %) was also added. Cells were lysed 16 h after the onset of stimulation by using reporter gene lysis buffer (Roche Diagnostics, Mannheim, Germany) and a constant-light-signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra Packard, Dreieich, Germany). Luciferase activities were normalized with respect to β -galactosidase activity and induction factors were calculated as the ratio of luciferase activity of the ligand-stimulated cells to that of the solvent control.

Limited-protease-digestion assay

³⁵S-labelled T₃R protein translated *in vitro* (2.5 μ l), alone or together with 2.5 μ l of RXR protein translated *in vitro* and 1 ng of unlabelled monomeric or dimeric RE (as indicated), was incubated with ligand for 15 min at 20 °C in 20 μ l of binding buffer. The buffer was adjusted to 150 mM by the addition of KCl. Trypsin (Promega; final concentration 8.3 ng/ μ l) was then added and the mixtures were incubated for a further 15 min at 20 °C. Digestion reactions were stopped by adding 25 μ l of protein gel loading buffer [0.25 M Tris/HCl (pH 6.8)/20 % (v/v) glycerol/5 % (v/v) 2-mercaptoethanol/2 % (w/v) SDS/0.025 % Bromophenol Blue]. Samples were denatured at 85 °C for 3 min and subjected to SDS/PAGE [15 % (w/v) gel]. Gels were dried and exposed to a Fuji MP2040S imager screen; individual protease-sensitive T₃R fragments were quantified on a Fuji FLA2000 bioimager.

RESULTS

We analysed, with the use of gel shift assays, complex formation of T₃R monomers on monomeric REs consisting of a single copy of a hexameric AGGTCA or AGTTCA motif and also that of T₃R–RXR heterodimers on REs consisting of two direct repeats of AGGTCA or AGTTCA motifs with four spacing nucleotides (DR4-type REs) (Figure 1). The amount of T₃R–RXR complex formation on dimeric REs was found not to depend on the hexameric motif sequence, whereas T₃R monomers formed complexes only on monomeric REs carrying an AGGTCA motif. In all cases, complex formation was not enhanced by the addition of T₃ (results not shown), which is consistent with a previous report [19].

To determine whether T₃R monomers can exist in parallel with T₃R–RXR heterodimers, gel shift assays were performed with an equimolar mixture of monomeric and dimeric RE and T₃R-to-RXR protein ratios that increased from 1:1 to 20:1 (Figure 2). The DR4-type element with AGTTCA motifs was chosen because no T₃R monomer binding is possible to this dimeric RE (see Figure 1). At an equimolar T₃R-to-RXR ratio hardly any T₃R monomer binding could be observed. However, at a T₃R-to-RXR ratio of 10 or more, we found maximal T₃R monomer complex formation on the monomeric RE, whereas the amount

Figure 4 Functional activity of the T₃R–LBD

(A) Luciferase reporter gene assays were performed with extracts from HeLa cells that had been transiently transfected with a reporter gene construct driven by three copies of the Gal4-binding site, an expression vector for a Gal4_{DBD}–T₃R_{LBD} fusion protein (or, as a control, a vector for Gal4_{DBD}) and an expression vector for full-length TIF2, SRC-1 or RAC3, as indicated. (B) Luciferase reporter gene assays were performed with extracts from Cos-7 cells that were transiently transfected with a reporter gene construct driven by one copy of a monomeric nuclear-receptor-binding site TGAGGTCA and expression vectors for full-length T₃R, TIF2, SRC-1 and RAC3, as indicated. In both cases, cells were treated for 16 h with saturating concentrations of T₃ (1 μ M; filled columns) or solvent [0.1 % (v/v) DMSO; open columns]. Induction of β -galactosidase-normalized luciferase activity was calculated in comparison with solvent control. Columns represent means from triplicate determinations; bars indicate S.D.

of T₃R–RXR heterodimer complex formation on the dimeric RE decreased in parallel. This indicates that T₃R monomers and T₃R–RXR heterodimers co-existed at limiting RXR concentrations.

The interaction of T₃R monomers and T₃R–RXR heterodimers with co-activator proteins was analysed by supershift assays (Figure 3). All three members of the p160 co-activator family (TIF2, SRC-1 and RAC3) were found to interact in a ligand-dependent fashion with T₃R monomers as well as with T₃R–RXR heterodimers. In the presence of saturating T₃ concentrations (1 μ M), co-activators were able to shift all DNA-bound T₃R monomers and T₃R–RXR heterodimers into more slowly migrating higher-molecular-mass complexes (supershifts). For T₃R–RXR heterodimers and all three co-activator proteins, these supershifts represent completely DNA-complexed T₃R proteins. In contrast, TIF2 bound 90 % of all DNA-complexed T₃R monomers, whereas SRC-1 and RAC3 each super-shifted

Figure 5 DNA-modulated and RXR-modulated stabilization of T₃R conformations

Limited-protease-digestion assays were performed by preincubating ³⁵S-labelled T₃R translated *in vitro*, both alone (**A**, **B**) and together with unlabelled RXR (**C**) in the presence of saturating concentrations of T₃ (1 μ M; filled columns) or solvent (open columns) as a control. T₃R was kept in solution (**A**) or protein–DNA complexes were formed on an unlabelled monomeric RE carrying one AGGTCA motif (**B**) or an unlabelled DR4-type RE containing two AGGTCA motifs (**C**) (see Figure 2). After digestion with trypsin, samples were subjected to SDS/PAGE [15% (w/v) gel]. Representative experiments are shown. The amounts of ligand-stabilized T₃R in conformations 1 (c1_{LDP}) and 2 (c2_{LDP}) were quantified on a bioimager in relation to T₃R input. Columns represent means from triplicate determinations; bars indicate S.D. The domain structures of full-length T₃R and its protease-resistant fragments are depicted schematically.

only approximately 40% of the T₃R monomer input. The apparent quantitative differences between the co-activator super-shifts are most probably related to differential trapping of probe input by the GST beads.

Functional activity of the monomeric LBD of T₃R was tested in mammalian one-hybrid assays in HeLa cells that had been transiently transfected with an expression vector for a fusion protein consisting of the Gal4 DBD and the T₃R LBD and a luciferase gene driven by Gal4-binding sites (Figure 4A). Saturating T₃ concentrations (1 μ M) induced Gal4_{DBD}–T₃R_{LB} fusion-protein-driven reporter gene activity 9.6-fold, whereas the Gal4_{DBD} control protein mediated only a very low, ligand-independent gene activity. In relation to the same solvent control, overexpression of TIF2, SRC-1 and RAC3 resulted in 26.4-fold, 40.9-fold and 28.6-fold activations respectively; that is, 2.8-fold, 4.3-fold and 3.1-fold superactivations. Functional activity of full-length T₃R monomers was analysed in Cos-7 cells that had been transiently transfected with an expression vector for T₃R α and a luciferase gene driven by one copy of a monomeric binding site (Figure 4B). In the absence of ligand, recruitment of co-repressors by T₃R halved the basal activity of the reporter gene construct. For reference, stimulation with 1 μ M T₃ induced reporter gene activity 3.4-fold. In relation to the same solvent control, overexpression of TIF2, SRC-1 and RAC3 resulted in 6.3-fold, 6.7-fold and 6.0-fold activations respectively; that is,

1.9-fold, 2.0-fold and 1.8-fold superactivations. Under the experimental conditions chosen here the overexpression of co-activator proteins did not significantly influence the simian virus 40 promoter-driven expression of T₃R [20].

Stabilization of T₃R conformations was investigated by limited-protease-digestion assays (Figure 5). Digestion of T₃-bound, ³⁵S-labelled T₃R translated *in vitro* provided two digestion products, c1_{LDP} and c2_{LDP}, with apparent molecular masses of 40 and 26 kDa respectively. As depicted schematically in Figure 5, these T₃R fragments contain the DBD and the LBD of the receptor (c1_{LDP}) or only the LBD (c2_{LDP}) and seem to represent two agonistic conformations. For T₃R monomers in solution, approx. 50% of the receptor molecules were stabilized in conformation c2_{LDP} by a saturating T₃ concentration, whereas in the absence of ligand only 2% of receptor input was stabilized in this conformation (Figure 5A). However, under these conditions only a very small proportion of all T₃R molecules (approx. 5%) was stabilized in conformation c1_{LDP}. When T₃R monomers were bound to DNA, the specific stabilization of c2_{LDP} remained identical but the quantity of T₃R molecules that were ligand-dependently stabilized in the c1_{LDP} conformation increased to 15% (Figure 5B). The pattern reversed when T₃R formed a heterodimer with RXR on a dimeric RE; under these conditions approx. 35% of the T₃R input was stabilized by T₃ in c1_{LDP} and only 15% in c2_{LDP} (Figure 5C).

Figure 6 Ligand sensitivity of T₃R conformations

Limited-protease-digestion assays were performed by preincubating ³⁵S-labelled T₃R translated *in vitro*, both alone (**A**, **B**) and together with unlabelled RXR (**C**) in the presence of graded concentrations of T₃. T₃R was kept in solution (**A**) or protein–DNA complexes were formed on an unlabelled monomeric RE carrying one AGGTCA motif (**B**) or an unlabelled DR4-type RE containing two AGGTCA motifs (**C**) (see Figure 2). After digestion with trypsin, samples were subjected to SDS/PAGE [15% (w/v) gel]. The amounts of ligand-stabilized T₃R in conformations 1 (c₁L_{DP}, ●) and 2 (c₂L_{DP}, ○) were quantified on a bioimager in relation to T₃R input. Results are means ± S.D. for triplicate determinations. EC₅₀ values for stabilization of c₁L_{DP} and c₂L_{DP} were determined from dose–response curves.

Finally, the dose-dependent stabilization of T₃R conformations c₁L_{DP} and c₂L_{DP} by T₃ was analysed by limited-protease-digestion assays under the same conditions as described for Figure 5 but in the presence of increasing T₃ concentrations (Figure 6). Under all three experimental conditions, namely T₃R monomers in solution (Figure 6A), T₃R monomers bound to DNA (Figure 6B) and T₃R–RXR heterodimers bound to DNA (Figure 6C), the proportion of T₃R input that was stabilized by saturation T₃ concentrations (1 μM) in c₁L_{DP} and c₂L_{DP} confirmed the results from Figure 5. Interestingly, the EC₅₀ for stabilization of c₂L_{DP} was 10 nM in all three experiments, whereas the EC₅₀ for

stabilization of c₁L_{DP} was not detectable for T₃R monomers in solution (Figure 6A) and was 1 nM for T₃R monomers and T₃R–RXR heterodimers bound to DNA (Figures 6B and 6C). Taken together, these results on T₃R conformations c₁L_{DP} and c₂L_{DP} show differential sensitivity to ligands; the relative importance of the more ligand-sensitive conformation c₁L_{DP} increased when the receptor formed heterodimers with RXR.

DISCUSSION

Physiological actions of thyroid hormone are initiated by the binding of T₃ to the LBD of T₃R [21]. To mediate gene activation, T₃Rs have to be transiently fixed to promoter regions of T₃ target genes. In the ligand-bound, agonistic conformation the last of the 12 α-helices of the LBD, helix 12, changes its orientation significantly with regard to the apo-conformation and closes the ligand cavity like a ‘lid’ [3]. This allows an LXXLL motif (single-letter amino acid codes) of co-activator proteins to interact specifically with a hydrophobic cleft on the surface of the LBD [22]. The co-activator then transfers the signal of the activated T₃R to the basal transcriptional machinery. For this multi-step process T₃Rs have to be competent on at least three levels: (1) they have to bind specifically to DNA, (2) they have to be very sensitively stabilized in their agonistic conformation, and (3) they have to interact specifically with co-activator proteins. It was widely assumed that T₃Rs meet these requirements only in a heterodimeric complex with RXR, i.e. that only in a heterodimer would T₃R be a competent ligand-activated transcription factor. However, in the present study we have demonstrated that T₃R also fulfils these requirements as a DNA-bound monomer.

The binding affinity of T₃R for monomeric binding sites has been determined previously as 2.1 nM, provided that the AGGTCA core-binding motif was 5′-flanked by the sequence TG [12]. In the present study we have shown that T₃R completely loses its affinity for monomeric binding sites if the third nucleotide in the hexameric core binding motif is changed from G to T, whereas in a heterodimeric complex with RXR this change seems not to affect DNA binding affinity. However, this selectivity made it possible to discriminate clearly between monomeric and heterodimeric complexes in the same experiment and to demonstrate that, at limiting RXR concentrations, DNA-bound T₃R monomers can co-exist with T₃R–RXR complexes bound to a DR4-type RE. Because RXR is involved in several nuclear hormone signalling pathways, the model of limiting concentrations of free RXR molecules is quite realistic and was shown previously to be the reason for squelching effects of nuclear receptors [23,24]. However, it was also shown previously [12] that only under optimized conditions, namely preferred 5′-flanking sequences, can T₃-induced gene activation by T₃R on a monomeric RE be as high as that of T₃R–RXR heterodimers on a dimeric element. In contrast, T₃R–RXR heterodimers are less sensitive to variations in the 5′-flanking sequences and can be boosted by co-induction with RXR ligands. Therefore, on average, T₃R–RXR heterodimers are at an advantage over T₃R monomers.

On the basis of protein–protein interaction assays performed in solution and in yeast and mammalian two-hybrid and one-hybrid assays with the use of T₃R fusion proteins, it has been known for some years that T₃R can interact with members of the p160 co-activator family [25]. However, in the present study we have shown for the first time that DNA-bound full-length T₃R monomers can interact in a ligand-dependent manner with TIF2, SRC-1 and RAC3 and that these co-activators can boost T₃R monomer-mediated gene activation. It has been accepted for a longer time that isolated T₃R LBDs, fixed via a Gal4–DBD

fusion to DNA, can mediate, without the contribution of RXR, gene activation in so-called mammalian one-hybrid assays (see Figure 4A). However, monomers or homodimers of full-length T_3 R have been considered to be incompetent in transcriptional activation and were thought to have a role only in repression processes [26]. In fact, in the absence of ligand, basal reporter gene activity was halved after the overexpression of full-length T_3 R (Figure 4B), indicating a recruitment of co-repressors by T_3 R monomers.

Limited protease digestion is a powerful method for monitoring ligand-induced conformational changes of nuclear receptors [27], but it had not been applied extensively to T_3 Rs. In the presence of ligand, T_3 R is clearly more stable to digestion with the endoprotease trypsin, which provides two receptor fragments that are representative of two agonistic T_3 R conformations, $c1_{LDP}$ and $c2_{LDP}$. It is assumed that at the moment of trypsin digestion the receptor pool is 'frozen' in its conformation ratio. Conformation $c1_{LDP}$ seems to be the 'true' agonistic conformation, in which helix 12 closes the ligand cavity and is able to interact with co-activators; that is, in $c1_{LDP}$ T_3 R seems to be fully competent for transactivation. In contrast, the exact nature of $c2_{LDP}$ is not yet known. Both conformations are stabilized in a dose-dependent manner by T_3 but the sensitivity of $c1_{LDP}$, with an EC_{50} of 1 nM, is clearly higher than that of $c2_{LDP}$, with an EC_{50} of 10 nM. Interestingly, in solution T_3 R was stabilized only in the $c2_{LDP}$ conformation, whereas in the presence of DNA T_3 R was stabilized in $c1_{LDP}$ too. A higher proportion of all T_3 Rs are found in $c1_{LDP}$ when they are complexed with RXR than when they are in monomeric form, but ligand sensitivity is the same under both conditions. This indicates that binding to DNA makes a certain proportion of T_3 R monomers competent for transactivation, but that complex formation with RXR increases the relative amount of competent T_3 Rs in the receptor pool.

In conclusion, this study has demonstrated that DNA-bound T_3 R monomers are competent on all three required levels to mediate ligand-dependent transactivation. This suggests that their role in the nuclear signalling of T_3 is currently underestimated and will need further investigation.

This work was supported by DFG-grant Ca229/1 and the Academy of Finland.

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Received 25 July 2001/7 September 2001; accepted 21 September 2001