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Retinoid X Receptor Isoforms α and β Differentially Regulate 3,5,3′-Triiodothyronine-induced Transcription

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Various heterodimers of the thyroid hormone receptor (TR) with other nuclear hormone receptors confer a wide range of transcriptional activities on thyroid hormone response elements (TREs) in the presence of the thyroid hormone (T_3). The present study analyzed the potential roles of retinoid X receptor (RXR) isoforms α and β in T_3-mediated transcription on a well characterized TRE, a direct repeat of AGGTCA separated by four nucleotides (DR4), using electrophoretic mobility shift assays and transient transfection in CV-1 cells. We demonstrated that RXRα supressed liganded TRα-induced transcription while RXRβ did not although both TRα/RXRα and TRα/RXRβ heterodimers were the predominant forms bound to the TRE-DR4 in the presence of T_3. We further demonstrated using Scatchard analysis that the two heterodimers had similar affinities for the TRE-DR4. All these observations suggest that the TRE-DR4 accomodates different types of TR/RXR heterodimers for a more finely tuned transcriptional response to T_3.

Normal growth and development as well as a variety of metabolic pathways are critically affected by thyroid hormones (T_3) (Evans, 1988). T_3 binds to thyroid hormone receptors (TRs), and controls the expression of specific target genes in a ligand-dependent manner (Lazar, 1993). TRs, in turn, bind to thyroid hormone-response-elements (TREs), which are composed of hexamer half-sites (AGGT(C/A)A) with a degeneracy in sequence, number, and orientation as well as spacing (Barettino et al., 1993). TRs bind to TREs as monomer and homodimers, as well as heterodimers with a T_3 receptor auxiliary protein (Darling et al., 1991), retinoic acid receptor (Evans, 1988), and retinoid X receptor (RXR) (Forrest et al., 1991; Rhee et al., 1995).

RXR mediates transcription in response to 9-*cis* retinoic acid (RA) (Levin et al., 1992; Heyman et al., 1992) via an RXR-response-element (RXRE) (Yu et al., 1991; Kliewer et al., 1992; Leid et al., 1992). There are major three isoforms of RXR; RXRα, RXRβ, and RXRγ (Yu et al., 1991; Kliewer et al., 1992; Leid et al., 1992). Furthermore, the transcriptional responsiveness of RXRs to 9-*cis* RA via RXRE varies depending upon the type of isoform (Mangelsdorf et al., 1992). RXRs heterodimerize with TRs on TREs, and these heterodimers regulate gene transcription in response to T_3 (Yu et al., 1991; Hallenbeck et al., 1992; Zhang et al., 1992). It has been known that both TRα/RXRα and TRα/RXRβ greatly enhance gene transcription via TRE-DR4, a direct repeat of AGGTCA separated by four nucleotides (DR4) of the myosin heavy chain gene promoter, and two copies of the palindromic TRE, respectively (Yu et al., 1991). However, Hallenbeck et al. have reported that the T_3/TRα-inducible expression of the myelin basic protein (MBP) gene is not enhanced by RXRβ, despite high affinity binding of the TRα/RXRβ to the MBP-TRE. Furthermore, RXR differentially augments thyroid hormone response in cell lines as a function of the response element and endogenous RXR content. Recently, Lazar group reported that RXR differentially recognizes the unliganded and liganded conformations of TR and that these differences appear to play a major role in the recruitment of corepressors to TR-RXR heterodimers. (Zhang and Lazar, 1997). Therefore, it seems that several factors, such as RXR isoforms, the structure of TREs, ligand, and cellular factors may participate in differentiating T_3-mediated gene transcription.

As an initial effort to examine the effect of RXR isoforms on TR-mediated transcription in response to T_3, we measured the transcriptional activities of TRα/RXRα and TRα/RXRβ heterodimers using a well-characterized TRE-DR4. We found that TRα/RXR heterodimers differentially transactivate DR4-TRE in response to T_3 in an RXR-isoform-specific manner.

Materials and Methods

Preparation of plasmid constructions

Plasmids were propagated in *Escherichia coli* HB101 and were isolated by alkaline lysis and purified on Qiagen 500 columns as described by the manufacturer. The luciferase (LUC) reporter construct pDR4-thymidine kinase(TK)-LUC was previously described (Yen et al., 1993) and was constructed by ligation of an oligonu-

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cleotide containing the TRE-DR4 (oligo DR4; 5′-AGC TACTTATTGAGGTCACATGAGGTCAAGTTACG-3′) with 5′-AGTC overhanging ends into a *Hind*III site at the multiple cloning site in front of the TK promoter of a LUC gene containing plasmid, pT109 (DeWet et al., 1987). The constructs, pMT2-TRα, pMT2- RXRα, and the control vector pMT2-UT were generous gifts of Dr. S. K. Karathanasis (Lederle Laboratories, Pearl River, NY) (Widom et al., 1992). pCMV-RXRα was produced by inserting the RXRα cDNA derived from pMT2-RXRα into the *Eco*RI site of the multiple cloning site of pCMV. pCMV-RXRβ was obtained from Dr. M. G. Rosenfeld (University of California at San Diego) (Yu et al., 1991).

Cell culture and transient transfection assays

CV-1 cells were grown in Dulbecco's modified Eagle's medium and 10% heat-inactivated (55℃, 1 h) fetal calf serum at 37℃. The serum was stripped of T_3 by constant mixing with 5% (wt/vol) AG1-X8 resin (Bio-Rad) twice for 12 h at 4℃ before ultrafiltration. The cells were seeded at 10^5 cells per well of a six-well plate (Libron, Flow Laboratories, Inc.) 24 h before transfection. Each well was transfected with expression (60 ng) and reporter (500 ng) plasmids as well as a Rous sarcoma virus-β-galactosidase (RSV-β-Gal) control plasmid (250 ng) (Edlund et al., 1985) by calcium phosphate coprecipitation as previously described (Widom et al., 1992). Cells were grown for 24 h in the presence and absence of T_3 (10^{-7} M) before harvesting. Cell extracts were analyzed for both LUC (DeWet et al., 1987) and β-Gal activity. Transcriptional activity is represented by LUC activity normalized to β-Gal activity.

Electrophoretic mobility shift assay (EMSA) and determination of dissociation constants (K_ds)

Whole-cell extracts for EMSA were prepared from COS-7 cells maintained in Dulbecco's modified Eagle's medium plus 10% heat-inactivated (55℃, 1 h) fetal calf serum. The cells were seeded at 10^6 per 100-mm dish 24 h before transfection and were transfected with 10 μg pMT2-TRα, pCMV-RXRα, or pCMV-RXRβ by the diethylaminoethyl-dextran procedure, essentially as previously described (Widom et al., 1992). After 48 h, cells were scraped from the dishes, pelleted by centrifugation, and resuspended in a fubber containing 20 mM HEPES (pH 7.8), 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (50 μl/dish). Cell extracts were produced by three freeze-thaw cycles and stored at -70℃. EMSA was perfomed as described previously by using oligo DR4 as a probe, which was radiolabeled by using T4 polynucleotide kinase and [γ-^{32}P] ATP.

To determine K_ds of TRα/RXRα and TRα/RXRβ heterodimers, EMSA was performed with constant amounts of proteins and increasing amounts of [^{32}P]oligo DR4. After electorphoresis, the gels were fixed, dried and exposed to X-ray films. Regions of the gel corresponding to heterodimer bound and free probe were excised and counted by liquid scintigraphy, and the K_ds constants were determined by the method of Scatchard (Scatchard, 1949).

Results

RXRα counteracts the T_3/TRα-induced transcription, but RXRβ does not

T_3-induced transcriptional activities of TR/RXR heterodimers vary depending upon the nature of the heterodimer formed on the TRE and the structure of TREs (Yu et al, 1991; Hallenbeck et al., 1992; Zhang et al., 1992). In this report, we analyzed the effect of RXR isoforms α and β on liganded TRα-induced transcription via a specific TRE, the TRE-DR4, which is composed of two DRs (AGGTCA) separated by four nucleotides (CATG). A single copy of the TRE-DR4 was inserted proximal to the basal promoter of the TK gene in a vector pT109 containing the LUC gene (pDR4-TK-LUC). The resulting constructs were cotransfected into CV-1 cells with vectors expressing TRα (pMT2-TRα), RXRα (pCMV-RXRα), RXRβ (pCMV-RXRβ), and β-Gal (pRSV-β-Gal) in the presence and absence of T_3 as shown in Fig. 1.

As shown in Fig. 1, unliganded-TRα repressed the basal transcription by 72%, whereas liganded TRα increased transcription as much as 21-fold in comparison with unliganded TRα. As expected, RXRα alone neither down-regulated the basal transcription nor activated the transcription in the presence of T_3 (Fig. 1). Cotransfection of pCMV-RXRα with pMT2-TRα did not alter the unliganded TRα-mediated basal transcription but reduced the T_3/TRα-induced transcription by 66% (Fig. 1). In contrast, RXRβ did not change

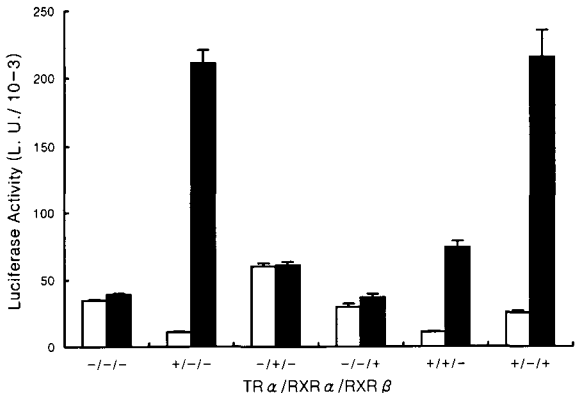

Fig. 1. T_3 responsiveness of TRα/RXRα and TRα/RXRβ heterodimers via the TRE-DR4. The plasmid pDR4-TK-LUC was transienly transfected into CV-1 cells with vectors expressing TRα, RARα, and/or RXRβ, at indicated amounts (-, 0 ng; +, 60 ng), in the presence (■) or absence (□) of 10^{-7} M T_3. Normalized LUC activities are depicted. Results represent the mean of duplicate determinations differing by less than 10% and are representative of five experiments.

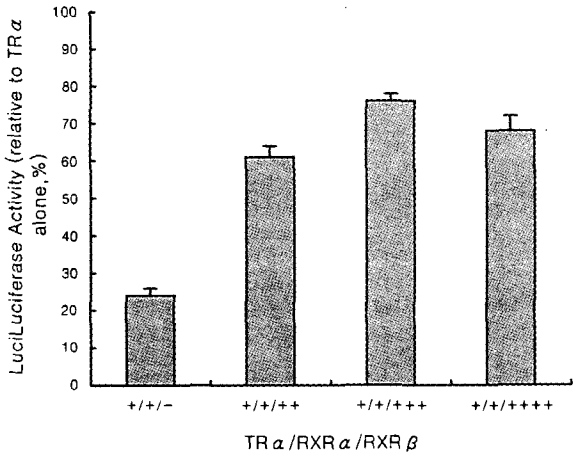

Fig. 2. Effect of RXRβ on TRα/RXRα heterodimer-mediated transcription in the presence of T_3. The plasmid pDR4-TK-LUC was transiently transfected into CV-1 cells at the indicated amount (-, 0 ng; +, 60 ng; ++, 120 ng; +++, 180 ng; ++++, 240 ng) of the plasmids expressing TR α, RXRα, and RXRβ. Normalized luciferase activities are depicted. Results represent the mean of duplicate determinations differing by less than 10% and are representative of three experiments.

the transcriptional activity of the T_3/TRα complex in the presence of T_3 (Fig. 1). Then, we examined the effect of RXRβ on the T_3/TRα/RXRα- induced transcription by conducting transient cotransfection assays; pCMV-RXR β was cotransfected with pMT2-TRα and pCMV-RXRα into CV-1 cells. RXRβ counteracted the blocking effect of RXRα on T_3/TRα-induced transcription by up to 3 fold (Fig. 2). However, 4 times excess the amount of RXRβ began to lose its rescuing effect on the T_3/TRα /RXRα-mediated transcriptional activity probably due to the quenching effect of its excess amount on general transcriptional factors (Fig. 2). Nonetheless, these data indicated that RXRβ heterodimerized with TRα on TRE-DR4 activated transcription in the presence of T_3, and that the two different heterodimer forms actually bound to TRE-DR4, but conferred different transcriptional activities.

TRα/RXRα and TRα/RXRβ, the major complexes formed on oligo DR4 in the presence of T_3

In order to examine whether the differential effects of the two RXR isoforms on T_3/TRα-mediated transcription resulted from their actual binding to the TRE-DR4, we carried out EMSA using COS-7 cell overexpressed receptors and radiolabeled oligo-DR4. Because the tested receptors were produced in COS-7 cells, we examined the possibility that a COS-7 cellular extract contained detectable amounts of various receptors that interacted with the TRE-DR4. As shown in Fig. 3, lane 1, 1 μl of COS-7 cellular extract, the maximum amount used for EMSA in this report, did not produce any detectable bands. TRα bound to oligo DR4 as a monomer and a homodimer (Fig. 3, lane 2) while treatment of T_3 (10^{-7} M) caused the dissociation of the TRα homodimer/oligo DR4 complex (Fig. 3, lane 3), a

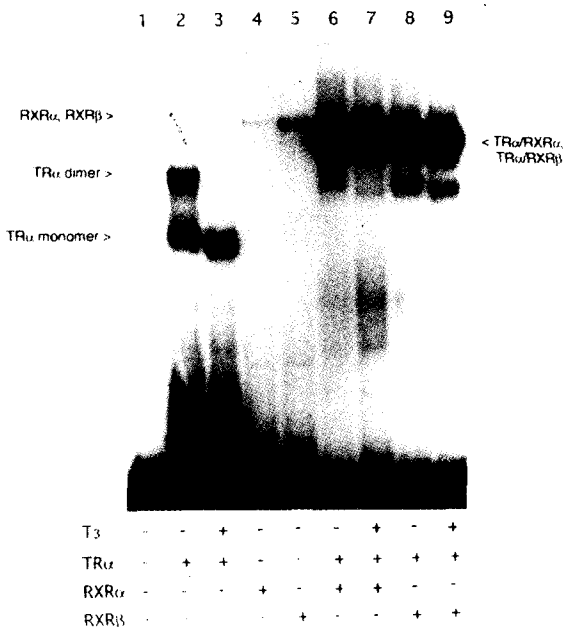

Fig. 3. Mobility shift assay of TRα heterodimer with RXRα or RXRβ. Reactions containing 0.5 ng of [^{32}P]oligo DR4, 1 μl of untransfected COS-7 cellular extract (lane 1), or COS-7-produced TRα, RXRα, and/or RXRβ were analyzed with EMSA using a 4% polyacrylamide gel.

well-documented phenomenon (Yen et al., 1992). Both RXRα and RXRβ bound as homodimers with low affinity (Fig. 3, lane 4, and 5). Both TRα/RXRα and TRα/RXRβ demonstrated strikingly high affinity for oligo DR4 in comparison to RXRs alone (Fig. 3, lanes 6 and 8). Furthermore, T_3 was unable to alter their binding affinities, confirming that the two heterodimer forms are the major complex formed on the TRE-DR4, particularly in the presence of T_3.

Similar binding affinities of TRα/RXRα and TRα/RXRβ

As an initial step to determine whether the different effects of RXRα and RXRβ on liganded TRα-mediated transcription is due to their different binding affinities, we measured the K_ds for their binding to the TRE-DR4. Specifically, constant amounts of TRα with either RXRα or RXRβ were incubated with increasing amounts of radiolabeled oligo DR4 and subjected to EMSA. Radioactivities of the two heterodimeric complexes (bound) were determined and plotted against the corresponding amounts of nonbound (free) oligo DR4 (data not shown). Subsequent Scatchard analysis of these data revealed that the K_ds of TRα/RXRα and TRα/RXRβ were 2.9 nM and 3.1 nM, respectively (Fig. 4), demonstrating that the two heterodimers bind to oligo-DR4 with similar affinities. Taken together with the data from transient cotransfection assays, it appears that the two heterodimers exert different transcriptional activites on the TRE-DR4 in response to T_3.

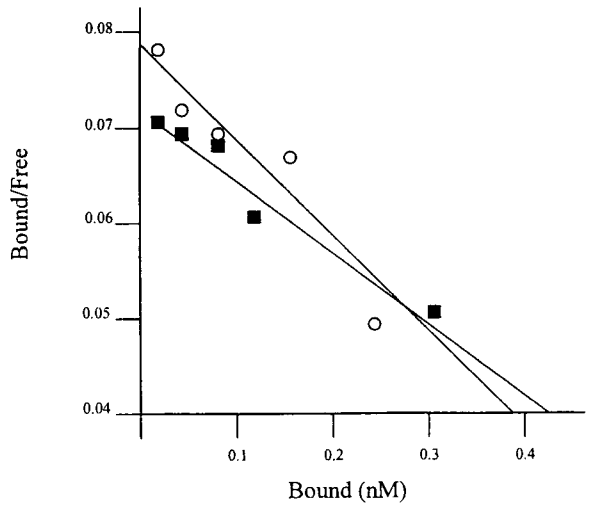

Fig. 4. Scatchard analysis of TRα/RXRα (○) and TRα/RXRβ (■) on oligo DR4. Receptors were prepared in COS-7 cells as described in Materials and Methods. One microliter of TRα was incubated with 1 μl RXRα or RXRβ and increasing amounts (0.25, 0.5, 1, 2, and 4 ng) of [^{32}P]oligo DR4 was loaded on a 4% polyacrylamide gel. After electrophoresis, areas corresponding to the TRα/RXRα and TRα/RXRβ heterodimers and free probe were counted as described in Materials and Methods and plotted by the method of Scatchard (Scatchard, 1949). The K_ds of TRα/ RXRα and TRα/RXRβ on oligo DR4 were 2.9 and 3.1 nM, respectively.

Discussion

Recent studies reported that the functions of TR/RXR heterodimers in gene transcription are diverse and depend on the nature of TREs. Hallenbeck et al. have shown that T_3/TRα- inducible expression of the MBP gene is not enhanced by RXRα, despite high affinity binding of TRα/RXRβ to the MBP TRE (Hallenbeck et al., 1993). In this report, we also showed that RXRα and RXRβ heterodimerize with TRα on the TRE- DR4 and mediate different transcriptional responses to T_3 on the same TRE-DR4. It is therefore conceivable that the specific nature of the TREs may determine the transcriptional activity of a battery of TR heterodimers formed with other nuclear hormone receptors.

The orientation and spacing of TRE half-sites are critical not only in dictating the nature of specific homodimer and heterodimer binding of receptors to the TRE but also in determining their transciprtional activities (Umesono et al., 1991; Naar et al., 1991). TRE-DR4s are physiologically relevant *cis*-acting elements that respond to the thyroid hormone at the transcriptional level. They have been found in several promoters, including those of the human myosin heavy chain-1 (Umesono et al., 1991), rat malic enzyme (Petty et al., 1990), and murine Moloney leukemia virus long terminal repeat gene (Sap et al., 1989). Two independent lines of evidence suggest that selective binding of TR/RXR heterodimers to their cognate DRs is achieved by a cooperative dimer interaction within the DNA-binding domains, and that this interaction establishes the polarity of heterodimer binding to DNA (Perlman et al., 1993; Kurokawa et al., 1993). Furthermore, Schrader et al. (1993) demonstrated that two different vitamin D response elements can dictate polarity of the binding of the vitamin D receptor/TR heterodimer, and that this polarity enables the heterodimer to respond to either vitamin D or T_3. It has been shown that TRα/RXRα preferentially binds to the downstream half-site of the TRE-DR4, and that this preferential binding is important in maximizing its transcriptional activity in the presence of T_3 (Ikeda et al., 1994). Two to three nucleotides flanking either end of the half-sites have been demonstrated to affect TRE activity profoundly and result in either strong or weak TRE half-sites (Kim et al., 1992; Hsu et al., 1995). These observations further support the idea that the structure of the TRE-DR4 may dictate different transcriptional responsiveness to T_3 on the TR heterodimers with RXRs. On the other hand, Lazar's group recently reported that TR CoR box and ninth heptad are required for RXR interaction and in turn for interaction with corepressor proteins N-CoR and SMRT, and that the addition of the thyroid hormone obviates the CoR box requirement for RXR interaction (Zhang et al., 1997). Furthermore, the fact that ligand-dependent heterodimerization of TR and RXR in solution is not a result of T_3-induced corepressor N-CoR (Collingwood et al., 1997; Kakizawa et al., 1997) suggests that nuclear receptor signaling may involve another level of cellular control.

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