Ligand-dependent Synergy of Thyroid Hormone and Retinoid X Receptors*

(Received for publication, June 10, 1992)

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The binding of thyroid hormone receptors to DNA is enhanced by heterodimerization with nuclear proteins. One such heterodimerization partner has recently been characterized as the retinoid X receptor. 9-cis-Retinoic acid has been identified as a natural ligand for retinoid X receptors, suggesting a potential receptor-mediated interaction between thyroid hormone and 9-cis-retinoic acid in the regulation of thyroid hormone-responsive genes. A transient cotransfection assay was used to test for such an interaction. When a complex thyroid hormone response element composed of both direct and inverted repeat hexamers was tested, these two ligands activated gene expression synergistically. In contrast, when the response element consisted only of directly repeated hexamers, unliganded retinoid X receptors enhanced thyroid hormone responsiveness, but 9-cis-retinoic acid induced no additional activation. The results suggest a unique mechanism to achieve differential thyroid hormone sensitivity of thyroid hormone-responsive genes within a cell. Genes with appropriate response elements will show amplification of the thyroid hormone response by 9-cis-retinoic acid in the presence of retinoid X receptors; other thyroid hormone-responsive genes will be influenced by retinoid X receptors, but not 9-cis-retinoic acid.

Thyroid hormone receptors (TRs) are ligand-dependent transcription factors that, along with the receptors for steroids, vitamin D, retinoic acid, and others, comprise the erbA superfamily (1). Although TRs can bind to triiodothyronine (T3) response elements (TREs) as monomers or homodimers (2), transcriptional activation may involve heterodimerization with other factors known as T3 receptor auxiliary proteins, or TRAPs. The TR/TRAP heterodimer binds to TREs with a higher affinity than does TR alone (3–5), and TR mutations that disrupt this heterodimerization severely compromise the ability of TRs to trans-activate target genes (6, 7).

Retinoid X receptors (RXRs) also are members of the erbA superfamily. RXRs were discovered in the process of cloning transcription factors that regulate murine major histocompatibility class I genes (8), and also by low stringency hybridization of cDNA libraries with a retinoic acid receptor (RAR) DNA binding domain probe (9). The ligand for RXRs was not immediately apparent; hence, these were considered to be orphan receptors. However, pharmacologic doses of all-trans-retinoic acid (RA) were capable of activating RXRs, suggesting that the genuine ligand may be an unknown retinoid, dubbed retinoid X (9). Recently, 9-cis-RA has been identified as retinoid X (10, 11).

Several investigators have shown that RXRs can heterodimerize with TRs on TREs and that RXRs can enhance the ability of TRs to trans-activate target genes (12–17). Thus, RXRs can function as TRAPs. However, these initial studies identifying RXRs as TRAPs were performed before the actual RXR ligand was characterized. The discovery of 9-cis-RA suggests a potential interaction not simply between TRs and RXRs, but also indirectly between T3 and 9-cis-RA in the regulation of T3-responsive genes. We now describe a complex receptor-mediated interaction between these two ligands. In the presence of TR and RXR (but not RAR), T3 and 9-cis-RA synergistically* trans-activate a gene that contains a TRE with an inverted repeat motif. However, 9-cis-RA is without effect on a gene that has a simple direct repeat TRE. On this TRE, unliganded RXR is capable of enhancing T3 induction of target gene expression.

MATERIALS AND METHODS

Cell Culture and Transfections—JEG-3 cells were grown in 90% Eagle’s medium plus 10% Calf Supreme (GIBCO), and were transfected using calcium phosphate precipitation (18). Rat TRα2 in the expression vector pCDM has been described (18). Rat RXRα (12) and RARα (19) were expressed from the same vector. High level TRβ transfections utilized 3 μg of pCDMTRβ, and low level transfections utilized 25 ng. Transfections also included 3 μg of pCDMRXRβ or 3 μg of pCM1ARα as indicated. The vector pCDM was added where needed to achieve a total of 8 μg of pCDM-based plasmid/transferrin. Three T3-responsive reporter plasmids were used at a dose of 4 μg/transfection. The reporter plasmid pTK35BA contains two copies of a modified rat growth hormone gene TRE (including the direct and inverted repeats) 5' to a basal thymidine kinase promoter directing chloramphenicol acetyltansferase (CAT) expression (20). The plasmid pTK14AA contains two copies of a directly repeated element 5' to the thymidine kinase promoter but no inverted repeats (18, 21), and pTK39 contains only the inverted motif (TGAGGTGATCACCTG). To control for transfection efficiency, each transfection also included 2 μg of pTKGH, in which the basal thymidine kinase promoter directs expression of human growth hormone (21). Cells in 60-mm Petri dishes were transfected in the presence of 10% charcoal-stripped Calf Supreme supplemented with 100 nM dexamethasone, and then cultured for 2 days ± T3 (0.2 or 100 nM) ± 9-cis-RA (100 nM). This dose of 9-cis-RA was an ED50 in transfection studies using RXRα and a specific RXR response element (11). Cells were harvested for CAT assay and media for human growth hormone assay as described (18). Results are expressed as -fold CAT induction, defined as CAT/human growth hormone for cells cultured with ligand divided by CAT/human growth hormone for cells cultured without

* This work was supported by National Institutes of Health Grant DK44195. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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†† The abbreviations used are: TR, thyroid hormone receptor; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; T3, triiodothyronine (thyroid hormone); TRAP, T3 receptor auxiliary protein; TRE, T3 response element.

‡‡ In this paper two ligands are considered to act synergistically if the response to both together is greater than the sum of their individual responses.
ligand. Results are the mean ± S.E. for at least 4 transfections/condition.

**Electrophoretic Mobility Shift Assay (EMSA)—[35S]Metioninelabeled and non-radiolabeled proteins (RXRβ and TRβ) were produced by translation in rabbit reticulocyte lysate. Protein DNA binding incubations included 15,000 cpm RXRβ or TRβ, 40 ng of rat growth hormone TRE or a palindromic TRE (22) and 1.4 μg of poly(dI.dC) in 35 μl of 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM dithiothreitol, 20% glycerol, 0.1% Nonidet P-40. To assess heterodimer formation, incubations included nonradiolabeled TRβ or RXRβ along with radiolabeled RXRβ or TRβ, respectively. In some experiments JEG cell extracts (7) were used in place of in vitro translated RXRβ or TRβ as a source of TRAP, which can function like RXRβ. Incubations were at room temperature for 40 min prior to loading onto an 8% polyacrylamide gel at 4 °C. Gels were dried and analyzed by fluorography (Autofluor™, National Diagnostics).

**RESULTS AND DISCUSSION**

To evaluate the potential receptor-mediated interaction between T3 and 9-cis-RA, JEG-3 cells were transiently cotransfected with a TRβ expression vector in the absence or presence of an RXRβ expression vector. The reporter plasmid used, pTK35BA, contains a CAT gene driven by a TRE derived from the rat growth hormone 5’-flanking region (20). When cells were transfected with high levels of TRβ but no RXRβ, a physiologic dose of T3 induced CAT expression a modest 4-fold (Fig. 1a, top). 9-cis-RA by itself was without effect, but the combination of T3 plus 9-cis-RA led to an 8-fold induction of CAT, possibly due to interaction with endogenous RXRs. Synergy between T3 and 9-cis-RA was more clearly observed when RXRβ was cotransfected with TRβ. Under these conditions, induction by T3 or 9-cis-RA alone was 4-6-fold, but the simultaneous addition of both ligands led to a 24-fold induction of CAT (Fig. 1a, middle). It is important to note that, in the absence of 9-cis-RA, RXRβ caused virtually no enhancement of the T3 induction of CAT (Fig. 1a, compare T3 bar of middle section with T3 bar of top).

Although this contrasts with the work of other investigators (12, 13, 16), we do find an effect of RXRβ in the absence of 9-cis-RA under other circumstances (see below).

Retinoic acid receptors represent a second class of 9-cis-RA receptors (11) that, in addition, are activated by all-trans-RA. Both RXRs (12-16) and RARs (24) heterodimerize with TRs in vitro, and all of these receptors bind to DNA elements that contain the hexameric consensus AGGTCA (12, 20, 21, 25). In a manner similar to that seen with RXRβ, 9-cis-RA will induce CAT 6-fold in the presence of RARα and TRβ (Fig. 1a, bottom). However, in marked contrast to the situation with RXRβ, the combination of RARα and TRβ does not support further enhancement of gene expression with T3 plus 9-cis-RA. A trivial explanation for the lack of ligand-dependent synergy in the presence of RARα would be squelching of crucial transcription factors by excess RARα. However, RARα did not inhibit expression of the cotransfected internal control plasmid pTKGH (data not shown). RARα may compete with TRβ for binding to the TRE, thus limiting the ability of T3 to induce CAT with or without 9-cis-RA.

Similar results were obtained using low levels of transfected TRβ. In these experiments, a receptor-saturating dose of T3 was used so that the effects of T3 alone could be measured reproducibly. In the presence of transfected TRβ plus RXRβ (Fig. 1b, middle), CAT was induced 12-fold by T3, 5-fold by 9-cis-RA, and 57-fold by both ligands together. When RARα was substituted for RXRβ, 9-cis-RA induced CAT almost 9-fold, but no further enhancement was seen with T3 plus 9-cis-RA (Fig. 1b, bottom). Once again, no enhancement of T3-induced trans-activation was noted when RXRβ was cotransfected with TRβ in the absence of 9-cis-RA.

When the reporter vector was transfected in place of TRβ, CAT induction with 100 nM T3 alone was only 3-fold, and no synergy was observed with 9-cis-RA (data not shown). The low level of T3 induction is consistent with the low level of endogenous TRβ in JEG cells (10 fmol/100 μg DNA) (26). In addition, if the TRE was omitted from the reporter plasmid, CAT induction by T3 and 9-cis-RA did not exceed 2-fold, even in the presence of high levels of TRβ and RXRβ (data not shown).

Several mechanisms could potentially account for the synergy between T3 and 9-cis-RA on this TRE. 9-cis-RA could enhance binding of TR/RXR heterodimers to the TRE. This was tested directly using an electrophoretic mobility shift assay (EMSA), but 9-cis-RA had no effect on the magnitude of DNA binding or on the mobility of the protein DNA complex (Fig. 2). A second possibility would be for 9-cis-RA to increase the amount of RXR in the cell, or to induce another protein of similar function. To test this, extracts were prepared from cells that had been transfected with RXRβ and then cultured with or without 9-cis-RA. Graded doses of these extracts were then incubated with TR and DNA, and heterodimer formation was assessed by EMSA (Fig. 3). Transfection with RXRβ increased the potency of the extracts in this assay, as expected (data not shown). However, cells cultured with 9-cis-RA had no more heterodimerization activity than cells cultured without 9-cis-RA. It also might be possible for 9-cis-RA to increase the amount of ligand-occupied TRs. This was excluded by finding no difference in nuclear [3H]T3 binding from cells that had been transfected with TRβ plus RXRβ and then incubated with or without 9-cis-RA (data not shown). In the absence of specific evidence to support any of the above mechanisms, it is likely that the ability of 9-cis-RA to synergize with T3 reflects a heterodimer conformation that is optimized for interaction with other transcription factors by the presence of both ligands.

The TRE used in the above studies is derived from the 5'-
flanking region of the rat growth hormone gene (20). This TRE contains three nearly perfect copies of the consensus hexamer, the most 3' of which is inverted relative to the other two. Mutational analysis clearly indicates all three hexamers are required for full T3 responsiveness of the rat growth hormone TRE (20). However, TRES can be constructed simply with directly repeated elements and no inverted repeats (18, 21). The nuclear hormone may contain a natural example of this class of TRE (27, 28). Therefore, it was of interest to examine the effects of T3, 9-cis-RA, and their receptors on expression of a reporter gene driven by a TRE that contains only directly repeated hexamers.

Surprisingly, the results show only minimal effects of 9-cis-RA on T3-induced gene expression, but substantial effects of RXRβ alone (Fig. 4). In the absence of cotransfected RXRβ, T3 induction of CAT was 17-fold. However, this rose to 56-fold with cotransfected RXRβ, using T3 as the only ligand. Although RXRβ showed a clear effect in the absence of 9-cis-RA, no further enhancement of gene expression was seen when this ligand was added along with T3. A similar trend, although quantitatively less impressive, was seen when pTK14AA was transfected under conditions identical to those of Fig. 1b (25 ng of pCDMTRβ ± 3 μg of pCDMORXβ and cultured ± 100 nM T3 ± 100 nM 9-cis-RA). Under these conditions T3 induction of CAT was 3.7-fold in the absence of cotransfected RXRβ and 9-cis-RA. When RXRβ and T3 were cotransfected, CAT induction was 6.2-fold with T3 as the only ligand and 6.4-fold with 9-cis-RA plus T3. Thus, in contrast to the rat growth hormone like TRE, this simple direct repeat TRE is not capable of showing ligand-dependent synergy between RXRβ and RXRβ. Also in contrast to the rat growth hormone-like TRE, T3 induction of this TRE is enhanced by RXRβ without 9-cis-RA. Perhaps on this direct repeat TRE the heterodimer conformation is optimized for interaction with other factors without 9-cis-RA, and hence this ligand has no additional effect.

To confirm that the ligand-dependent synergy observed in Fig. 1 with the complex TRE did indeed depend on the inverted repeat motif, similar studies were performed with the reporter plasmid pTK39, in which the TRE contains only an inverted repeat. As predicted, synergy was observed in the absence of 3 μg of TRβ and RXRβ cDNAs; ligand induction of CAT was 3.3-fold for T3 alone, 3.1-fold for 9-cis-RA, and 15.3-fold for both ligands together.

Thyroid hormone regulates a vast number of metabolic processes. The degree of T3 response varies greatly from one gene to another, and some genes are highly T3-responsive in one tissue but not another, even though both may contain TRs (29, 30). The mechanisms underlying this diversity and specificity of T3 action are not well understood, but the studies reported here suggest one possible explanation. Two TRES that are functionally identical in the presence of TRs may behave differently if 9-cis-RA and/or RXR are present within the cell. The unoccupied RXR can heterodimerize with TRs and enhance the ability of T3 to induce expression from certain T3-responsive genes; other genes will show a synergistic enhancement of T3 response only in cells that contain both RXR and 9-cis-RA. Given this, it will be important to determine the factors that regulate intracellular RXR and 9-cis-RA levels. If 9-cis-RA is produced enzymatically there may be important metabolic factors that regulate its production. (Precedent for enzymatic isomerization of retinoids is found in the visual system, where an isomerase catalyzes conversion of all-trans to 11-cis-retinal; see Ref. 31.)

The important discriminating effects of 9-cis-RA on T3 action may be specific for RXRs, since a second class of 9-cis-RA receptors, the RARs, do not mediate similar effects when studied under identical conditions. These studies also may bear relevance to the syndrome of generalized resistance to thyroid hormone. Patients with this condition require supraphysiological levels of T3 to remain clinically euthyroid. Most cases appear to be due to mutations that impair ligand binding of TRβ (32, 33). Our studies would predict that mutations in an RXR gene might lead to a clinical syndrome
with some features that overlap those of thyroid hormone resistance. Mutations that impair 9-cis-RA binding to an RXR might lead to diminished T3 induction of a subset of T3-responsive genes in those organs that contain RXR and 9-cis-RA. However, mutations of TRδ or an RXR that impair heterodimerization might inhibit T3 induction of a wider array of genes. Clearly, the ultimate level of hormonal effect in vivo is the result of complex interactions between multiple receptors and ligands which are just beginning to be understood.

Acknowledgments—We thank Chris Glass and Michael G. Rosenfeld for RXRδ, Ron Evans for RARα, P. Reed Larsen for the reporter plasmids, Joseph Grippo for 9-cis-RA, and William Pratt for helpful discussions.

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Ligand-dependent synergy of thyroid hormone and retinoid X receptors.
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J. Biol. Chem. 1992, 267:22010-22013.

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