Regulatory functions of a non-ligand-binding thyroid hormone receptor isoform

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Gene regulation by thyroid hormones is mediated through multiple nuclear receptors. Only some of these thyroid hormone receptor (TR) isoforms become transcriptional enhancers in the presence of the thyroid hormone T_3 . Here we analyze the regulatory function of the human TR α 2 isoform. This protein does not bind T_3 and is not a transcriptional activator of thyroid hormone-responsive elements (TRE). Transfected TR α 2 functions as a constitutive repressor of the transcriptional activators TR α 1 and TR β 1 but also represses heterologous receptors, including the retinoic acid receptor and the estrogen receptor, which can activate TRE-controlled genes. TR α 2 protein showed strongly reduced DNA binding to a palindromic TRE when compared with the active TRs. Hybrid receptor analysis revealed that the special properties of the TR α 2 protein, including its repressor function and DNA binding characteristics, are intrinsic properties of its carboxyterminus and can be transferred to other receptors. Although it has been shown that the active TRs can act as repressors and silencers due to their strong DNA binding in the absence of hormone, our data show that TR α 2 is unlikely to inhibit TRs and other receptors through a competitive DNA binding mechanism. Antibody gel shift experiments suggest that repression by TR α 2 might result from interaction with active receptors. Thus, the receptor-like TR α 2 isoform differs from typical nuclear receptors in its DNA-binding and ligand-binding properties and appears to regulate the activity of other receptors via protein-protein interaction.

Introduction

Thyroid hormone receptors (TR) belong to a large family of nuclear receptors that also include the steroid hormone and retinoic acid receptors (RAR). TRs are encoded by two genes,

TR α and TR β , from which several isoforms can be expressed. Two TR β isoforms with different aminoterminal regions have been described (Weinberger *et al.*, 1986; Koenig *et al.*, 1988; Hodin *et al.*, 1989), and at least four isoforms of TR α that all differ in their carboxyterminal sequences have been isolated (Sap *et al.*, 1986; Benbrook and Pfahl, 1987; Thompson *et al.*, 1987; Mitsuhashi *et al.*, 1988; Nakai *et al.*, 1988a). Both TR β isoforms are ligand-dependent transcriptional activators, but only one of the α -receptor isoforms, TR α 1, is a true receptor and functions as a ligand-dependent transcriptional enhancer. The other TR α isoforms are the result of differentially spliced mRNAs encoding various forms of an alternative carboxyterminal domain. These carboxyterminal variants are receptor-like proteins that do not bind T_3 (Mitsuhashi *et al.*, 1988; Lazar *et al.*, 1989; Schueler *et al.*, 1990), and little is known about their functions and physiological roles.

It is now well established that TR α 1 and TR β 1 have dual regulatory roles; in the presence of hormone they act as transcriptional enhancer proteins, whereas in the absence of ligand they repress transcription from promoters containing thyroid hormone-responsive elements (TRE). TRE-containing promoters can also be activated by several heterologous receptors (Umesono *et al.*, 1988; Graupner *et al.*, 1989, 1991), including RARs and the estrogen receptor (ER). Numerous experiments have shown that TR α 1 and TR β 1 bind the TRE in the absence of ligand in vitro and in vivo and with higher affinity than the heterologous receptors. This enables TR α 1 and TR β 1 to repress RAR and ER activity on a TRE effectively by a competitive DNA-binding mechanism (Graupner *et al.*, 1989, 1991; Zhang *et al.*, 1991). In addition, these TRs can also function as silencers on TRE-containing promoters and repress constitutive transcription that is TRE independent (Damm *et al.*, 1989; Banihmad *et al.*, 1990; Zhang *et al.*, 1991).

The biological significance of the TR repressor function is exemplified in the viral *v-erbA* oncoprotein, a mutated TR α isoform that does not bind T_3 and has lost its transcriptional enhancer

Figure 1. Regulation of TRE activity by TR α -isoforms. CV-1 cells were cotransfected with 2 μ g of TRE₂-tk-CAT alone or together with pECE-TR α 2 (1 μ g) or pECE-TR α 1 (1 μ g). Cells were treated with 50 nM T₃ (□) or no hormone (■) and 24 h later assayed for CAT activity (CAT activity is normalized for β -galactosidase activity). The results show the means ($\pm$ SE) of two independent experiments.

function. However, *v-erbA* has maintained its TRE-specific repressor activity (Damm *et al.*, 1989; Sap *et al.*, 1989). Thus, *v-erbA* can act as a constitutive repressor or silencer protein of thyroid hormone-responsive genes and, when coexpressed with TR α 1, eliminates the response to the thyroid hormone. Both activities are directly related to the ability of *v-erbA* to bind the TRE with similar affinity as TR α 1 or TR β 1. The carboxyterminal variants of TR α could therefore represent functional cellular homologues to the *v-erbA* oncoprotein, their major physiological role being to bind TREs and to repress transcription from thyroid hormone-responsive genes.

The carboxyterminal isoform TR α 2 was first isolated from a human testis cDNA library (Benbrook and Pfahl, 1987) and has also been found in the rat (Lazar *et al.*, 1988; Mitsunashi *et al.*, 1988). A prominent locus of its expression is the brain, where it represents the major TR isoform during postnatal development and in the adult animal (Wills, *et al.*, 1991; Bradley *et al.*, 1989). The finding that the altered carboxyterminus of the TR α 2 isoform has been highly conserved from rat to human and the high level of its expression in several tissues (Sakurai *et al.*, 1989) suggest important regulatory functions for this "receptor-like" protein. Here we analyze the functional activities of the human TR α 2 isoform (previously termed *c-erbA-T* [Benbrook and

Pfahl, 1987]) in comparison with the transcriptional activators TR α 1 and TR β 1. It has been reported that TR α 2 can act as a weak repressor of active TRs (Koenig *et al.*, 1989; Lazar *et al.*, 1989). We found that, in addition, TR α 2 represses the heterologous receptors ER and RAR, which are co-expressed with TR α 2 in several tissues. The repression pattern was different from that observed for TR α 1 or TR β 1. In addition, repression was also observed with a TR α 2 mutant that lacked the DNA-binding domain. Furthermore, TR α 2 showed a strongly reduced binding to the TRE when compared with TR α 1 and in addition lacked silencer activity. Thus, a repression mechanism based on competitive DNA interaction is unlikely. This was supported by band-shift experiments in which TR α 2 was mixed with TR α 1 and a TR α 2-specific antibody. Overall these data suggest that TR α 2 may function primarily by protein-protein interaction and does not follow the classical protein-DNA interaction mechanism of the ligand-binding nuclear receptors.

Results

TR α 2 is not a transcriptional enhancer or silencer protein

To investigate the regulatory properties of TR α 2, we cotransfected an expression vector carrying the TR α 2 coding sequence with a thyroid hormone-responsive reporter gene (TRE₂-tk-CAT into CV-1 cells). This reporter construct has a partially constitutive promoter, allowing for analysis of both silencer and activator functions of the cotransfected receptors. As control, an analogous TR α 1 expression vector was transfected in parallel. Whereas TR α 1 was a highly efficient T₃-dependent activator of the reporter gene, TR α 2 did not respond to T₃ (Figure 1). To investigate the possibility that TR α 2 is activated by a different ligand, several other compounds, including T₄ and reverse T₃ (a thyroid hormone antagonist), and organic extracts from rat brain and liver were tested. None activated TR α 2 (data not shown). Moreover, although TR α 1 in the absence of T₃ showed a remarkable silencing effect on the TRE reporter, no such activity was observed for TR α 2 (Figure 1).

To determine which portions of the TR α 2 protein contributed to the loss of ligand-dependent transcriptional activation, we constructed a number of hybrid receptors that contained the DNA- or hormone-binding domains of the ER and hormone- or DNA-binding domains of either TR α 2 or TR α 1 (Figure 2A). When these hybrid

Figure 2. Hybrid receptor analysis. (A) The schematic representation shows the human TR α 2, TR α 1, and ER; the derived hybrid receptors ER-TR α 2, TR α 2-ER, and ER-TR α 1 (aminoterminal-carboxyterminal); and the FLAG-TR α 2 hybrid. Numbers above and below bars represent amino acids of the parental receptors that constitute the aminoterminal and carboxyterminal portion of the hybrid constructs. The junction contains two amino acids originating from the *Bam*HI site created by PCR. The aminoterminal antibody epitope (FLAG) of FLAG-TR α 2 is shown in single letter code. (B) Transcriptional activity of hybrid receptors. CV-1 cells were cotransfected with 5 μ g of the reporter plasmids (ERE-tk-CAT or TRE₂-tk-CAT) and 5 μ g of the expression vectors pECE-ER-TR α 1, pECE-ER-TR α 2, or pECE-TR α 2-ER. Cells were treated with hormone as indicated (E₂, 10 nM; T₃, 50 nM; control, no hormone) and 24 h later assayed for CAT activity (CAT activities are normalized for β -galactosidase activity). A representative experiment is shown.

receptors were analyzed in the transient transfection assay, we observed that the TR α 2 DNA-binding domain was functional, as indicated by the estradiol-inducible activation of the TR α 2-ER hybrid receptor. However, the ligand-binding domain, i.e., the ER-TR α 2 hybrid, could not activate transcription (Figure 2B). In contrast, the corresponding ER-TR α 1 hybrid receptor, serving as a control, showed strong T₃-dependent transcriptional activation. The carboxyterminal domain of the TR α 2 protein thus abolishes transcriptional activation when transferred to a heterologous DNA-binding domain.

TR α 2 represses activation of TRE controlled promoters by TRs and heterologous receptors

It has been reported that TR α 2 inhibits TRE-dependent transcription by active TRs (Ko-enig *et al.*, 1989; Lazar *et al.*, 1989). Because

it is known that TREs can also respond to heterologous receptors, like the RAR (Graupner *et al.*, 1989), we investigated whether those receptor activities could also be effectively repressed by TR α 2. When TR α 2 was cotransfected together with either TR α 1, TR β 1, or RAR β , we observed that TR α 2 repressed the activity of all three receptors (Figure 3A). However, whereas partial repression was readily observed, complete repression could not be obtained, even when an excess of TR α 2 expression vector over the other receptors was used. The incomplete repression by TR α 2 is distinct from the repression pattern of RAR β and ER by TR α 1 and TR β 1, as described previously (Graupner *et al.*, 1989, 1991; Zhang *et al.*, 1991). These data, together with the lack of silencer activity, suggest that the repressor activity of TR α 2 results from a different mechanism.

Figure 3. Receptor-dependent TRE activation is repressed by TRα2. (A) Repression of TRβ1, TRα1, and RARβ by TRα2. CV-1 cells were cotransfected with 5 μg of TRE₂-tk-CAT and either one of the expression vectors—pECE-TRβ1 (1 μg), pECE-TRα1 (3 μg), or pECE-RARβ (2 μg)—together with pECE-TRα2 in the indicated amounts (controls [C] received no receptor plasmids). Cells were treated with hormone (□, 50 nM T₃ [TRβ1, TRα1], 600 nM retinoic acid [RARβ]), or no hormone (■). CAT-activity was determined by the classical method (protein extracts were normalized according to their β-galactosidase activity) and spots were quantified by liquid scintillation counting. The results shown represent the means (±SE) of two independent experiments. (B) Activation of thyroid hormone-responsive genes by ER is controlled by TRα2 and TRα1. CV-1 cells were cotransfected with 2

These conclusions were further supported by data obtained from cotransfections with an ER expression vector. The TRE and the estrogen response element (ERE) of the vitellogenin promoter are closely related (Glass *et al.*, 1988), and we have recently reported that the ER is also an efficient activator of the TRE (Graupner *et al.*, 1991). Figure 3B shows the activation of the TRE reporter gene by ER and repression of this activation by TRα2. As observed previously for RAR, the repression of ER activity was not released by T₃ (data not shown). A 50% repression of ER activity was observed when equal amounts of TRα2 and ER expression vectors were cotransfected with the reporter gene. When the TRα2 expression vector was used at a higher concentration, only a moderate increase in repression was observed. TRα1 was a more efficient repressor of ER than TRα2 but only in the absence of T₃. When we investigated the hybrid receptor ER-TRα2 for its ability to repress ER, we observed that this receptor behaved similarly to TRα2. This suggests that the TRα2 carboxyterminal domain maintains its distinct inhibitory properties when connected to the DNA-binding domain of another member of the nuclear receptor family.

To test whether a DNA-binding function is necessary at all for the repression, we constructed a vector expressing only the hormone binding/carboxyterminal domain (HBD) of TRα2 and transfected it together with the ER expression vector and the reporter gene. Figure 3C shows that the HBD-TRα2 construct repressed the activation of the TRE-reporter by the ER, suggesting that repression by TRα2 does not require DNA binding. The relatively high efficiency of repression observed for HBD-TRα2 may result from higher expression levels of the HBD-TRα2 protein because this construct con-

μg of TRE₂-tk-CAT, and 1 μg pECE-ER alone, or together with the indicated amounts of either pECE-TRα2, pECE-ER-TRα2, or pECE-TRα1 (controls [C] received no receptor plasmids). Cells were treated with 10 nM E₂ (□) or no hormone (■) and 24 h later assayed for CAT activity (CAT activity is corrected according to β-galactosidase activity). The results shown represent the means (±SE) of two independent experiments, each performed in duplicate. (C) Repression of ER activity by TRα2 does not require a functional DNA binding domain. CV-1 cells were cotransfected with 2 μg of TRE₂-tk-CAT and 0.5 μg pECE-ER alone or together with the indicated amounts of pECE-HBD-TRα2. Cells were treated with 10 nM E₂ (□) or no hormone (■) and 24 h later assayed for CAT activity (CAT activity is corrected according to β-galactosidase activity). The results shown represent the means (±SE) of a representative experiment performed in triplicate.

tains a perfect Kozak consensus sequence (Kozak, 1989).

DNA binding of TR α isoforms and hybrid receptors

To analyze further the mechanism of repression by TR α 2, we compared its DNA-binding properties with those of TR α 1. In vitro DNA-binding studies were carried out using the band-shift technique. Receptor proteins were synthesized by in vitro transcription/translation and incubated with 32 P-labeled TRE₂. As previously shown (Graupner *et al.*, 1989; Zhang *et al.*, 1991), TR α 1 bound effectively to the TRE₂ (Figure 4A). Binding to the TRE₂ was T₃-independent and specific, because it could be competed by unlabeled TRE₂, but not by a 50-fold excess of nonspecific DNA (Graupner *et al.*, 1989; Zhang *et al.*, 1991). However, under the same conditions, significant binding of the TR α 2 could not be detected (Figure 4A). This difference was not due to varying translation efficiencies, as revealed by sodium dodecyl sulfate-polyacryl-

amide gel electrophoresis (SDS-PAGE) analyses of 35 S-labeled translation products (data not shown). Moreover, when we investigated the binding of ER-hybrid receptors, we observed that the ER-TR α 1 hybrid that contained a normal receptor ligand-binding domain bound specifically to the TRE, whereas the ER-TR α 2 hybrid containing the altered carboxyterminus did not (Figure 4B). The same results were obtained when the binding of the hybrid receptors to the ERE was investigated (not shown). Thus, in analogy to the results from the transfection experiments, the carboxyterminal domains of TR α 2 abolished DNA binding even when transferred to other receptors.

To be able to probe specifically for the TR α 2 protein, we constructed a TR α 2 derivative that contained a nine-amino-acid peptide (FLAG) fused to its aminotermus. This derivative (FLAG-TR α 2) maintained the repressor function of TR α 2 (data not shown) and, like TR α 2, revealed no significant DNA binding (Figure 4A). However, when a specific monoclonal antibody directed against FLAG (anti-FLAG) was included

Figure 4. Binding of TR α 2, TR α 1, FLAG-TR α 2, and the hybrid receptors ER-TR α 1 and ER-TR α 2 to the TRE. In vitro synthesized receptor proteins were incubated with 32 P-labeled TRE₂ in the presence of T₃ (10 nM). Anti-FLAG antibody and specific competitor (50-fold excess of nonlabeled TRE₂) were present as indicated. Protein/DNA complexes were analyzed by gel retardation. Controls (none) show DNA binding of translation extracts that did not contain receptor protein.

in the DNA-binding reaction, strong DNA binding of the FLAG-TR α 2 protein was observed (Figure 4A). The complex displayed a strongly reduced mobility, indicating an antibody-FLAG-TR α 2-DNA complex. These observations revealed that the DNA-binding domain of the in vitro translated TR α 2 protein was principally functional and that DNA binding was stabilized in the presence of anti-FLAG antibody directed against an aminoterminal peptide of the receptor.

TR α 2 interacts with TR α 1

Significant DNA binding activity of TR α 2 and of the hybrid receptor ER-TR α 2 could not be detected by the band-shift assay (Figure 4). The repressor activities of TR α 2 and ER-TR α 2 could consequently not be explained by a competitive DNA-binding mechanism. We therefore investigated whether TR α 2 could interact with other receptors thereby interfering with their transcriptional enhancing activity. When FLAG-TR α 2 was mixed with TR α 1 before incubation with the TRE $_2$, the binding of TR α 1 was unchanged (Figure 5). However, when anti-FLAG antibody was included in this reaction, a major part of the prominent TR α 1-DNA complexes disappeared (Figure 5, compare lanes 5 and 6, open arrows) and was shifted up (Figure 5, filled arrow). The shifted band was much stronger than the one observed in the presence of FLAG-TR α 2 (Figure 5, compare lanes 3 and 6, filled arrows) alone, implying that TR α 1 was part of this "upshifted" complex. Because TR α 1 did not interact with anti-FLAG (not shown), this result strongly suggests that TR α 2 and TR α 1 interact and can form a DNA-binding complex.

Discussion

It has been reported that TR α 2 is not a transcriptional activator of thyroid hormone-responsive promoters (Izumo and Mahdavi, 1988; Lazar *et al.*, 1989). Our hybrid receptor analysis shows that this characteristic is an intrinsic function of the carboxyterminal domain. This domain, when transferred to other receptors, renders them transcriptionally inactive, as shown for the ER-TR α 2 hybrid, whereas the analogous ER-TR α 1 hybrid was transcriptionally active. The molecular basis for this lack of transcriptional activity has not yet been determined. Obviously, the alternative carboxyterminus interferes on several levels with TR function, i.e., hormone binding, DNA binding, and transcriptional activation. Originally, it was reported that

Figure 5. TR α 2 interacts with TR α 1. Proteins were synthesized in vitro and incubated either separately or as mixtures with 32 P-labeled TRE $_2$. All samples were adjusted to the same protein concentration by addition of lysate. Anti-FLAG antibody was present as indicated. Complexes were analyzed by gel retardation. Controls (none) show binding of translation extracts that did not contain receptor protein. Open arrows mark complexes specific for TR α 1 and the closed arrow indicates the complex formed with FLAG-TR α 2 in the presence of anti-FLAG antibody.

in vitro translated TR α 2 binds T $_3$ (Benbrook and Pfahl, 1987; Nakai *et al.*, 1988b). A number of more recent studies showed that this is apparently not the case and that the original data might have resulted from the in vitro system (Mitsuhashi *et al.*, 1988; Lazar *et al.*, 1989; Schueler *et al.*, 1990). In this context, it is interesting to note that the *v-erbA* protein, which does not respond to thyroid hormones in vertebrate cells, does show a response to T $_3$ in yeast (Privalsky *et al.*, 1990). It cannot yet be excluded that TR α 2 responds to a different ligand, although neither the compounds investigated here nor the organic solvent extracts

from rat brain or liver showed transcriptional activation.

Compared with TR α 1, the DNA-binding properties of cell-free translated TR α 2 are greatly impaired, consistent with reduced TR α 2 binding observed by others in cell extracts (Rentoumis *et al.*, 1990) or different assay systems (Lazar *et al.*, 1988). From our current understanding, there are several possible explanations for this finding: (1) the longer carboxyterminus of TR α 2 might mask its DNA-binding domain and directly interfere with DNA binding; (2) the active DNA binding forms of receptors are dimers, and the altered carboxyterminus of TR α 2 prevents efficient homodimer formation; (3) a combination of (1) and (2), in which the TR α 2 carboxyterminus interferes with both DNA binding of the monomer and its dimerization. Our observation that the antibody induced DNA binding of FLAG-TR α 2 is consistent with the second hypothesis but does not exclude completely the third possibility. Artificial dimerization of FLAG-TR α 2 via the two antigenic binding sites of the IgG can mimic natural dimerization and therefore restore high-affinity DNA binding. Comparable antibody-induced DNA binding has recently been reported for the ER, which is unable to dimerize in the presence of the anti-estrogen ICI 164384. The use of a specific antibody directed against the ER aminoterminal region allowed DNA binding in the presence of the ICI 164384 anti-estrogen (Fawell *et al.*, 1990).

Data from a detailed deletion analysis of TR β 1 show that the carboxyterminal region of this receptor is necessary for dimerization and DNA binding (Zhang *et al.*, 1991). Deletion of 17 amino acid residues from TR β 1 resulted in reduced DNA binding, and deletion of additional 19 amino acid residues abolished DNA binding completely. Because the carboxyterminal regions show considerable homology between TR α 1 and TR β 1 (but not with TR α 2), loss of these regions through alternative splicing is therefore likely to result in altered dimerization or oligomerization properties of TR α 2. Modulation of DNA binding and dimerization properties by alternative splicing has also been observed for other DNA-binding proteins, notably the products of the E2A gene (Sun and Baltimore, 1991). A third possibility, that significant binding of TR α 2 could not be detected due to inefficient translation in the *in vitro* system, is unlikely because SDS-PAGE analysis showed a comparable translation efficiency of TR α 2 and TR α 1. A careful analysis of mutations present in the *v-erbA* protein defined a region of 9 amino

acids in the very carboxyterminus of TR α 1 that is necessary for transcriptional activation (Zenke *et al.*, 1990). This region is replaced in TR α 2 by the altered carboxyterminus, which offers an explanation for the loss of its transcription activation function.

Silencing activity of active TRs and the *v-erbA* oncogene product has been attributed to the strong DNA binding of these proteins (Damm *et al.*, 1989; Sap *et al.*, 1989; Baniahmad *et al.*, 1990; Zhang *et al.*, 1991). No significant silencing effect on the constitutive reporter used here could be observed for TR α 2, consistent with its impaired DNA binding. However, TR α 2 repressed activation of the TRE reporter by other active receptors. For repression of TR β 1, TR α 1, and RAR β , an excess of TR α 2 was necessary, whereas ER activity was repressed by 50% at an equimolar concentration. Similar repression was obtained with the hybrid receptor ER-TR α 2. As observed for TR α 2, this fusion protein did not show significant binding to the TRE. Therefore, we concluded that the TR α 2 carboxyterminus abolishes strong DNA binding but retains its repressing activity even when fused to a different DNA-binding domain. The observation that the carboxyterminal domain of TR α 2 by itself can cause repression of ER activity reveals that for this function DNA binding is not required. In addition, these data suggest an interaction between ER and the carboxyterminus of TR α 2.

It has been proposed that the repression observed with TR α 2 could be due to its interaction with a limiting factor necessary for the transcriptional activity of TR α 1 (Rentoumis *et al.*, 1990). However, our data showing that DNA complexes with TR α 1 can be upshifted with an antibody directed specifically against TR α 2 point to a more direct action of TR α 2, in which this receptor variant forms heterodimers with TR α 1. Thus, both isoforms could form heterodimers that still bind to DNA but have reduced transcriptional activity. We are currently investigating this possibility in more detail and whether the mechanism of repression by TR α 2 is common for all the receptors it represses (TRs, ER, and RAR). The fact that receptors of at least three different classes can activate TRE-controlled genes points to the broad specificity of those elements. Repression of heterologous receptors by TR α 2 could increase the specificity of TREs without silencing basal-level transcription of associated promoters. In addition, TR α 2 could modulate the response of active TRs and thereby fine-tune the T₃ response. TR α 2 is

highly transcribed together with TR α 1 in several brain tissues during postnatal development and in the adult (Bradley *et al.*, 1989; Wills *et al.*, 1991), tissues that depend critically on thyroid hormones for normal development.

All data on the repression function of TR α 2 obtained so far were derived from transient transfection studies, and it may be possible that repression is not the only or main function of this protein. Recent immunohistochemical studies (Strait *et al.*, 1991) using antisera directed specifically against TR α 2 proved that not only the message but also the TR α 2 protein is highly expressed in rat brain and in testis, strongly suggesting an important function for this protein. More specifically, among the testicular cells TR α 2 was shown to be expressed exclusively in the nuclei of dividing spermatogonia and disappeared after maturation into spermatids. Notably, our human TR α 2 cDNA clone was obtained from a testis library, and it will be interesting to assess whether the observed repressor function of TR α 2 can also be found for the endogenously expressed protein and whether it plays a regulatory role in spermatogenesis.

Methods

Plasmid construction

cDNA sequences of all receptors used in this study were excised from their cloning vectors by appropriate restriction digests and inserted into the multiple cloning site of the eukaryotic expression vector pECE (Ellis *et al.*, 1986). pECE-TR α 2 was constructed by inserting a *Bam*HI-*Kpn*I DNA fragment derived from clone T-1-4 (Benbrook and Pfahl, 1987) into pECE. pECE-ER was derived by transferring an *Eco*RI fragment that contained the ER coding region from B1-ER (Benbrook and Pfahl, 1987) fragment into pECE. pECE-TR α 1, pECE-TR β 1, and pECE-RAR have been described previously (Graupner *et al.*, 1989). The RAR used throughout this work is the RAR β or ϵ (Benbrook *et al.*, 1988; Brand *et al.*, 1988). The TRE-reporter plasmid, TRE₂-tk-CAT, contains a double repeat of the synthetic palindromic thyroid-responsive element (Glass *et al.*, 1988). It was obtained by insertion of two copies of the TRE sequence 5'-GATCTCAGGTCAT-GACCTGA-3' (containing *Bgl* II linkers) into the *Bam*HI site of pBL-CAT2 (Luckow and Schütz, 1987). The ERE-tk-CAT contains a single palindromic ERE of the *Xenopus* vitellogenin gene (Klein-Hitpass *et al.*, 1986).

To provide a vector that allows expression of various FLAG-fusion proteins, we cloned into a *Bgl* II-*Sa*I I-digested pECE the double-stranded, synthetic fragment

encoding the FLAG-sequence (Hopp *et al.*, 1988) under control of the Kozak consensus sequence (Kozak, 1989). The resulting vector (pECE-FLAG) allows fusion of cDNA sequences to FLAG via various sites and the easy transfer of the resulting constructs via the internal *Not* I and *Nco* I sites.

Hybrid receptors and truncated receptors

Hybrid receptor genes were constructed by fusion of the aminoterminal and the carboxyterminus of two different receptors in the hinge regions. Specific oligonucleotide primers complementary to sequences in their hinge regions were designed that carried at their 5'-ends an additional *Bam*HI recognition sequence. By low-cycle polymerase chain reaction (PCR)—using standard vector primers (T7, T3, and SP6) together with the specific ones—the corresponding receptor fragments were obtained. The fragments were ligated at the introduced *Bam*HI site and cloned into Bluescript (Stratagene, La Jolla, CA) and pECE. The resulting hybrid genes contained an artificial *Bam*HI site in the hinge region. The PCR mixture (100 μ l) contained 1 μ g of the parental receptor plasmid, oligonucleotides at 1 μ M each, dNTPs at 200 μ M each, and 2.5 U of Taq-polymerase (Perkin Elmer-Cetus Norwalk, CT) in reaction buffer (50 mM KCl, 10 mM tris(hydroxymethyl)aminomethane pH 8.3, 1.5 mM MgCl₂, 0.01% [wt/vol] gelatin). After 10 cycles of amplification (denaturation: 1.5 min, 94°C; annealing: 2 min, 37°C; extension: 3 min, 72°C), reaction products were purified on agarose gels, digested, ligated, and cloned into Bluescript and pECE. To check the hybrid protein for correct size, we synthesized proteins by *in vitro* transcription and translation in the presence of ³⁵S-methionine and analyzed them by SDS-PAGE. The following specific oligonucleotide primers were used in the construction of hybrid receptor genes (*Bam*HI site underlined):

5'-TCTGGATCCGATCCCACTCTTCAG-3' for the aminoterminal of TR α 2,

5'-TTGCGGATCCTCATGTCTCCAGCA-3' for the aminoterminal of ER,

5'-AAGGGATCCATCTGATCCACATTG-3' for the carboxyterminus of TR α 1 and TR α 2,

5'-GCGGATCCGAGCTGCCAACCTTTG-3' for the carboxyterminus of ER.

The same strategy was used to construct FLAG-TR α 2. In this case, the TR α 2 cDNA was amplified starting from the codon corresponding to the second amino acid using the oligonucleotide 5'-GAAGATCTTGAACAGAAGCCAA-3' (*Bgl* II site underlined) as specific primer. The fragment obtained was digested, inserted into pECE-FLAG, and from there transferred into *Not* I-*Kpn* I-digested Bluescript. *In vitro* synthesized ³⁵S-labeled FLAG-TR α 2 was checked for correct size and antigenic specificity by immunoprecipitation with anti-FLAG monoclonal antibody (M2) followed by SDS-PAGE analysis.

The expression vector for HBD-TR α 2 was obtained by cloning the TR α 2 carboxyterminus into the *Bgl* II-digested pECE-FLAG vector such that an in-frame translational start site was provided.

5'-GATCCGCGGCCGCCACCATGGATTACAAGGACGACGACGAT AAGATCTG-3'

3'-GCGCCGCGGGTGGTACCTAATGTTCTGCTGCTGCTATTCTAGACAGCT-5'

Transient transfection and CAT-assay

CV-1 cells were plated at a density of $1.3\text{--}1.7 \times 10^6$ cells per dish (10 cm) 16–24 h before transfection in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). One to 3 h before transfection, cells were fed with 10 ml DMEM supplemented with 10% charcoal-treated FCS. Cells were transfected by a modified calcium phosphate precipitation procedure (Chen and Okayama, 1988). The precipitates contained 2 μ g reporter plasmid, 1–7 μ g receptor expression vector, 3 μ g β -galactosidase control plasmid (pCH110, Pharmacia, Piscataway, NJ), and Bluescript plasmid (to obtain a total amount of 23 μ g DNA). The transfections were carried out as described (Pfahl *et al.*, 1990). CAT activity was determined by either the classical assay (Gorman *et al.*, 1982) or a modified assay using ^3H -acetyl-coenzyme A as substrate (Nielsen *et al.*, 1989). Counts per minute corrected for β -galactosidase activity (to normalize for transfection efficiency [Herbomel *et al.*, 1984]) were expressed as relative CAT activity.

DNA-binding analysis

In vitro synthesis of receptor proteins. Receptor protein was synthesized by in vitro transcription/translation as described (Graupner *et al.*, 1989; Pfahl *et al.*, 1990). To determine quantity and size of the proteins, we did translations in the presence of ^{35}S -methionine in parallel and analyzed synthesized proteins by SDS-PAGE.

Gel retardation assay. In general, the assay was carried out as described previously (Graupner *et al.*, 1989). One to 10 μ l of in vitro translated receptor protein was incubated with double-stranded, ^{32}P -labeled TRE-dimer (5'-GATCT-CAGGTCATGACCTGAGATCTCAGGTCATGACCTGA-3') in a 20- μ l reaction mixture containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl₂, 10% glycerol, and 1 μ g poly(dI-dC) at 25°C for 20 min. In experiments in which different proteins were mixed, all samples were adjusted to the same protein concentration by addition of reticulocyte lysate. Anti-FLAG antibody (0.2 μ g) was incubated with the protein mixtures for 15 min at 25°C before addition of the DNA. The reaction mixture was analyzed on a 4% nondenaturing polyacrylamide gel containing the electrophoresis buffer (10 mM HEPES, 3.3 mM sodium acetate, pH 7.9). Electrophoresis was performed at 4°C (140 V, continuous circulation).

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