A Thyroid Hormone Receptor-Dependent Glucocorticoid Induction

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Glucocorticoid and thyroid hormones exert their effects in many body tissues by binding to their respective receptors. The search for possible cross-talking mechanisms in overlapping target cells led to the discovery of synergism between a thyroid hormone receptor-binding site and a cryptic glucocorticoid-responsive element. Glucocorticoid responsiveness could only be detected in the presence of thyroid hormone and its receptor. This synergism requires the glucocorticoid receptor (GR) DNA-binding domain and is mediated by the trans-activation domains. We found that synergism also occurs when the thyroid hormone receptor is replaced by the retinoic acid receptor or the GR is replaced by the progesterone receptor. Synergism is qualitatively independent of the type of thyroid hormone receptor-binding site and promoter. In several combinations of promoter and response elements, including a retinoic acid response element, T3 induction was only seen in the presence of the cryptic glucocorticoid-responsive element, GR, and glucocorticoids. (Molecular Endocrinology 8: 440-447, 1994)

RESULTS

Functional Responsive Elements Reveal the Presence of a Cryptic GRE

To study the combined gene regulation of GR and TR, we cotransfected expression vectors coding for the rat TRα and the human GRα. Their effect on gene regulation with or without the corresponding ligands was determined using a reporter gene consisting of the thymidine kinase (tk) promoter and the chloramphenicol acetyltransferase (CAT) gene. Binding sites for the receptors were inserted in front of the tk promoter (see Fig. 2C). Transfections were performed using the human mammary carcinoma cell line T47D, which is capable of eliciting high hormonal responses.

Expression of the tkCAT gene construct containing the chicken lysozyme T3RE (GREc-T3RElys-tkCAT; see below) was induced 6-fold by the addition of T3 hormone (Fig. 1a) in the presence of rat (r) TRα and human (h) GRα. In contrast, the addition of dexamethasone (dex) alone had no effect. The combination of both hormones resulted in a very high (90-fold) induction of transcription, suggesting a synergistic action between the two hormone receptors. Detailed sequence analysis of the reporter plasmid revealed the presence of an element resembling a GRE (see also Fig. 2C) located 265 bp upstream of the T3RElys. Binding assays using a consensus palindromic GRE, the 38-bp NcoI fragment containing the GRE-like element, or various other vector fragments as competitors demonstrated GR binding to the NcoI fragment (see also Fig. 2). We termed the element located in this fragment a cryptic
Thyroid Hormone Synergism

Fig. 1. T3RElys Synergizes with a cGRE

T47D cells were transfected with 0.1 pmol hGRα and 0.1 pmol rTRα expression vector together with 1 pmol of different tk-CAT reporter plasmids, as indicated. Hormones were added 2 h after transfection at the following concentrations: 0.1 μM L-T3 and 0.5 μM dex (a synthetic glucocorticoid). Cells were harvested after 2 days. CAT activities are expressed as the percent CAT conversion, and the so from three independent experiments is indicated. For activities higher than 70% CAT conversion, the assay was repeated using less cell extract, and the value was normalized to the weakly active extracts.

GRE (GREc), because it is unable to mediate glucocorticoid responsiveness in the absence of T3 hormone. Deletion of up-stream sequences including the GREc did not abrogate T3 inducibility, but abolished the synergistic GR effect (Fig. 1b), indicating that the cryptic binding site is required for GR/TR synergism. In fact, the addition of dex leads to a weak decrease in T3 induction, possibly due to a squelching effect in the presence of both hormones. As the ligand-free TR is able to repress transcription, we investigated whether the observed T3/dex effect is due to stimulation or to a release of repression. Therefore, we used a reporter plasmid containing a mutation in the T3RE so that the T3 receptor cannot bind (GREc-T3REmut-tkCAT; see Materials and Methods). The basal level in the absence of hormone was similar to that obtained with GREc-T3RElys-tkCAT, indicating that this low basal level is not due to repression via the T3RElys. Neither the separate addition of each hormone nor the combination of both hormones resulted in a detectable induction of GREc-T3REmut-tkCAT (Fig. 1c), stressing the absolute requirement of the T3RE for the observed effects.

Our data indicate a synergistic effect between T3RElys and GREc. The synergism requires both binding sites, although the GREc is nonfunctional when tested alone.

The relative orientation of the two receptors or the distance between the binding sites is not crucial for the synergistic effect. A reporter construct (GREc+4-T3RElys-tkCAT) containing a 4-bp insertion, i.e. about half a helical turn, between GREc and T3RElys displayed the same inducibility as the original construct (Fig. 1d). This result suggests that a strict spatial ar-

Fig. 2. GREc Weakly Binds the DNA of the GR

Bacterially expressed GR-DBD is incubated with the indicated DNA probes, and the complexes formed are analyzed on a 5% native polyacrylamide gel electrophoresis. A, A GREpal (lanes 1–3) or GREc (lanes 4–6) probe was incubated in the presence of 0.4 or 4 μg bacterial extracts, respectively. Lanes 1 and 4, extracts from bacteria nontransformed with expression vector but induced with IPTG were used. Lanes 2 and 5 show the results obtained using transformed but noninduced bacterial extracts. Lanes 3 and 6 show the complexes formed using GR-DBD-containing extracts. B, A 10-fold longer exposure of the gel is shown, displaying lane 6 from A (lane 1) and the results obtained after incubation of a GAL-4 DNA-binding site containing probe with 4 μg extracts from nontransformed (lane 2), noninduced (lane 3), and transformed plus induced bacteria (lane 4). C, Schematic representation of the reporter plasmid GREc-T3RElys-tkCAT. Boxes represent the tkCAT gene with its transcriptional start site (+1); the relative positions of the regular response element (TRE and RARE) and the GREc are indicated. The bottom shows a sequence comparison of GREc with the canonical GREpal and a consensus negative GRE (nGRE).

rangement of the GR or TR is not required for GR/TR synergism.

Cryptic GRE Weakly Binds the Receptor

To ensure that the cryptic GRE is able to bind GR, we performed gel retardation experiments. As the GREc is unable to mediate GR inducibility on its own, and the homology to known GREs is less than 60% (Fig. 2C), we expected the binding to be weak compared to that of a consensus palindromic GRE. Therefore, we used
the bacterially expressed DNA-binding domain (GR-DBD; amino acids 370–503) of hGR, which is present in large amounts in crude bacterial extracts. This protein contains the zinc finger domain of the GR, which is sufficient for specific GRE binding of the receptor (16–19). Incubation of extracts containing GR-DBD with a labeled GREpal probe led to the formation of a protein-DNA complex (Fig. 2A, lane 3). This complex is specific for the expressed GR-DBD, as neither extracts from nontransformed bacteria nor extracts from transformed bacteria in the absence of inducer (IPTG) produced a similar complex (Fig. 2, lanes 1 and 2, respectively). Similarly, a specific complex was formed using a GREc probe and the GR-DBD-containing extracts (lane 6), but not with control extracts (lanes 4 and 5). As expected, this complex was much weaker than that obtained with GREpal. Densitometric scanning analysis of the autoradiograms revealed roughly a 400- to 500-fold lower affinity of the GR-DBD for GREc compared to GREpal. It should be stressed that for GREpal, the amount of protein extract used was 10-fold less than that for GREc. The complexes shown are not due to nonspecific DNA binding of the GR-DBD, as incubation of the same extracts with an unrelated DNA sequence containing a GAL-4-binding site did not give rise to any complex, even after a 10-fold longer exposure of the gel (Fig. 2B, lanes 2–4).

Taken together, these results show that the GREc sequence at position −265 is able to bind the GR weakly. Interestingly, the complex formed with GREc migrates faster in the gel than the GREpal complex, despite the fact that the probe is somewhat longer, suggesting a different arrangement of the GR on this weak binding site.

Synergism Depends on the Transactivation Function of the GR and Requires the DBD of the Receptor

We wanted to localize the GR domains required for synergism. In the first set of transfection experiments, mutants of the GR were cotransfected with 0.1 pmol TR expression plasmid. Their ability to synergize with TR (fold synergism) was compared to their transactivation capacity (fold induction), determined by cotransfecting the mutants with the reporter GREc-(GREpal)x2-tkCAT (18) containing a highly inducible glucocorticoid-responsive unit. The hormone-binding domain of the GR was deleted (Fig. 4), generating constitutively active transcription factors (17). Thus, the addition of dexamethasone can be avoided, and possible effects of the endogenous GR can be excluded. Synergism was measured by comparing the T3-induced CAT values without and with cotransfection of the GR mutants. As demonstrated in Fig. 3A, deletion of the hormone-binding domain results in proteins functional in synergism. Deletion of the r2 transactivating domain (hGR 1–515) reduces both transactivation and synergism. Deletion of r1 in hGR 262–550 and hGR 262–515 results in similar activities as the r2 deletion, although the r1 deletion more strongly affected transactivation (100-fold, compare 1–515 to 262–515) than the deletion of r2 (10-fold; compare 1–550 to 1–515 or 262–550 to 262–515). The GR/TR synergism mediated by GR mutants containing only one transactivation domain was similarly low. When the complete DBD of the GR mutants was replaced by that of the yeast transcription factor GAL4, the resulting fusion protein completely lost its synergizing capability (Fig. 3A). To assess more specifically the role of the GR DNA-binding function, a
DNA binding-deficient mutant was tested. Mutant 1–550/G441 carries a deletion of the hormone-binding domain and a C to G conversion at position 441 located in the first zinc finger, which completely abolishes the DNA-binding ability (17). Figure 3B shows that this mutant is unable to mediate both transactivation and synergism with TR, in contrast to the constitutive activity of mutant 1–550.

These data show that transactivation and synergism with TR mediated by GR require the same GR domains. On the other hand, the absolute requirement for the DBD of GR agrees with the involvement of the cryptic binding site GREc.

Progestosterone and Retinoic Acid (RA) Receptor (RAR) Can Replace GR or TR, Respectively

To assess the general validity of the observed synergism, we first tested whether the participating hormone receptors can be replaced by other members of the steroid receptor superfamily. First we asked whether the progesterone receptor (PR), which normally displays the same binding specificity as the GR, would be able to synergize with TR. The data presented in Fig. 4a show that the GR can functionally be replaced by the endogenous PR of the T47D cells (addition of progestin R5020 at 0.01 μM) to reproduce the synergistic effect. Thus, like the GR, the PR is unable to mediate induction via the GREc alone, but strongly synergizes with the T3-activated TR. Additionally, a reporter plasmid containing a naturally occurring RA response element (RARE), DR5 (20, 21), was used to test whether the hRARα (22) is able to substitute for TR in synergism with GR. Cotransfection of GR and hRARα expression vectors with GREc-DR5-tkCAT produced a 30-fold induction of transcription by RA alone (Fig. 4b). The addition of both ligands, dex and RA, led to a 3-fold increase in induction.

Several Different TREs Are Able to Mediate Synergism

In the next set of experiments we investigated whether the synergistic response obtained by the combined action of TR and GR is restricted to the inverted palindromic structure of the chicken lysozyme T3RE. Thus, we compared the different TR-binding sequences T3RElys, T3REpal (23), T3RElap (24), and the direct repeat DR4 (20, 21) for their ability to confer synergism in combination with TR and GR (0.1 pmol rTRα and 0.1 pmol hGRα). The results shown in Fig. 5 clearly demonstrate that the orientation of the palindromic T3RE half-sites does not play a major role, resulting in only a marginal quantitative difference. The high transcriptional activity of GREc-DR4-tkCAT in the presence of both hormones probably results from the already high T3 response of this construct.

Different Promoters Respond to GR-TR Synergism

To test whether the HSV-tk promoter sequences are required for the GR/TR synergistic response, we con-
constructed a set of four additional reporter plasmids. The tk promoter sequences were replaced by two other viral core promoters [simian virus-40 (SV40) early and adenovirus major late promoters], by the basal promoter of the human α-globin gene, or by the human metallothionein-II gene (Fig. 6A). The chicken lysozyme T₃RE was inserted in the SalI site of the polylinker, and all constructs were tested for synergism using identical conditions (0.1 pmol rTRα and 0.1 pmol hGRα). The results from this set of transfection experiments are presented in Fig. 6B. All promoters produced a synergistic response, extending the general validity of GR/TR synergism. These experiments demonstrate the difference in the magnitude between single induction and synergism. Two of the T₃RE promoter combinations tested (GREc-T₃RElys-glob-CAT and GREc-T₃RElys-adeno-CAT) did not respond to the addition of thyroid hormone or dex, respectively. Only the combined administration of both hormones resulted in an induction of approximately 250-fold, in contrast to the about 28-fold synergistic induction (T₃ vs. dex plus T₃) observed for the three other promoters. This result defines a new type of glucocorticoid-responsive unit:

**Synergism Occurs in Different Cell Lines**

To test whether the synergism between GR and TR is a cell-specific effect, the reporter plasmid GREc-T₃RElys-glob-CAT was transfected together with expression plasmids for TR and GR into additional cell lines. In MCF-7 cells, a human mammary carcinoma line, a strong synergism was seen compared to the basal level obtained with single hormone addition, although CAT activity in the presence of both hormones was lower than that in T47D cells (Fig. 7). In CV1 cells, the CAT activity was much lower, but a clear T₃ induction, depending on the presence of dex, was detected.

**Synergism Alters the Specificity of Responsive Elements**

As shown in Fig. 8a, the RA-induced activation of the RAR on a DR5, a naturally occurring RARE, containing reporter plasmid could be increased 3-fold by simultaneous addition of Dex. We were interested in the effect of TR on such a reporter plasmid. It had been shown that TR is able to bind to a DR5 element, but cannot transactivate this RARE (20, 21). We cotransfected a T₃RE containing promoters insensitive to T₃ induction functionally respond to glucocorticoids in the presence of T₃ and a cryptic GRE.

**Fig. 6. Promoter-Specific Responsiveness to Synergism**

Cotransfection of rTRα and hGRα expression plasmids with various reporter constructs. The reporter constructs are depicted in A; the position of the inserted T₃RElys fragment is indicated by a triangle. B, CAT activities of transfection experiments. Hormone additions are explained in the inset. The lower part of the figure is magnified so as that any small effects by individual hormones can be seen.

**Fig. 7. Synergism Is Detected in Different Cell Lines**

GREc-T₃RElys-globCAT was transfected into T47D, MCF-7, and CV1 cells along with expression vectors for GR and TR. CAT conversion in the presence of the indicated hormones is shown.
**Thyroid Hormone Synergism**

**Hormone A:**

**Hormone B:**

**Receptor A:**

**Receptor B:**

**Reporter:**

\[ \text{GREc} \quad \text{GREc} \quad \text{GREc} \]

\[ \text{DR5} \quad \text{DR5} \quad \text{DR5} \]

\[ \text{tkCAT} \quad \text{tkCAT} \quad \text{tkCAT} \]

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**Fig. 8.** The Specificity of a Hormone Response Element Can Be Changed by Synergism

A. Cotransfection of 1 pmol GREc-DRS-tkCAT with 0.1 pmol hRARα and 0.1 pmol hGRα expression plasmid. B. Cotransfection of 1 pmol GREc-DRS-tkCAT with 0.1 pmol rTRα and 0.1 pmol hGRα expression plasmid. Induction with hormone was performed as described in Fig. 4.

GREc-DRS5-tk-CAT reporter plasmid with expression plasmids for rTRα and hGRα (Fig. 8b). As expected, no T₃ induction could be seen. The addition of glucocorticoids alone could not activate transcription, as shown in all previous cases. Induction of both receptors resulted in the activation of transcription comparable to that of the RAR shown in Fig. 8a.

By the synergistic action of two receptors, which alone are unable to activate this gene, the specificity of the RARE is overruled, and TR can mediate activation through this element in the presence of GR.

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**DISCUSSION**

The effects of steroids and thyroid hormones on development, differentiation, and gene regulation have been intensively studied. Thyroid hormones and the glucocorticoid cortisol were the first hormones to be purified and characterized. In fact, most body tissues respond to these hormones. Therefore, the simultaneous action conferred by the receptors for both hormones is the rule rather than an exception.

Here we show that the presence of a cryptic GRE is able to mediate a strong synergism between TR and GR. Several lines of evidence illustrate the requirement for specific DNA binding of GR in synergism. Changing the DNA binding specificity of the GR by switching to the GAL4 DBD or destroying the DNA binding of the GR by a point mutation abolished the effect. Deletion of the GREc in the CAT vector resulted in a loss of synergistic activation. Finally, we could show that this DNA element binds the GR in vitro, although with a very low affinity. It is unclear whether the slightly different electrophoretic mobility of the GR/GRE complex compared to that of the GR/GREpal reflects a different binding geometry of the receptor to these elements, as was described for a negative GRE in the POMC gene (25). Very importantly, the presence of the GREc could only be functionally detected in the presence of a consensus receptor-binding site, a T₃RE or RARE. This underscores the importance of weak binding sites that show strong activities in the presence of other elements, in our case mediating a thyroid hormone-dependent glucocorticoid induction.

We believe that the phenomenon of synergism explains the cooperative effect of the two hormones seen in several cases. For example, the rat phosphoenolpyruvate carboxykinase gene requires both hormones for induction (26). Furthermore, synergism may explain how T₃ induction of the GH gene can be potentiated by glucocorticoids. Either T₃ or RA is required to mediate glucocorticoid induction of the GH gene (27, 28). Multiple T₃REs have been found in the rat GH gene (29), whereas a GRE has not been identified. Also, the concentration of TR is not changed by glucocorticoid induction (30). Thus, GH induction may be mediated by synergism of a cryptic GRE with the T₃RE sequences.

In addition to increasing the T₃ stimulus, synergism increases the number of GR-inducible genes, as T₃-inducible genes containing cryptic GREs may be inducible by glucocorticoids as well. This effect is very striking for promoters that contain a T₃RE but are minimally T₃ responsive, as we have demonstrated in two cases. In addition, we showed that an otherwise unresponsive TR-binding site (DR5), which is a specific RA-responsive element, is turned to a thyroid hormone-responsive element in the presence of GREc. These observations suggest that the response pattern of a gene to various stimuli is influenced by very weak binding sites for some transcription factors.

An additional complexity can be introduced by the apparent cell specificity of GR/TR synergism. Although the effect is seen in different cell lines, it is much stronger in the mammary tumor cell lines T47D and MCF-7. It is unclear whether this observation is due to the generally more active transcription machinery in these cell lines or whether it might be related to the mammary carcinomal origin of the cells.

Possible interplays between identical or different signal transduction pathways have been demonstrated between nuclear receptors and nonreceptor transcription factors binding to adjacent sites (1, 3, 13, 14). Depending on the factors involved, different domains of the GR are required for synergism: the DBD (18), the transactivating domain, or the steroid-binding domain (31). Analysis of GR deletion mutants revealed that the transactivating domains are required for synergism with TR mediated by GREc. The hormone-binding domain
is not involved, in contrast to the previously described synergism of a consensus GRE with a CACCC box (31).

MATERIALS AND METHODS

Plasmids

Expression vectors for the different receptors were kindly provided by R. Evans (10, 22, 32). Expression vectors for GnR mutants have been previously described (17, 31) as well as the vector for GAL-GR and the bacterial expression vector for Gn RD (19). Previously described reporter plasmids contain the vector-based GRE and were, therefore, renamed GREc-TRElys-tkCAT, GREc-TREmut-tkCAT, GREc-TREpal-tkCAT, and GREc-TREIAP-tkCAT correspond, respectively, to F2, TREmut-, TREpal- and TREpal-tkCAT in Ref. 24. GREc-GREpalx2-tkCAT and GREc-GRE-tkCAT were described previously (14, 18) as pGpal29 Gpal tkCAT and pGpal29 Gpal tkCAT, respectively. GREc-(GREpal)x2-tkCAT and GREc-GRE-tkCAT were constructed as follows (compare Fig. 7). The SV40 core promoter sequences in front of the CAT-coding region were constructed as follows (compare Fig. 7). The SV40 core promoter sequences were isolated as a HindIII (filled-in)/BglII fragment from pA10CAT2 (33) and inserted into the XhoI (filled-in)/BglII sites of pBLCAT2 (34). The promoter sequences, including a part of the polylinker, were excised as a HindIII (filled-in)/PstI fragment and reinserted into the BamHI (filled-in)/PstI sites of pBLCAT. Finally, transactivating up-stream vector sequences were removed by a HindIII/BglII digestion. The construct hMT-IIA-CAT was made by ligation of the HindIII (filled-in)/BglII metallothionein promoter fragment of MCAT2 (gift from P. Mitchel and R. Tjian), a HindIII/BglII polylinker fragment of pBLCAT, and the vector ptkCATAH/N (24) from which the HSV-tk promoter was deleted by EcoRI (filled-in)/HindIII digests. The construct h-globin-CAT was made by replacing the HSV-tk promoter of ptkCAT3/H/N (BamHI (filled-in)/BglII (filled-in)) by the Xmal (filled-in)/NcoI (filled-in) promoter fragment of the plasmid pX-globin (gift from B. Ondek and W. Herr). In the reporter plasmid adenoML-CAT the HSV-tk promoter of the parental CAT construct ptkCAT3/H/N was replaced by a synthetic oligonucleotide and using the adenovirus major late promoter sequences (35) from 5′-53 to 41 bp plus half of the BamHI and XhoI restriction sites. The correct sequences were verified by sequencing. Derivatives of the CAT constructs described above, containing in addition the chicken lysozyme thyroid response element T3RElys (F2) (24), were generated by replacing the HindIII/BamHI polylinker of the respective parental plasmids by the HindIII/BamHI polylinker fragment of F2tkCAT. In this fragment, the lysozyme T3RE was previously described (38) with minor modifications (36). All cells were harvested 2 days after transfection. Diagnostic cotransfections with a control plasmid showed the reproducibility of the transfections. Therefore, the CAT activities achieved did not have to be corrected, rather the mean and so from independent triplicate experiments are presented.

Bacterial Extracts and Gel Retardation

Extracts were made from bacteria transformed with an expression vector coding for the hGRa DBD (residues 370–502). After induction of expression by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside, bacteria were processed as previously described (10, 19). Gel retardation experiments were performed (18). The probes were obtained as follows. GREc was a NcoI fragment spanning nucleotides 49–84 from GREc-TRElys-tkCAT. GREpal was a HindIII/XbaI fragment from GREc-GREpalx2-tkCAT. Both were purified by polyacrylamide gel electrophoresis and eluted from the gel. The UAS probe was a synthetic oligonucleotide previously described (18). The probes were 32P labeled using polynucleotide kinase.

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