Multiple Domains of the Glucocorticoid Receptor Involved in Synergism with the CACCC Box Factor(s)

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Steroid induction of responsive genes functions through the synergistic activity of steroid receptor-binding sequences with adjacent transcription factor-binding sites. To analyze the mechanism of synergy we tested different human glucocorticoid receptor mutants for synergistic function with another transcription factor in comparison with intrinsic *trans*-activation obtained with a single receptor binding site (glucocorticoid response element). Multiple domains were found to be involved in synergistic activity of the glucocorticoid receptor with the CACCC box factor. Deletions within the N-terminal receptor half affected simultaneously intrinsic *trans*-activation and synergism. However, deletion of the hormone-binding domain mainly impaired synergism rather than intrinsic *trans*-activation, clearly showing that this domain synergizes by a mechanism independent of intrinsic activation. A chimeric protein where the DNA-binding domain of the glucocorticoid receptor was replaced by that of the yeast GAL4 protein also showed functional synergism. These data suggest that some of the receptor domains outside the DNA-binding domain synergize by their intrinsic *trans*-activating property, but the hormone-binding domain contributes to synergism by a different mechanism. (Molecular Endocrinology 5: 1498–1503, 1991)

INTRODUCTION

Regulatory elements distant from the transcriptional start site are often composed of multiple binding sites for different transcription factors cooperating for full activity (see Ref. 8 for review). In the case of steroid hormone-responsive genes, the central transcription factor involved is the hormone receptor, which binds to a specific DNA sequence. This hormone-responsive element consists of a short palindromic sequence (see Ref. 3 for review). Synergistic activity has been demonstrated both between two adjacent palindromic receptor-binding sites (1, 7, 15, 25) and between a steroid receptor-binding site and any other transcription factor recognition sequence tested (25–27). Thus, the final transcriptional activity of a hormone-dependent gene results from two effects: the intrinsic *trans*-activation mediated by the glucocorticoid receptor alone and the synergism resulting from adjacent binding of a second receptor or another transcription factor. For duplicated glucocorticoid or progesterone receptor-binding sites, increased DNA-binding affinities have been demonstrated (22, 24, 30), a mechanism that is also found in some cases for the estrogen receptor (15, 19, 22). Binding cooperativity apparently is not involved in the synergism of the glucocorticoid receptor with other transcription factors (20), although one exception has been found (4).

The requirement for a stereospecific alignment of the synergizing factors suggests direct or indirect interaction between the proteins (22, 24–26). According to recently proposed models, functional synergism (not based on DNA-binding cooperativity) may involve the interaction of *trans*-activating domains of transcription factors with a common target, intermediary, or adaptor protein which transmits the regulatory signal to the transcription initiation complex (see Ref.16 for review). *Trans*-activating domains of synergizing factors may touch a common adapter more efficiently than a single transcription factor (23) or synergizing transcription factors may activate different stages in the initiation of transcription (29). Such a mechanism predicts that synergism is mediated by the regular intrinsic *trans*-activation mechanism of the factors involved. Alternatively, synergism may use a distinct mechanism mediated by direct or indirect interaction of the receptor with the neighboring transcription factor independent of its intrinsic *trans*-activation domains.

To find out whether synergism and intrinsic *trans*-activation act on a common target, we determined both activities for several receptor mutants. Both functions

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should be altered concomitantly if they are closely linked. We now show that domains mediating *trans*-activation and synergism are overlapping, but not always identical.

RESULTS

Receptor Domains Mediating Synergism with the CACCC Box

We wanted to determine the contributions of different parts of the human glucocorticoid receptor to synergism with different transcription factors. Therefore, we cotransfected an expression plasmid coding for the human glucocorticoid receptor in its wild-type or mutant form along with a reporter plasmid into T47D cells [which lack functional glucocorticoid receptor (6)]. The transcriptional activity of a reporter gene regulated by a receptor-binding sequence depends on several properties of the receptor: DNA binding, hormone binding, nuclear translocation, dimerization, protein stability, *trans*-activation, and synergistic interaction with transcription factors bound on adjacent sequences. To analyze only the synergistic function of receptor mutants, we compared the activity of a reference (control) reporter gene (pG29C*tkCAT) to that of a test reporter gene (pG29CtkCAT). The test reporter gene (pG29CtkCAT) contained a single imperfect palindromic receptor-binding site, as found in the long terminal repeat of the mouse mammary tumor virus, fused to a functional CACCC sequence originating from the rat tryptophan oxygenase gene (26). A distance of 29 basepairs (bp) separating the two binding sites had been shown to be optimal for synergism (26). This hormone-responsive unit was linked at position −105 to the thymidine kinase (tk) promoter, which, in turn, controlled the expression of the chloramphenicol acetyl transferase (CAT) gene (Fig. 1) (26). The reference (control) reporter gene (pG29C*tkCAT) contains a mutagenized CACCC (C*) replacing the wild-type sequence. The C* sequence does not bind nuclear factors, nor does it functionally synergize (26). In addition, the glucocorticoid response element (GRE; −186 bp upstream of the transcriptional start site) does not synergize with transcription factors bound to the tk promoter (26). Thus, transcriptional activity of this gene is due to the action of one isolated GRE and represents the intrinsic *trans*-activation activity of the tested receptor. CAT activities of these reporter constructs always reflected the amount of correctly initiated RNA (not shown).

DNA transfections were performed using constant amounts of reporter genes while increasing the concentration of the respective receptor expression plasmid, leading to increasing CAT activities. In this way the slope within the linear range of the curve (Fig. 3) achieved with the test reporter gene could be compared to the slope seen with the control reporter gene. Synergism was measured as the ratio between the two

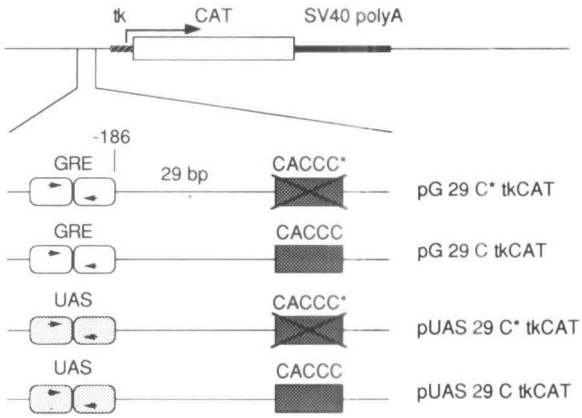

Fig. 1. Schematic Representation of Reporter Plasmids
The reporter plasmids with the tk promoter, the CAT-coding region, and various receptor (GRE)- and nonreceptor-binding site combinations are shown. The nonreceptor-binding sites (24) are a CACCC box and an up-stream activator sequence (UAS) element. The mutagenized nonbinding CACCC box is indicated (*).

slopes. This strategy enables analysis even of those receptors that lead to only weak transcriptional stimulation and corrects for varying amounts of different mutated receptor species present within the cells. Within a wide range of expression plasmid concentrations, reporter gene activity increased linearly. Intrinsic *trans*-activation of wild-type and mutant receptors was determined with the control reporter gene (pG29C*tkCAT) at a constant amount of expression plasmid and agreed with the published values (13). The characterized domains of the receptor are indicated in Fig. 2: *trans*-activating domains τ_1 and τ_2 (13), the DNA-binding domain (DNA), and the hormone-binding domain (HBD) (12).

Cotransfections of the wild-type receptor expression plasmid with reporter plasmids pG29C*tkCAT and pG29CtkCAT confirmed the synergistic effect of the CACCC sequence on steroid induction (Fig. 3 and Table 1). The receptor deletion mutants can be categorized into four groups according to their synergizing properties, a prototype for each of these categories is shown in Fig. 3. Only the wild-type receptor belongs to the first category, showing high activities in both synergism and intrinsic *trans*-activation. The second category (mutant 1–550) shows a high intrinsic *trans*-activation, as high as the wild-type receptor, but only a moderate synergistic activity. This mutant is a clear example that intrinsic *trans*-activation and synergism do not necessarily colocalize on the receptor. Another example of this kind is demonstrated by the third category, which shows the same moderate level of synergism as the second category, but exerts about a 10-fold reduced intrinsic *trans*-activation. A prototype of this category is mutant 1–515 (Fig. 3D), which shows effects similar to those of mutants Δ77–262, Δ262–418, 418–777, and 1–488 (Table 1). The fourth category is characterized by a very low intrinsic *trans*-activation (although clearly

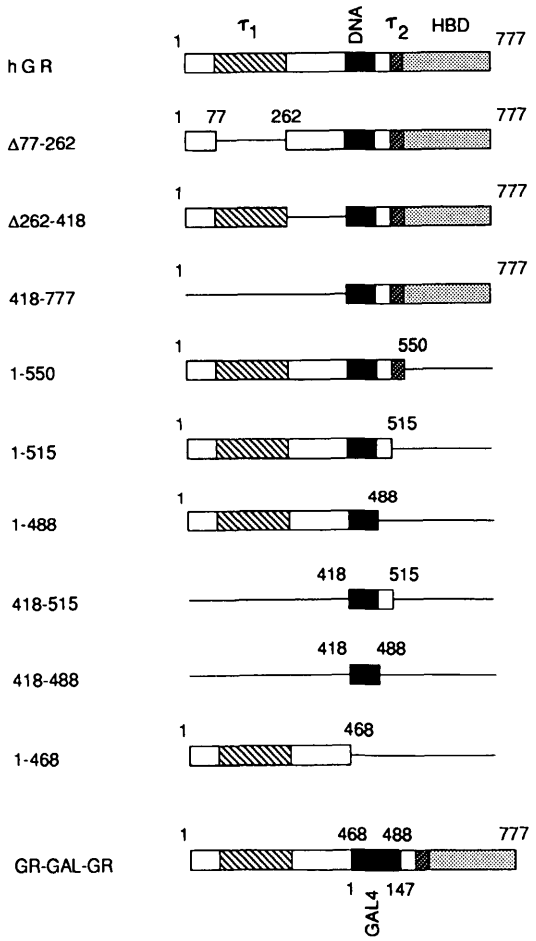

Fig. 2. Schematic Representation of Expression Plasmids

The different domains of the wild-type human glucocorticoid receptor are shown: the hormone-binding domain (HBD), *trans*-activation domains τ1 and τ2, and the DNA-binding domain (DNA). The regions deleted in each mutant are indicated with a *line*, and the deletion end points are given. GR-GAL-GR is shown, and the end points of the human glucocorticoid receptor parts are indicated as well as the GAL4 DNA-binding domain (GAL4).

measurable compared with the nonbinding mutant 1–468; Table 1) and no detectable synergism (Fig. 3B and Table 1; mutants 418–515 and 418–488).

In conclusion, all of the tested deletion mutants of the glucocorticoid receptor affect the synergism with a neighboring CACCC box factor. Intrinsic *trans*-activation is clearly impaired as well, but both functions do not change coordinately in some mutants. This is an indication that intrinsic *trans*-activation and synergism do not necessarily follow identical mechanistic pathways.

Domains outside the DNA-Binding Domain Contribute to Synergism

As all of the mutants tested above showing synergism still contained the DNA-binding domain, the question arises of whether this domain might be responsible for the observed synergism. Such a function may be undetectable in mutant 418–488, containing only the DNA-binding domain, since its intrinsic *trans*-activation is very low. To address this question, we decided to construct a chimeric protein where the DNA-binding domain of the receptor was replaced by the DNA-binding domain of the GAL4 protein (GR-GAL-GR). Similar fusion proteins have been used before to test for function of isolated domains of DNA-binding proteins (13, 18, 31). Amino acids 1–147 of the GAL4 protein contain all of the properties necessary for dimerization (5) and specific binding to the palindromic UAS sequence, but do not lead to *trans*-activation (14) or cooperative binding to a duplicated binding site (10). When only this domain is expressed in T47D cells, no effect whatsoever was detectable on any UAS-containing reporter plasmid (data not shown). When the fusion protein GR-GAL-GR was expressed, a strong intrinsic *trans*-activation was seen using the reporter gene UAS-C* containing a single GAL4-binding site. When the reporter UAS-C was used, harboring a UAS element adjacent to a CACCC box, a strong synergism was seen (Fig. 4). Comparison of the slopes of the curves obtained with the control reporter (UAS-C*) and the test reporter plasmid (UAS-C) showed a 5-fold synergism of GR-GAL-GR with the neighboring CACCC factor. This observation clearly shows that domains outside the receptor DNA-binding domain contribute to funtional synergism.

DISCUSSION

Models for synergistic *trans*-activation have been suggested previously. They involve interaction of the *trans*-activating domains with a protein called target, adaptor, or intermediary factor (see Ref. 16 for review), transmitting the regulatory signal to the transcriptional start site. In one model two transcription factors would simply provide a larger surface for such an interaction (23). Another model suggested that different *trans*-activating domains would activate different stages in the initiation of transcription and would, therefore, be able to synergize (29). Both models are similar in predicting that synergism is conferred by the *trans*-activating pathway. An alternative model involves an additional protein contacting specific domains of the two synergizing factors.

To test whether synergism is mediated by the intrinsic *trans*-activating pathway, we tested several receptor deletions for their synergistic capacity with the CACCC box. Most of the receptor deletions affect both the synergism and the *trans*-activation even when regions outside the τ1 and τ2 domains are deleted. This confirms the notion that the glucocorticoid receptor may comprise at least four *trans*-activating functions (28). The synergism and *trans*-activation observed with receptor mutants was not due to the receptor DNA-binding domain alone, present in all of the mutants, as the replacement of this domain with the DNA-binding

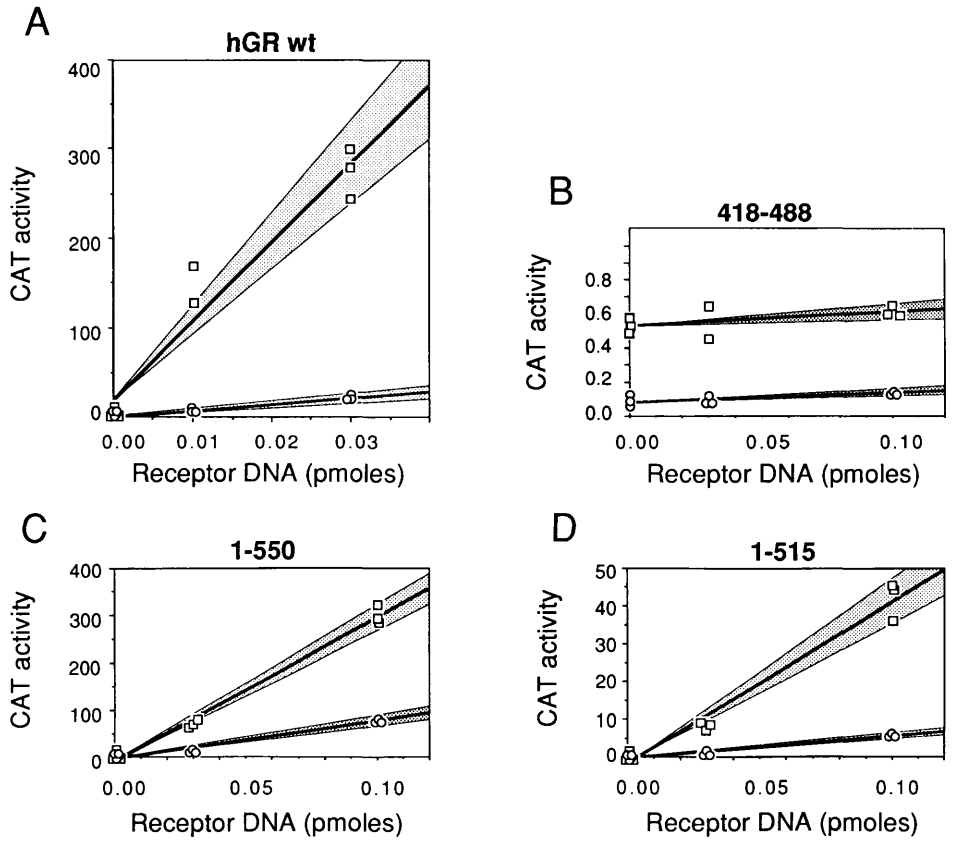

Fig. 3. Synergism of Glucocorticoid Receptor Mutants with the CACCC Box Binding Protein

The graphs show CAT activities achieved after transfection of increasing amounts of wild-type or mutant receptor expression vector (picomoles of receptor DNA per transfection) together with constant amounts of pG29C*tkCAT (○) or pG29CtkCAT (□) reporter plasmids. CAT activity is expressed as picomoles of acetylated chloramphenicol per mg protein/min. The expression plasmids used are the wild-type receptor (A; hGRwt) or mutant 418–488 (B), mutant 1–550 (C), and mutant 1–515 (D). The graph was calculated by linear regression, and the SD (*shaded area*) was determined according to standard procedures (21).

Table 1. Synergism of Glucocorticoid Receptor Mutants with the CACCC-box Binding Protein

Receptor Mutant	Fold Synergism[a]	*Trans*-Activation (%)[b]
hGR	15.2	100 ± 12
Δ77-262	5.0	10 ± 3
Δ262-418	7.1	3 ± 0.5
418-777	6.9	6 ± 0.9
1-550	5.3	79 ± 0.8
1-515	5.9	6 ± 0.5
1-488	4.0	3 ± 0.6
418-515	1.1	0.05 ± 0.005
418-488	0.9	0.05 ± 0.005
1-468	NA	<0.01 ± 0.005

[a] Fold synergism is defined as the ratio between the slopes achieved with pG29CtkCAT and pG29C*tkCAT (see Fig. 3). NA, Not applicable.

[b] Intrinsic *trans*-activation is determined with 0.1 pmol receptor expression plasmid and 1.5 pmol pG29C*tkCAT.

domain of the yeast protein GAL4 results in a protein that is able to both *trans*-activate and synergize. We cannot rule out the possibility that the DNA-binding domain of the receptor might contribute to synergism, since the construct GR-GAL-GR is less efficient in synergizing than the wild-type receptor and since the DNA-binding domain of the receptor shows some (weak) *trans*-activation if tested alone. On the other hand, this difference in synergism may be due to non-optimal conformation of the receptor in the GAL fusion protein. In addition to the colocalization of *trans*-activating and synergizing receptor properties, one important exception was found, deletion of the hormone-binding domain reduced the synergism with the CACCC box factor by about 3-fold, whereas the intrinsic *trans*-activation of this receptor mutant tested in isolation is only marginally reduced (~0.8-fold). Such a finding argues for the presence of an additional factor, which may contact the hormone-binding domain and the CACCC box protein simultaneously.

In summary, the puzzling finding that every combination of the glucocorticoid receptor with other transcription factors led to a synergistic glucocorticoid in-

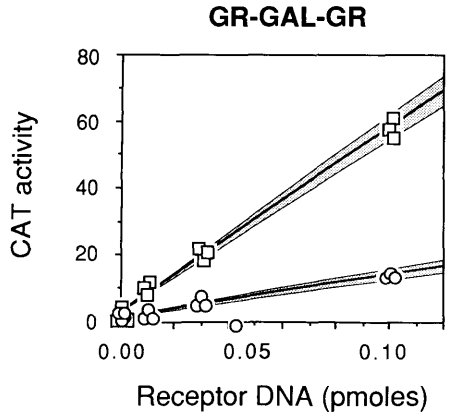

Fig. 4. Synergism of a GAL4-Glucocorticoid Receptor Fusion Protein with the CACCC Box Binding Protein

The graph shows CAT activities achieved after transfection of increasing amounts of pRS GR-GAL-GR (picomoles of expression vector DNA per transfection) together with constant amounts of pUAS29C*tkCAT (○) or pUAS29CtkCAT (□) reporter plasmids. For calculations, see Fig. 3.

duction (25–27) may be solved by the fact that particular combinations may synergize via specific functions of the multiple receptor domains. These functions include cooperative binding in the case of receptor/receptor synergism, the synergism mediated by several independent *trans*-activating receptor functions, and a synergizing property of the hormone-binding domain not using a strong *trans*-activating function.

MATERIALS AND METHODS

Plasmids

The constructs containing the indicator gene tk-CAT (CAT gene fused to the tk promoter) and carrying binding sites for the glucocorticoid receptor and the CACCC box have been described previously (24). The pUAS 29 C*tkCAT and pUAS 29 CtkCAT were generated in an analogous fashion by replacing the GREs by the binding site CGGAGGACTGTCCTCCG (UAS) for the yeast GAL4 protein (10), with the corresponding *Hin*dIII/*Sma*I restriction sites at the 5′ and 3′ ends.

The expression vectors for wild-type human glucocorticoid receptor (pRShGR) and for the receptor mutants Δ77–262, I488, 1–515 (I515*), 1–550 (I550*), and 1–77/262–515 (GR15) were kindly provided by R. M. Evans (9, 13). Mutant 418–777 was generated by replacing a *Hin*fI/*Hin*dIII fragment (amino acids 1–426) from pRShGR with a synthetic oligonucleotide duplex (5′-ACTCCAG-CAGCCAGATCTGACCTCCCCCAAA-CTCTGCCTGGTGTGCTCTGATGA-3′), thereby restoring the otherwise deleted part of the DNA-binding domain (amino acids 418–426). Mutant 1–488 was constructed by linearizing I488 with *Bam*HI, filling in with Klenow enzyme, followed by religation, thereby inducing a frame shift in the natural sequence leading to 26 unrelated codons until a stop codon is reached. Mutant 1–468 was constructed by linearizing pRShGR with *Cla*I, filling in with Klenow enzyme, followed by religation. Mutants 418–515, 418–488, and 418–777/I488 were obtained by replacing the *Kpn*I/*Hin*dIII fragment in 1–515, 1–488, and I488, respectively with the *Kpn*I/*Hin*dIII fragment from 418–777. Mutant Δ262–418 was constructed by inserting the *Bgl*II/*Bgl*II (amino acids 77–262) fragment from pRShGR into the *Bgl*II site of 1–78/418–777.

The GAL4 DNA-binding domain expression vector pRS GAL1–147 was constructed by joining an oligonucleotide duplex $^{5'}$GATCTGAAGCTACTGTCTCTATCGAACAAGCATG$^{3'}$ to the *Sph*I site of the *Sph*I/*Bam*HI fragment of pMA424 (17), thereby restoring the first 10 amino acids of GAL1–147, followed by insertion into the *Bgl*II/*Bam*HI sites of mutant 418–777/I488. The resulting plasmid contains linker sequences, coding for 26 unrelated amino acids after amino acid 147.

The pRS GR-GAL-GR was generated by fusing the *Kpn*I/*Hin*dIII fragment (filled in with Klenow), containing the first 426 amino acids of human glucocorticoid receptor, into the *Kpn*I/*Bgl*II sites (filled in with Klenow) of pRS GAL1–147. In addition, the *Eco*RI and *Bam*HI linker sites of pRS GAL1–147 were cut, filled in with Klenow enzyme, and religated, leading to a frame shift and thereby restoring the whole C-terminal part (amino acids 488–777) of human glucocorticoid receptor.

Cell Culture and Transfection

T47D cells were transfected as previously described (2). All test plasmids (1.5 pmol) were cotransfected with different amounts of receptor expression vector, as indicated. Cells were seeded in charcoal-depleted medium, and 5×10^{-7} M dexamethasone was added. Endogeneous receptors did not influence the results, since T47D cells do not have functional glucocorticoid receptors (6), and the use of antihormone together with receptor mutants lacking hormone-binding domain did not change the synergism. Cells were harvested and tested for CAT activity 40 h after transfection (11). All transfections were performed in triplicate. The complete series of transfections was performed at least twice. Cell extracts were heat treated (10 min; 60 C) before carrying out the CAT assay. CAT assay conditions were chosen to yield converson rates between 0.5–60% of chloramphenicol and ranged from incubation of 5 μg cell protein for 30 min to 150 μg for 16 h.

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