Repressor to activator switch by mutations in the first Zn finger of the glucocorticoid receptor: Is direct DNA binding necessary?

(interleukin 1 inducibility/dexamethasone modulation/DNA-binding domain mutants/interleukin 6 and *c-fos* promoters)

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ABSTRACT Transfection of HeLa cells with cDNA vectors expressing the wild-type human glucocorticoid receptor (GR) enabled dexamethasone to strongly repress cytokine- and second messenger-induced expression of cotransfected chimeric reporter genes containing transcription regulatory DNA elements from the human interleukin 6 (IL-6) promoter. Deletion of the DNA-binding domain or of the second Zn finger or a point mutation in the Zn catenation site in the second finger blocked the ability of GR to mediate repression of the IL-6 promoter. Unexpectedly, deletion of the first Zn finger, a point mutation in the Zn-catenation site in the first finger, or one in the steroid-specificity domain at the base of the first finger converted GR into a dexamethasone-responsive activator that enhanced basal and interleukin 1-induced IL-6 promoter function. These first-finger mutants of GR also mediated dexamethasone-responsive enhancement of expression of the herpesvirus thymidine kinase-chloramphenicol acetyltransferase (TK₋₁₀₅-CAT and TK₋₈₀-CAT) reporter genes but not of the murine mammary tumor virus long terminal repeat-CAT or the *c-fos*-CAT (pFC700) reporter genes. Wild-type GR was able to specifically bind to DNA fragments containing glucocorticoid response element sequences in both the murine mammary tumor virus and IL-6 promoters, albeit weakly to the latter, in a sequential DNA-binding immunoprecipitation assay. The first-finger mutants of GR, however, were inactive in this assay. Thus, mutations in the first Zn finger unmask unusual promoter-specific activation properties of GR that may not require direct high-affinity binding of the mutant GR to target DNA.

Gene activation or repression by proteins in the steroid receptor superfamily, all of which contain two Zn fingers in the highly conserved DNA-binding domain (see Fig. 1), is thought to involve cooperative DNA binding by steroid receptor dimers at specific target nucleotide sequence motifs in the target promoters coupled with interactions of the steroid receptor with appropriate transcription factors (1–5). All previous studies of the relationship between the structure of the glucocorticoid receptor (GR) and its function in hormone-dependent promoter activation or repression using deletion, point-mutation, and domain-swap analyses have led to the inference that both the Zn fingers in the DNA-binding domain are indispensable for transcriptional modulation (4, 6–12). Repression of gene expression by steroids, which is now drawing increasing attention, may occur either by binding of GR to functional DNA elements in the promoter (13–17), by protein–protein interactions between receptor and nonreceptor transcription factors (18–23), or both.

The inhibition of interleukin 6 (IL-6) gene expression by glucocorticoids represents an important regulatory link between the endocrine and immune systems (17, 24–26). In

previous experiments in HeLa cells transfected with cDNA vectors constitutively expressing the wild type (wt) but not the inactive carboxyl-terminal truncation mutants of GR, we observed that dexamethasone (Dex) strongly repressed the induction by interleukin 1 (IL-1), tumor necrosis factor, phorbol ester, or forskolin of IL-6–chloramphenicol acetyltransferase (CAT) constructs containing IL-6 DNA from –225 to +13. Dex also repressed induction of IL-6–thymidine kinase (TK)–CAT chimeric constructs containing a single copy of the IL-6 DNA segment from –173 to –151 (MRE I) or from –158 to –145 (MRE II), which derive from within the multiple cytokine- and second messenger-responsive enhancer (MRE) region irrespective of the inducer used (17, 27). MRE I contains the typical GACGTC cAMP/phorbol ester-responsive (CRE/TRE) motif; mutations at CG in this CGTCA motif block induction by cAMP and phorbol ester but not by serum, IL-1, or tumor necrosis factor (17, 27). The other DNA element, MRE II, contains an imperfect dyad repeat and bears little resemblance to a typical CRE/TRE motif but is nevertheless also induced by cAMP, phorbol ester, IL-1, and tumor necrosis factor (17); it is also a binding site for the CCAAT enhancer binding protein family of transcription factors (28). Overall, the MRE enhancer region in IL-6 bears strong nucleotide sequence and functional similarity to the *c-fos* serum response element (17, 29). The induction by pseudorabies virus of an IL-6 construct containing the TATA box and the RNA start site [initiator element (Inr) motif] but not the MRE region was also repressed by Dex in a wt GR-specific manner (17). DNase I footprinting showed that the purified DNA-binding fragment of GR bound across the MRE, the TATA box, and the Inr site in the IL-6 promoter (17). These observations suggested that ligand-activated GR repressed IL-6 gene function by occlusion not only of the IL-6 MRE enhancer region but also of the basal promoter elements (17).

To further explore the relationship between GR structure and IL-6 promoter repression, we studied the repression of human IL-6 promoter constructs by Dex in HeLa cells cotransfected with expression plasmids producing wt or mutants of the DNA-binding domain (DBD) of GR.

MATERIALS AND METHODS

Cell Culture and DNA Transfection Assays. HeLa cells were cultured in Dulbecco's modified Eagle's medium as described (30). Procedures for transfection were essentially as described (17, 27) except that the plasmid pCH110 (3 μ g), a

Abbreviations: IL-1, interleukin 1; IL-6, interleukin 6; CAT, chloramphenicol acetyltransferase; TK, herpesvirus thymidine kinase; wt, wild type; GR, glucocorticoid receptor; Dex, dexamethasone; DBD, DNA-binding domain; MRE, multiple cytokine and second messenger responsive enhancer; MTV-LTR, mouse mammary tumor virus long terminal repeat; GRE, glucocorticoid response element.

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constitutive expression vector for β -galactosidase, was included in the DNA mixture added to cells in every 100-mm Petri dish as a marker for transfection efficiency. Extracts prepared from the transfected cells were first assayed for β -galactosidase activity using chlorophenyl red β -D-galactoside as the substrate. The CAT assay was carried out as described (17, 27) using amounts of each extract that contained a defined amount of β -galactosidase activity.

Plasmids. The various IL-6, *c-fos*, and herpesvirus TK-CAT promoter constructs used have been described (17, 27). pAR12TKC was constructed by cloning a double-stranded oligonucleotide containing IL-6 promoter sequences from -173 to -141 encompassing the entire IL-6 MRE sequence (17, 27) at the *Xba* I site of pTK₋₁₀₅-CAT. pMTV-CAT and the constitutive GR expression vectors were kindly provided by S. Hollenberg and R. Evans (7). pIL-6225 was obtained by cloning IL-6 sequences between the *Nhe* I and *Hind*III sites from pIC225 between the *Xba* I (filled in) and *Hind*III sites of pGEM-4Z (Promega). Western blots were used to verify the production of mutant proteins in the transfected HeLa cells.

DNA-Binding Immunoprecipitation Assay. Extracts of HeLa cells transfected with individual GR constructs and pCH110 were prepared and used in DNA-binding immunoprecipitation assays using antiserum α GR135 (rabbit antibody to the GR peptide residues 144–172) and ³²P-end-labeled DNA fragments generated by digesting plasmid pMTV-CAT with *Dra* I or plasmid pIL-6225 with a mixture of *Nde* I, *Bam*HI, and *Hind*III using procedures described in ref. 6.

RESULTS

To evaluate the relationship between GR structure and its ability to repress the human IL-6 promoter, we tested the ability of a series of deletion and point mutants in the DNA-binding domain of GR (Fig. 1) to exhibit Dex-mediated repression of the IL-6 (-225 to +13)-CAT chimeric construct pIC225. Fig. 2A and Table 1 show that in transfected HeLa cells treated with Dex, both wt GR (RSVhGR α) and mutant G442 repressed induction by IL-1 of the IL-6 promoter in the

FIG. 1. Point and deletion mutants in the segment between amino acid residues 419 and 490 in the DNA-binding domain region of the human GR (7) used in this study. The indicated mutants were evaluated for their effects on the expression of IL-6 and other promoter constructs in transient transfection assays in HeLa cells. Mutants in the first Zn finger: G421 (Cys → Gly conversion), Δ 420–451 (deletion of the first Zn finger), and G442 [Lys → Gly conversion in the "P" or steroid-specificity box (31) at the base of the first finger]. Mutants in the second Zn finger: G457 (Cys → Gly conversion) and Δ 450–487 (deletion of the second Zn finger). Δ 428–490 is a mutant devoid of both the Zn fingers (deletion of the entire DNA-binding domain).

plasmid pIC225. Deletion of the entire DNA-binding domain (Δ 428–490), deletion of the second Zn finger (Δ 450–487), or a point mutation in the second Zn finger that disrupts Zn catenation (G457) blocked the ability of GR to repress IL-1-induced expression from pIC225 (Fig. 2A). In contrast, deletion of the entire first Zn finger (Δ 420–451) or a mutation in the Zn-catenation region (G421) enhanced the ability of GR to increase both the basal and the IL-1-induced expression of pIC225 in a Dex-responsive manner (Fig. 2A). The transcription activation ability of the first Zn-finger mutants of GR was best demonstrated when cells transfected with pIC225 and either G421 or Δ 420–451 were treated with Dex and a suboptimal inducing concentration of IL-1 α (0.5 ng/ml) [Fig. 2A (ii); Table 1].

Our analysis of repression of the IL-6 promoter by different DNA-binding domain mutants of GR showed a switch in the behavior of the first Zn-finger mutants of GR. We tested whether this unusual transcription enhancing activity of the mutant GR proteins could also be observed with other promoters. Fig. 3A and Table 2 show that wt GR and G442, both of which repressed the IL-6 promoter in pIC225 (Fig. 2A and Table 1), stimulated the murine mammary tumor virus long terminal repeat (MTV-LTR) construct pMTV-CAT. It has been previously shown that both wt GR and G442 bind to the glucocorticoid response element (GRE) in MTV-LTR, although only the former activates it in CV-1 cells (7). Mutants G421 and Δ 420–451, which activated pIC225 (Fig. 2A and Table 1), were unable to activate pMTV-CAT (Fig. 3A and Table 2). The inability of G421 and Δ 420–451 to activate pMTV-LTR in HeLa cells conforms to the previous phenotypic characterization of these mutants in CV-1 cells (7). Thus, in HeLa cells, the ability of GR and its mutants to repress the IL-6 promoter in pIC225 correlated with their ability to activate MTV-LTR; mutants unable to activate MTV-LTR did not repress the IL-6 promoter. Nevertheless, two of the mutants in the first Zn finger, which were unable to activate pMTV-CAT (G421 and Δ 420–451), did markedly activate the pIC225 IL-6 construct.

We then examined the ability of the wt and the first Zn-finger mutants of GR to modulate expression from two additional IL-6 promoter constructs (Figs. 2B and 3) from two different herpesvirus TK promoter constructs (Fig. 3A) and from the *c-fos* promoter (Fig. 3A). The wt GR mediated repression of basal and IL-1-induced activation of pAR12TKC, which contains the entire IL-6 MRE enhancer region from -173 to -141 linked to TK₋₁₀₅-CAT (Fig. 2B and Table 1). Again, first-finger GR mutants enhanced expression from pAR12TKC in HeLa cells treated with Dex and IL-1 α (Fig. 2B and Table 1). The observation that mutant G442, which repressed pIC225, enhanced expression from pAR12TKC points to target promoter-specific differences in the function of mutant GR proteins. All three first-finger GR mutants tested, but not the wild-type GR, enhanced expression of the IL-6 construct pIC110 (which lacks the MRE enhancer but contains the NF- κ B, TATA box, and Inr sites) and of pTK₋₁₀₅-CAT but not that of pFC700 in a hormone-dependent manner (Fig. 3A and Table 2). Similar data were obtained by using pTK₋₈₀-CAT, a plasmid that does not contain a CAAT box present in pTK₋₁₀₅-CAT (data not shown). In conformity with earlier observations (13, 15), the chimeric genes pTK₋₁₀₅-CAT and pTK₋₈₀-CAT, which do not contain any known GR-binding DNA motif, did not respond to Dex in the presence of wt GR. Nevertheless, all three first-finger mutants of GR (G421, G442, and Δ 420–451) were able to mediate Dex-responsive upregulation of even these basal TK promoter constructs (Fig. 3A and Table 2). However, this is not a promiscuous upregulation of all RNA polymerase II promoter function in the transfected HeLa cells because two of these three mutants (G421 and Δ 420–

FIG. 2. Repression or activation of IL-6 promoter constructs is a function of the fine structure of the DNA-binding domain of GR. HeLa cells ($2-3 \times 10^6$ cells) in 100-mm plates were transfected with the IL-6-CAT reporter plasmid pIC225 (10 μ g) (A) or the IL-6-tk-CAT plasmid pAR12TKC (2.5 μ g) (B) together with constitutive human GR expression vectors containing cDNAs for the intact wt GR (RSVhGR) or mutants in the first, the second, or both Zn fingers (see Fig. 1) in the DNA-binding domain of GR (5 μ g) and pCH110 (3 μ g) by the calcium phosphate coprecipitation procedure (27, 30, 32). At 16–18 hr after transfection, the precipitate was washed off and the plates were replenished with serum-free medium in the presence or absence of IL-1 α at 0.5 ng/ml [A(i)] or at 5 ng/ml [A(ii)] or 1 μ M Dex in different combinations. The cells were harvested 24 hr later and extracts were assayed for β -galactosidase activity. Extracts normalized to equal β -galactosidase activity were assayed for CAT activity (17, 27, 30). The CAT activity data are enumerated in Table 1. (C) Schematic representation of the IL-6-CAT reporter plasmid constructs. This figure is a composite of several different experiments.

451) did not upregulate MTV-LTR and all three failed to upregulate pFC700 (*c-fos*-CAT) (Fig. 3A and Table 2).

To investigate the possibility that the observed activation properties of the first Zn-finger mutants of GR involved direct high-affinity DNA binding, we performed sequential DNA-binding immunoprecipitation assays (6, 33). Extracts of HeLa cells transfected with wt GR, G421, and Δ 420–451

Table 1. Dex-induced repression or activation of IL-6-CAT constructs by Zn-finger mutants of the human glucocorticoid receptor

Plasmid		CAT activity in the presence of Dex, % of Dex-free control	
Reporter	GR construct	Without IL-1	With IL-1
pIC225			
	Exp. 1		
	RSVhGR	54	7
	G421	160	200
	Δ 420–451	150	140
	G442	62	17
	G457	153	90
	Δ 450–487	80	75
pAR12TKC			
	Exp. 2		
	RSVhGR	61	10
	G421	206	321
	Δ 420–451	125	1187
	G442	299	287
	Δ 420–451	324	454
	G442	221	318

Numerical data relate to experiments illustrated in Fig. 2. In all experiments, the plasmid pCH110 carrying the β -galactosidase gene linked to the simian virus 40 promoter was cotransfected to provide an internal control for differences in transfection efficiency. Where added, cells received 5 ng of IL-1 α per ml Exp. 1, which gave a 30-fold increase in CAT activity (23% absolute conversion of [14 C]chloramphenicol), or 0.5 ng of IL-1 α per ml Exp. 2, which resulted in a 4-fold increase in CAT activity (2% absolute conversion of [14 C]chloramphenicol).

were tested by this assay for their ability to specifically recognize and bind DNA fragments containing GR-binding motifs (GRE) in the MTV and IL-6 promoters. Fig. 4A is a control experiment that reproduces the earlier observation of Hollenberg and Evans (7) showing that cell extract containing wt GR but not the first Zn-finger mutants G421 and Δ 420–451 could specifically bind to and precipitate a DNA fragment containing the MTV GRE from a mixture of DNA fragments. Fig. 4B demonstrates that cell extracts containing wt GR can specifically precipitate an IL-6 DNA fragment (–225 to +13 and linker sequences) containing GRE sequences. In comparison to the high-affinity binding of wt GR to the GRE sequences in the MTV promoter, wt GR bound only weakly to IL-6 GRE sequences (Fig. 4B). However, extracts made from cells transfected with either G421 or Δ 420–451 completely failed to precipitate the GRE-containing IL-6 DNA fragment. Thus, using this sequential DNA-binding immunoprecipitation assay we were unable to detect any high-affinity DNA binding by the first Zn-finger GR mutants to GRE sequences in either the MTV or the IL-6 promoter, raising the possibility that direct DNA binding may not be necessary for transcriptional activation of the IL-6 and other promoters by these first Zn-finger mutants of GR.

DISCUSSION

The DBD of GR contains two Zn fingers (7, 34–36). Detailed molecular studies have demonstrated the functional requirement of this segment in the modulation of gene expression by GR. The DBD has been shown to be necessary for both the trans-activation and the trans-repression functions of GR (reviewed in refs. 11 and 37). Extensive mutational analyses have pointed to different hormone-specific functional roles for the conserved and variant amino acids within this domain (6, 7, 10, 34). However, it remains unclear whether the structural determinants in the DBD necessary for gene activation are identical to those required for gene repression. Some studies on the repression of expression of genes like the α subunit of glycoprotein hormone, proopiomelanocortin, proliferin, and prolactin (13–16) have suggested that the

FIG. 3. Effect of cotransfected wild-type or mutant GR expression plasmids on Dex-responsive expression of CAT activity from different promoters. (A) HeLa cells were transfected with the reporter constructs pMTV-CAT (5 μ g), the IL-6-CAT construct pIC110 (10 μ g), pTK₋₁₀₅-CAT (2.5 μ g), or the c-fos-CAT construct pFC700 (2 μ g), the indicated GR expression plasmid (5 μ g), and pCH110. Cells were harvested and assayed for β -galactosidase and CAT activities as described in the legend to Fig. 2. The CAT activity data are enumerated in Table 2. (B) Schematic representation of the reporter constructs used in this experiment. This figure is a composite of several different experiments.

predominant mechanism of repression might be steric interference with binding of positive transcription factors by direct binding of GR to the target DNA. More recently, studies on repression of the collagenase promoter, the proliferin promoter, as well as synthetic AP-1 site CAT constructs (19, 20, 22, 23) have demonstrated the involvement of protein-protein interactions in the repression of these promoters; nevertheless, this repression required the presence of an intact DBD. Although the repression of the collagenase and proliferin promoters *per se* is receptor and target specific in the sense that an AP-1 site is required for repression by GR, the target gene specificity of the DBD itself does not appear to be important for the glucocorticoid and GR-mediated repression of these promoters because swapping the DBD of

Table 2. Effect of first Zn-finger mutants of GR on Dex-induced expression from different target promoters expressed relative to that of wt GR (RSVhGR)

Reporter	Plasmid		Dex-responsive change in CAT activity, % of control
	GR construct		
pMTV-CAT	G421		<1
	Δ420-451		<1
	G442		60
pIC110	G421		500
	Δ420-451		1071
	G442		457
pTK ₋₁₀₅ -CAT	G421		371
	Δ420-451		275
	G442		756
pFC700	G421		91
	Δ420-451		108
	G442		110

Numerical data relate to experiments illustrated in Fig. 3. All CAT activities are normalized to β -galactosidase activity and are expressed as % CAT activity relative to that in the extract prepared from cells that received wt GR and the appropriate reporter plasmid.

GR with those of other receptors of the same family (e.g., retinoic acid receptor or thyroid hormone receptor; ref. 22) did not interfere with the glucocorticoid repression of AP-1 site-driven CAT constructs, suggesting that the structural requirement for an intact DBD may reside in its participation in protein-protein interactions and not DNA binding *per se* (22).

Our earlier analysis of the repression of the IL-6 promoter by GR had established a dependence on wt GR in order to elicit repression (17). Footprinting studies that revealed that the DNA-binding fragment of GR bound across both the enhancer and basal elements in the IL-6 promoter suggested that negative regulation by GR involved exclusion of both

FIG. 4. DNA-binding immunoprecipitation assay of the ability of wt and mutant GR proteins to directly bind DNA. Cell extracts were used to bind to and immunoprecipitate GRE-containing and control DNA fragments lacking GRE from either the MTV (A) or IL-6 (B) promoters. The probe (lanes P) in each case is a mixture of end-labeled DNA fragments. The position of the fragment containing the MTV GRE is $\approx$ 520 base pairs (bp) and that containing the IL-6 GRE sequences is 250 bp (arrowheads). The 250-bp IL-6-GRE-containing fragment is the lower fragment in the doublet; the larger 260-bp fragment is derived from the vector. Samples in all lanes were immunoprecipitated with α GR135 antibody. Immunoprecipitated DNA was analyzed by polyacrylamide gel electrophoresis.

basal and positive transcription by competitive binding to overlapping sites in the IL-6 promoter (17). The present study establishes that an intact DBD in GR is required for repression of the IL-6 promoter. However, specific mutations in the DBD of GR can convert GR from a repressor to an activator of IL-6 expression. Expression from the IL-6 promoter can be repressed, activated, or left unaffected by Dex and GR depending on the fine structure of the DBD. Since the DNA-binding immunoprecipitation assay only picked up specific binding to IL-6 DNA by wt GR, it appears possible that the first Zn-finger mutants (G421 and Δ 420–451) and mutation at the base of the first finger (G442) upregulate IL-6 promoter function through protein–protein interactions with other transcription factors, perhaps via the intact second Zn finger (36), and may no longer need to bind directly to the target promoter DNA in order to activate gene expression. Interestingly, G442 was also reported to enhance the activity of the AP-1 site in phorbol ester response element–CAT constructs that are ordinarily repressed by wt GR (21, 23). The weak binding by wt GR to IL-6 sequences raises the possibility that a combination of DNA-binding and protein–protein interactions may account for efficient repression of the IL-6 promoter by wt GR. G442 represses induced expression from IL-6 enhancer sequences when linked to its own core promoter, while it stimulates expression when coupled to the TK promoter. This raises the possibility of the interplay of additional transcription factors that interact with the TK promoter in the activation by G442 of pAR12TKC.

Our experiments show that the first Zn-finger mutants of GR can elicit the most dramatic activation when the promoter is only weakly induced by a suboptimal concentration of IL-1 [Fig. 2A(ii) and Table 1]. The functional synergistic interaction of wt GR with transcription factors such as CP1, Sp1, OTF, and NF1 has been reported (38, 39). Interestingly, the degree of synergism was found to be inversely related to the strength of the GRE target used (38). GR has been shown to interact directly with *c-jun* but not *c-fos* (19, 20, 22, 23), an interaction that is dependent on the integrity of the leucine-zipper region in *c-jun* (22). That the marked activation of pIC225 and pAR12TKC by first Zn-finger mutant GR proteins is not observed with Dex alone but requires weak activation of the IL-6 promoter by suboptimal inducing concentrations of IL-1 points to a synergism between the mutant GR and one or more IL-1-activated transcription factors such as the IL-1-activated human transcription factor called NF-IL6 (28), which (i) belongs to the leucine-zipper containing the CCAAT enhancer binding protein family of transcription factors [ref. 40; other members of this family are IL-6DBP and AGP/EBP, which appear to be the rat and murine homologs of NF-IL6 (41, 42)] and (ii) binds to the imperfect dyad repeat between –158 and –145 in the IL-6 DNA element AR12. That CCAAT enhancer binding protein family members may directly interact with GR is enhanced by the observation that NF-IL6 participates in the IL-6- and glucocorticoid-synergized induction of acute-phase plasma protein gene transcription (43).

Our data raise the possibility that naturally occurring point mutations in or deletions of the first Zn finger of GR (which is encoded by a separate exon) or of other steroid receptors may unleash aberrant transcriptional activity leading to a dysregulation of cellular function.

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