Role of Activation Function Domain-1, DNA Binding, and Coactivator GRIP1 in the Expression of Partial Agonist Activity of Glucocorticoid Receptor−Antagonist Complexes

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ABSTRACT: The determinants of the partial agonist activity of most antisteroids complexed with steroid receptors are not well understood. We now examine the role of the N-terminal half of the glucocorticoid receptor (GR) including the activation domain (AF-1), the DNA binding site sequence, receptor contact with DNA, and coactivator binding on the expression of partial agonist activity in two cell lines for GRs bound by five antiglucocorticoids: dexamethasone mesylate (Dex-Mes), dexamethasone oxetanone (Dex-Ox), progesterone (Prog), deoxycorticosterone (DOC), and RU486. Using truncated GRs, we find that the N-terminal half of GR and the AF-1 domain are dispensable for the partial agonist activity of antiglucocorticoids. This contrasts with the AF-1 domain being required for the partial agonist activity of antisteroids with most steroid receptors. DNA sequence (MMTV vs a simple GRE enhancer) and cell-specific factors (CV-1 vs Cos-7) exert minor effects on the level of partial agonist activity. Small activity differences for some complexes of GAL4/GR chimeras with GR- vs GAL-responsive reporters suggest a contribution of DNA-induced conformational changes. A role for steroid-regulated coactivator binding to GRs is compatible with the progressively smaller increase in partial agonist activity of Dex-Mes > Prog > RU486 with added GRIP1 in CV-1 cells. This hypothesis is consistent with titration experiments, where low concentrations of GRIP1 more effectively increase the partial agonist activity of Dex-Mes than Prog complexes. Furthermore, ligand-dependent GRIP1 binding to DNA-bound GR complexes decreases in the order of Dex > Dex-Mes > Prog > RU486. Thus, the partial agonist activity of a given GR−steroid complex in CV-1 cells correlates with its cell-free binding of GRIP1. The ability to modify the levels of partial agonist activity through changes in steroid structure, DNA sequence, specific DNA-induced conformational changes, and coactivator binding suggests that useful variations in endocrine therapies may be possible by the judicious selection of these parameters to afford gene and tissue selective results.

Antisteroids, or steroid antagonists, have proven to be extremely useful clinical agents in endocrine therapies to combat many unwanted conditions such as hypertension (*1*), some cases of Cushing's syndrome (*2*), conception (*2*, *3*), and hormone-dependent cancers (*4*, *5*). Initially, compounds were sought that were pure antisteroids and devoid of any agonist activity. However, such compounds often produce undesirable side effects due to the suppression of all genes induced by the steroid that they antagonize. Subsequently, it has become apparent that there are advantages with steroids that block only some of the actions of an endogenous steroid and retain partial or complete agonist activity for other regulated genes, thereby reducing the number of unwanted side effects. These agents have been called selective receptor modulators (SRMs).[1] One essential characteristic of SRMs is that they display partial agonist activity with at least some responsive genes. Among the most notable SRMs are the anti-estrogens tamoxifen and raloxifene, which block estrogen receptor (ER) actions in breast but are estrogenic in bone, with tamoxifen but not raloxifene also being estrogenic in the uterus (*6*). Over the last several years, a variety of candidate SRMs have been reported for androgen (ARs) (*7*), glucocorticoid (GRs) (*8*−*13*), mineralocorticoid (*14*), and progesterone receptors (PRs) (*15*−*17*).

More recently, it has been reported that the amount of partial agonism of a given antisteroid varies not only among genes but also for the same gene under different conditions (reviewed in ref *18*). Thus, the partial agonist activity of the antiglucocorticoid dexamethasone 21-mesylate (Dex-Mes) changes for the rat tyrosine aminotransferase (TAT) gene with cell density (*19*) and for stably and transiently trans-

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[1] Abbreviations: GRs, glucocorticoid receptors; ARs, androgen receptors; ERs, estrogen receptors; PRs, progesterone receptors; Dex-Mes, dexamethasone mesylate; Dex-Ox, dexamethasone oxetanone; Prog, progesterone; DOC, deoxycorticosterone; SRMs, selective receptor modulators; GRE, glucocorticoid response element; TAT, tyrosine aminotransferase; LBD, ligand binding domain; DBD, DNA binding domain; ABCD assay, avidin−biotin coupled DNA binding assay; MMV, mouse mammary tumor virus; UAS, upstream activating sequences.

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fected reporters containing assorted segments of the TAT promoter (*20*, *21*). Similarly, the partial agonist activity of antiglucocorticoids with transiently transfected reporters is affected by the concentration of several factors including proteins binding to a specific modulatory element of the TAT gene, coactivators, corepressors, comodulators, Ubc9 (a human homologue of the yeast E2 ubiquitin-conjugating enzymes), and Sur2 (a component of the human homologue of the yeast modular transcription complex Mediator) in addition to GRs themselves (*9*−*11*, *13*, *22*−*25*). This modulatory activity of many of these factors is not limited to GRs but, in combination with unidentified cell-specific factors, has also been observed with all of the classical steroid receptors (*18*, *26*).

The mechanism(s) responsible for the expression of partial agonist activity remains obscure. Structural features of the steroids are clearly important but have resisted generalization. Bulky substituents are thought to be important for the relocation of helix 12 of the receptor to a conformation that blocks the binding of coactivators (*27*, *28*). However, deacylcortivazol is an extremely large molecule (*29*) with a very bulky substituent fused to the A-ring of the steroid but still is the most potent glucocorticoid described (*30*). Conversely, other steroids are antagonists in the absence of bulky substituents, such as the antiestrogen genistein (*31*) and the antiglucocorticoids progesterone and deoxycorticosterone (*32*). These observations have led to the hypothesis that the DNA-bound receptor−steroid complexes possess different conformations of the ligand binding domain (LBD), which causes differential interactions with coregulators, coadaptors, and the transcriptional machinery (*17*, *27*, *33*).

Conformational variations of the LBD can be caused by ligand-induced conformational changes (*34*−*36*). Alternatively, DNA-induced changes in the receptor DNA binding domain (DBD) may be transmitted to the LBD to modify the binding of coactivators and corepressors, thereby altering the total levels of gene expression (*37*−*41*). DNA-induced conformational changes of proteins are well documented both for transcription factors in general (*42*−*44*) and for steroid receptor DBDs (*37*, *45*−*47*). DNA-induced conformational changes have also been reported to affect the AF-1 domain of receptors (*48*, *49*).

It is thought that the AF-1 domain regulates the transcriptional activity of antisteroid complexes of steroid receptors such as PR (*50*), AR (*51*), and ER (*52*−*57*) even though the interactions of coactivators and corepressors with receptors are mediated by the AF-2 domain, which is absolutely required for the ligand binding activity of receptors. This is believed to result from the binding of p160 coactivator proteins to the AF-1 domain. Thus, the human coactivator TIF2 (and the mouse homologue GRIP1) increased the partial agonist activity for tamoxifen in the initially cloned ER (ERG400V) (*58*) by enhancing AF-1 activity (*59*). TIF2 and SRC-1 also augmented the constitutive transactivation activity of GRs lacking the LBD (*60*). For this reason, we were surprised to find recently that the AF-1 domain of GR is not required for the expression of the partial agonist activity of the antiglucocorticoid Dex-Mes (*61*). However, this behavior could be unique to Dex-Mes. Not all antiglucocorticoids display the same amount of partial agonist activity with the same reporter/cell system (*12*). Studies with several different antisteroids indicated that the nature of the steroid-induced conformational changes in the receptor LBD is not the same when assayed by their sensitivity to protease digestion (*62*, *63*). Similarly, the ability of various receptor−antagonist complexes to bind selected peptides is different (*64*).

The focus of this study, therefore, is to see if the partial agonist activity of a variety of antiglucocorticoids is independent of the GR AF-1 domain as has been observed for Dex-Mes (*61*). For this purpose, we examine a variety of antiglucocorticoids in two cell lines with two different GREs. This last comparison allows us to examine the role of DNA-induced conformational changes in the expression of partial agonist activity for GR complexes. Finally, we ask whether the effect of coactivators on different GR−antagonist complexes is the same. We find that the expression of partial agonist activity is independent of the GR AF-1 domain. DNA-induced conformational changes in GRs are shown to constitute a contributing mechanism for modulating the activity of several, but not all, receptor−antagonist complexes. Finally, steroid-dependent changes in coactivator binding to GRs participate in determining the final amount of partial agonist activity.

MATERIALS AND METHODS

Unless otherwise indicated, all operations were performed at 37 °C.

Chemicals and Antibodies. Dex, Prog, and DOC were from Sigma (St. Louis, MO). Dex-Ox (*65*) and Dex-Mes (*66*) were prepared as described. Restriction enzymes and digestions were performed according to the manufacturer's specifications (New England Biolabs, Beverly, MA). Anti-GR antibody BUGR-2 was purchased from Affinity Bioreagents (Golden, CO), and anti-HA (HA-probe, F-7) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antiglucocorticoid receptor antibody aP1 is from Bernd Groner (Frankfurt am Main, Germany).

Preparation of Plasmids. Renilla null luciferase reporter was purchased from Promega (Madison, WI), and pM vector was from Clontech (Palo Alto, CA). pFR-Luc reporter, which contains five repeats of the GAL4 binding element fused upstream of a basic TATA promoter and the Luciferase reporter, was from Stratagene (La Jolla, CA). GREtkLUC (*12*), GAL/GR and VP16/GR (*23*), GAL/GR-525C, GAL/GR-407C, and human serum albumin (hSA)/pSG5 (24) are described elsewhere. GR-407C (pC7/GR407C) was donated by Didier Picard (University of Geneva, Switzerland). pSVL-GR was received from Keith Yamamoto (UCSF, San Francisco). pHA-GRIP and pSG5-HA were gifts from Michael R. Stallcup (USC, Los Angeles). pHA-TIF2.4 was constructed by Ajian He by inserting the *Eco*RI/*Bam*HI fragment of pGAL/TIF2.4 (*13*) into the *Eco*RI/*Bam*HI site of pSG5-HA (AH4-77). MMTVLUC (pLTRLUC) was kindly provided by Gordon Hager (NIH, Bethesda, MD).

Cell Culture and Transient Transfection. Cos-7 and CV-1 cells were maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen/Life Technologies, Carlsbad, CA) supplemented with 5% or 10% of fetal bovine serum (Biosource International, Camarillo, CA), respectively. Cells were seeded at a density of 2×10^4 cells/well in 24-well plates (Corning Inc., Corning, NY). Unless specified otherwise, all transfections were in triplicate in 24-well plates.

After overnight culture, a total of 0.3 μg of plasmids containing 0.1 μg of reporter plasmid, 0.01 μg of Renilla internal control plasmid, and other expression plasmids (see figure captions) were mixed with Fugene solution (0.7 μL of Fugene per 0.3 μg of DNA in serum-free DMEM), incubated at room temperature for 30 min, and added to the culture. One day later, transfected cells were induced with 1 μM of the appropriate steroids for 24 h. The cells were lysed and assayed for reporter gene activity using the luciferase assay reagent according to the manufacturer's instructions (Promega). Luciferase activity was measured in an EG&G Berthold luminometer (Microlumat LB96P). The data were normalized for Renilla null luciferase activity to correct for differences in transfection efficiency. The partial agonist activity of a steroid A (expressed as percent) is defined as follows: 100 × [(the activity with 1 μM steroid A) − (the basal level seen in the absence of hormone)]/[(the activity with 1 μM Dex) − (the basal level seen in the absence of hormone)]. The amount of each receptor plasmid used is determined from titration experiments to be less than that required for maximal gene induction, thus ensuring that the receptor protein is limiting for each condition (*11*).

Preparation of Cos-7 Cell Cytosol Containing Overexpressed Proteins. Cos-7 cells were seeded at a density of 1 × 10^6 cells/100-mm dish and incubated overnight. Expression plasmids (1 μg of pSVL-GR, pHA-GRIP, or pHA-TIF2.4) plus 4 μg of carrier DNA (pBSK+) in 0.8 mL of DMEM were mixed with 27 μL of Fugene in 0.8 mL of DMEM, incubated at rt for 30 min, and added to the cells. The medium was change at 24 h, and the cells were harvested at rt after 48 h, washed three times with ice cold PBS, and rapidly frozen on dry ice for at least 15 min. Cells were lysed in one pellet volume of TAPS buffer (25 mM TAPS, pH 9.5, 1 mM EDTA, 10% glycerol) with brief vortexing. Cytosol was obtained after centrifugation (15000*g* for 15 min at 4 °C) and used directly or stored at −80 °C.

Modified ABCD Assay. Biotinylated sense (5′-Biotin-GATCCTGTACAGGATGTTCTAGCTACA) and anti-sense (5′-Biotin-TGTAGCTAGAACATCCTGTACAGGATC) GRE oligonucleotides were obtained from Invitrogen/Life Technologies. Equal amounts of complementary oligonucleotides (10 nmol each in a total volume of 0.2 mL of TES buffer [10 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl]) were heated at 85 °C for 5 min and cooled over 2−3 h to below 40 °C. The annealing efficiency was checked by running samples on a 20% native PAGE, and the resulting double stranded oligonucleotides were used directly without further purification. Streptavidin-agarose beads (Sigma) were pre-equilibrated by washing once with HEPES buffer (10 mM HEPES, pH 7.5, 1 mM EDTA, 10% glycerol) and resuspended in one bed volume of HEPES buffer. Annealed GRE oligonucleotides (2.5 nmol in 25 μL) were added to 0.5 mL of 50% slurry and incubated at 4 °C for 2 h with constant agitation in a Belco rotating drum at 11 rpm. The DNA-bound beads were washed once with HEPES buffer containing 10 mM NaCl and twice more with HEPES buffer without NaCl. Mock or GR-containing Cos-7 cytosol was diluted with HEPES buffer to 30% and bound with 1 μM steroid (2.5 h at 0 °C). After activation (30 min at 20 °C), cytosols (50 μL) were treated with 20 μL of the 50% slurry containing immobilized DNA oligonucleotides (4 °C for 16 h on a Belco rotating drum at 11 rpm). Unbound GR was removed by centrifugation, and 100 μL each of mock, GRIP- or TIF2.4-containing Cos-7 cytosol was added to the beads and incubated for 4 h more under the same conditions. The samples were washed three times with HEPES buffer and resuspended in a final volume of 20 μL. Bound proteins were eluted by adding 20 μL of 2X SDS loading buffer and boiling for 5 min.

Western Blotting. Proteins were transferred from SDS-polyacrylamide gels to PROTRAN nitrocellulose membranes (Scheicher & Schuell GmbH, Dassel, Germany) using Xcell II transblot modules (110 mA overnight; Invitrogen) as described (*62*) except that the membranes were incubated with 5% Carnation nonfat dry milk in TBS (Quality Biologicals Inc.) containing 0.1% Tween-20 (Bio-Rad Laboratories, Hercules, CA). Primary antibodies were diluted in TBS containing 0.1% Tween-20 (1:5000 to 1:15000 for BUGR-2 and 1:200 for anti-HA) and followed by biotinylated secondary antibodies (anti-mouse IgG for BuGR2 and anti-HA, and anti-rabbit IgG for aP1) and ABC reagents (Vector Laboratories, Burlingame, CA). The signals were detected by enhanced chemiluminescence using the recommended protocol of the supplier (Amersham Pharmacia Biotech). The magnitude of the Western blot signals was quantitated by a Bio-Rad GS-800 calibrated densitometer.

Statistical Analysis. Unless otherwise indicated, all transient transfection experiments were performed in triplicate several times. The values of *n* independent experiments were then analyzed for statistical significance by the two-tailed Student *t* test using the program InStat 2.03 for Macintosh (GraphPad Software, San Diego, CA). When the difference between the S.D.s of two populations is significantly different, then the Mann−Whitney test or the Alternate Welch *t* test is used.

RESULTS

Properties of Assay Systems To Determine Partial Agonist Activities. To examine the role of different GR domains in the expression of the partial agonist activity of antiglucocorticoids, a variety of GR constructs were prepared (Figure 1A). The role of the first 406 amino acids and the AF-1 domain can be determined from the activity of the truncated GR (GR407C) vs full length GR, when bound by four different antagonists (Dexamethasone mesylate [Dex-Mes], Dex oxetanone [Dex-Ox], deoxycorticosterone [DOC], and progesterone [Prog]), with two Luciferase reporters (GREtkLUC and MMTVLuc). To elucidate the role of the GR LBD, which does not bind to DNA, we fused the GR LBD to another DNA binding molecule (GAL4 DBD) to make GAL/GR525C (Figure 1A) and looked at its ability, when bound by the same antiglucocorticoids, to induce a Luciferase reporter controlled by five upstream GAL4 binding elements (FRLuc). We prepared GAL/GR407C (Figure 1A) in order to address several additional questions. By quantitating the activities of GAL/GR407C on MMTVLuc vs FRLuc, and of GAL/GR407C vs GAL/GR525C on FRLuc, we can evaluate the role of the fused GAL4 DBD (GR407C vs GAL/GR407C on MMTVLuc), the DNA binding sequence (GAL/GR407C on MMTVLuc vs FRLuc), and the GR DBD (GAL/GR407C vs GAL/GR525C on FRLuc). In each case, the responses were determined in two cell lines (CV-1 and Cos-7) to investigate possible cell-specific effects.

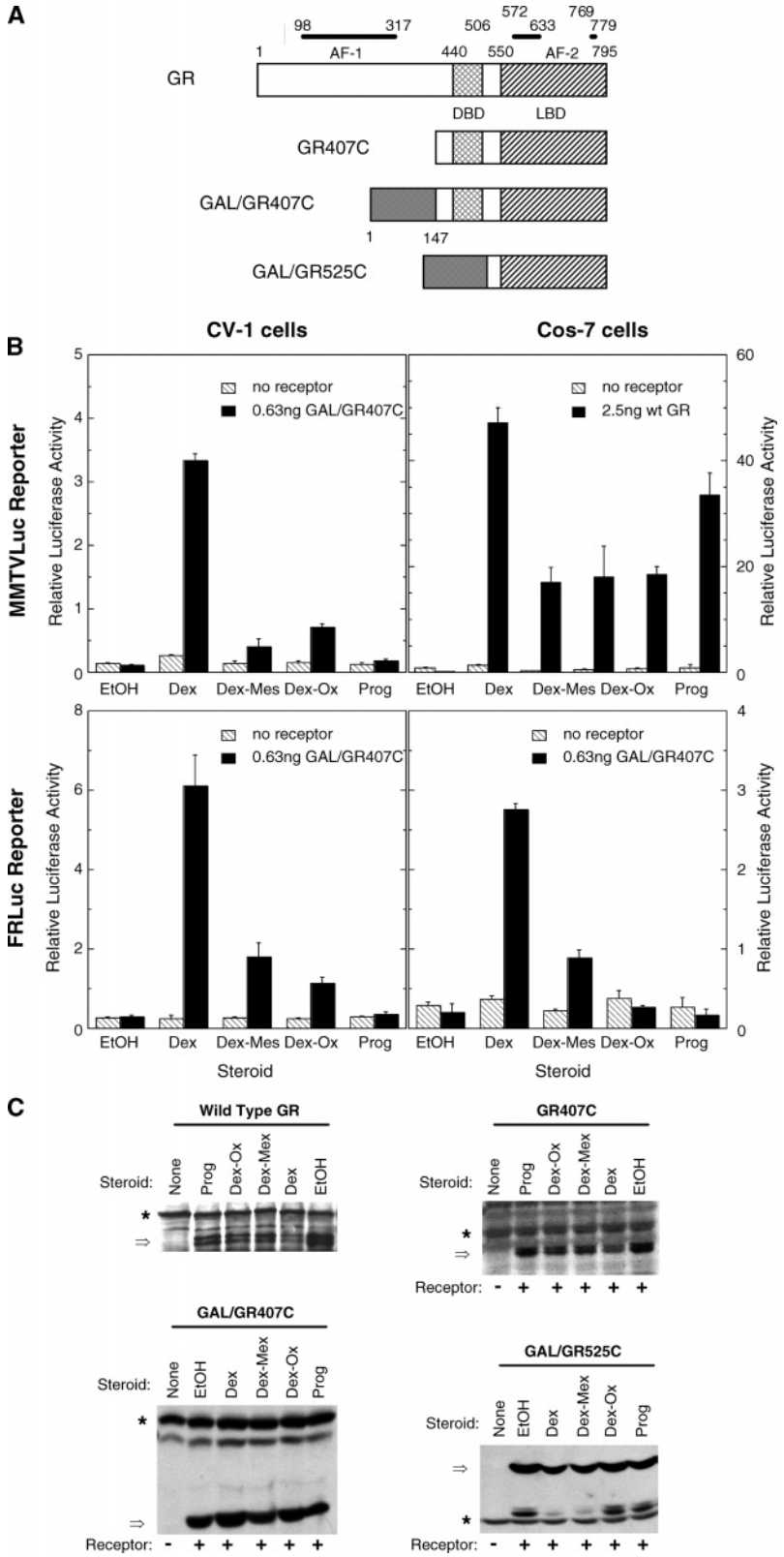

FIGURE 1: Properties of the assay system used to determine the partial agonist activity of GR complexes. (A) Cartoon of GR constructs used. DBD (amino acids 440−506) and LBD (amino acids 550−795) domains of GR are indicated by the crosshatched and diagonally striped boxes. The regions of the AF-1 and AF-2 (562−633 and 769−779) domains are indicated by the solid bars above the cartoon for GR. The GAL DBD is depicted by the shaded box. (B) Requirement of transfected GRs for activity of antiglucocorticoids. Cells were transiently transfected with or without the indicated amount of GR plasmid, 100 ng of MMTVLuc or FRLuc reporter, and Renilla control plasmid, and induced with EtOH ± 1 μM of the indicated steroids. The relative luciferase activities, normalized to the internal Renilla control values, were plotted ± SD of the triplicate samples. (C) Receptor stability in the presence of steroids. Cos-7 cells were transfected with 0.63 ng of GAL/GR407C plasmid without reporter plasmid or 100 ng of reporter plasmid (GREtkLUC or FRLuc) plus 2.5 ng GR, 10 ng GR407C, or 0.1 ng GAL/GR525C and incubated with 1 μM steroid for 24 h. Equal amounts of cell lysate were separated by SDS−PAGE, and the receptors (⇒) were visualized by Western blotting with BUGR2 anti-GR antibody or anti-GAL antibody. The loading of the same amount of protein in each lane was confirmed by the presence of equal amounts of a nonspecifically detected protein (*).

First, we conducted several control experiments. The percent agonist activity of each steroid is not significantly greater at 10 μM than at 1 μM for either the full length GR or GAL/GR525C in CV-1 or Cos-7 cells (data not shown). Therefore, despite differences in steroid affinity for GRs, 1 μM of each steroid is sufficient for full or nearly full occupancy of receptors. We therefore elected to conduct all future studies with a maximum of 1 μM steroid. Experiments without transfected receptors, some of which are shown in Figure 1B with the MMTVLuc and FRLuc reporters, established the absence of any endogenous, functional receptors that might induce either reporter and thus complicate the interpretation of results with transfected GRs in either cell line. The necessity of specific GRE sequences for GR-induced transactivation from the GREtkLUC reporter has been previously documented (*67*). To assess the nonspecific transactivation of the FRLuc reporter by a receptor without a GAL-DBD, we determined the activity of VP16/GR, which should induce transcription ± steroid upon binding anywhere near the promoter. The activity of 85 ng of VP16/GR with FRLuc without (0.06 units) and with (0.22 units) 1 μM Dex is significantly less than the 9.4 units for 0.63 ng of GAL/GR407C with 1 μM Dex, thus indicating that the GAL4-DBD is needed to activate the FRLuc reporter and that nonspecific transactivation by GRs is low. Dex-Mes is an affinity labeling steroid (*68*, *69*) but the partial agonist activity of Dex-Mes is the same with wild type GR and a mutant GR that cannot be covalently labeled (*12*), thus establishing that the covalent labeling of GR does not alter the amount of agonist activity of Dex-Mes. Finally, we asked whether the level of receptor protein is altered by 24 h of incubation with a variety of steroids. As shown in Figure 1C, the receptor protein levels after 24 h of incubation with each antisteroid are the same either in the absence (GAL/GR407C) or in the presence (wt GR, GR407C, or GAL/GR525C) of reporter plasmids. Therefore, it is unlikely that any steroid-dependent differences in partial agonist activity will result from differential effects of the various antiglucocorticoids on receptor turnover.

Amino Terminal Half of GR Is Not Required for Partial Agonist Activities. Consistent with previous results in CV-1 cells (*9*, *11*, *12*, *24*), each of the above four antisteroids displays a low to intermediate amount of agonist activity in CV-1 cells with the full length GR and a GREtkLuc reporter (Figure 2A, top left panel). Similar results are seen with the MMTVLuc reporter in CV-1 cells (bottom left panel) and, except for the higher activity for Prog, with both reporters in Cos-7 cells (right-hand panels). As shown in Figure 1B, these results are not compromised by the endogenous GRs of CV-1 cells (*9*), or any other receptors, because of their negligible functional activity.

Deletion of the AF-1 domain and the amino terminal half of GR, to give GR407C (Figure 1A) reduces the total amount of gene activation of GREtkLUC and MMTVLuc reporters in CV-1 cells by 4- and 20-fold respectively as has been previously observed (*11*, *70*). This confirms the importance of the amino terminal half of GR, and the AF-1 domain, as a determinant for gene induction. Surprisingly, this same deletion has little effect on the amount of partial agonist activities with either reporter in either cell line (Figure 2B vs 2A). If anything, the amount of partial agonist activity is increased by this truncation of GR. The different rank order

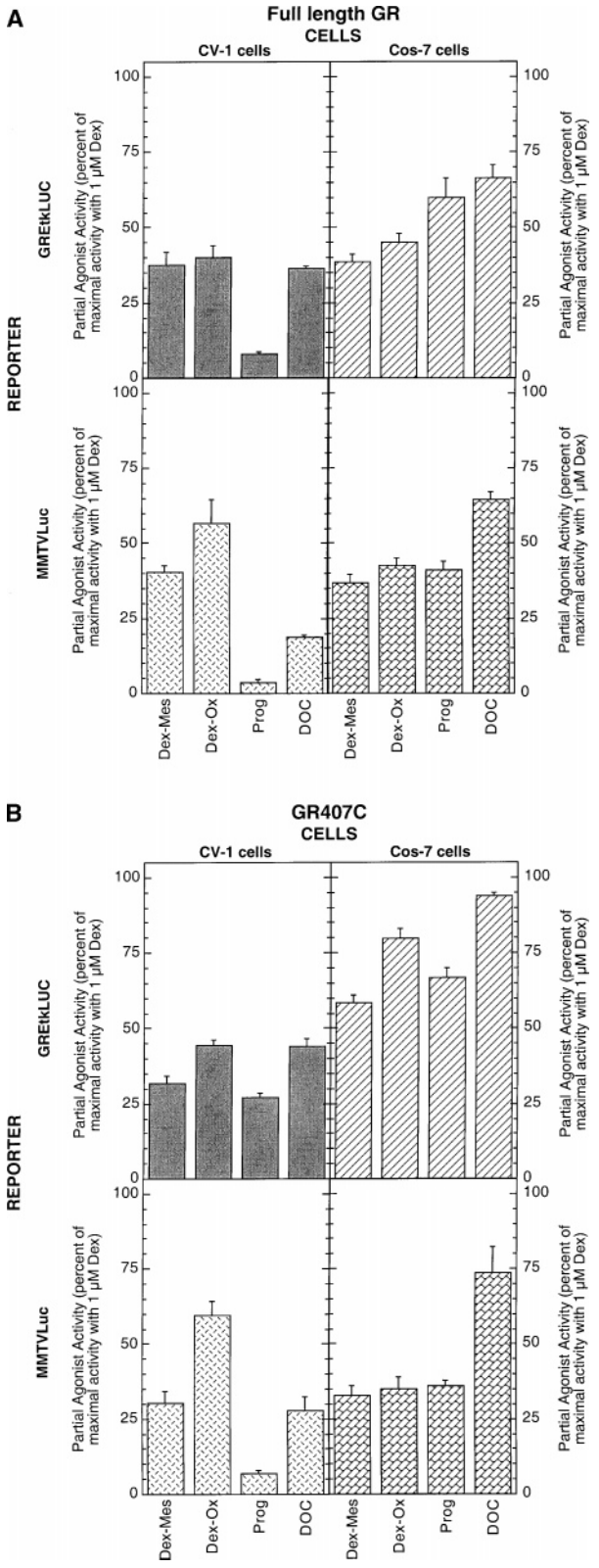

FIGURE 2: Partial agonist activity of antiglucocorticoids with (A) full length GR and (B) GR407C on different reporters in different cells. Cells were transiently transfected with full length GR (2.5 ng of pSVLGR) or GR407C (10 ng), 100 ng of GREtkLUC or MMTVLuc reporter, and Renilla control plasmid, and induced with EtOH ± 1 μM Dex or 1 μM antisteroid (Dex-Mes, Dex-Ox, Prog, or DOC). The luciferase activities, normalized to the internal Renilla control values, were expressed as percent of the maximal response with 1 μM Dex as described in Materials and Methods to give the partial agonist activity. The plotted values represent the average ± SEM of 3−7 independent experiments.

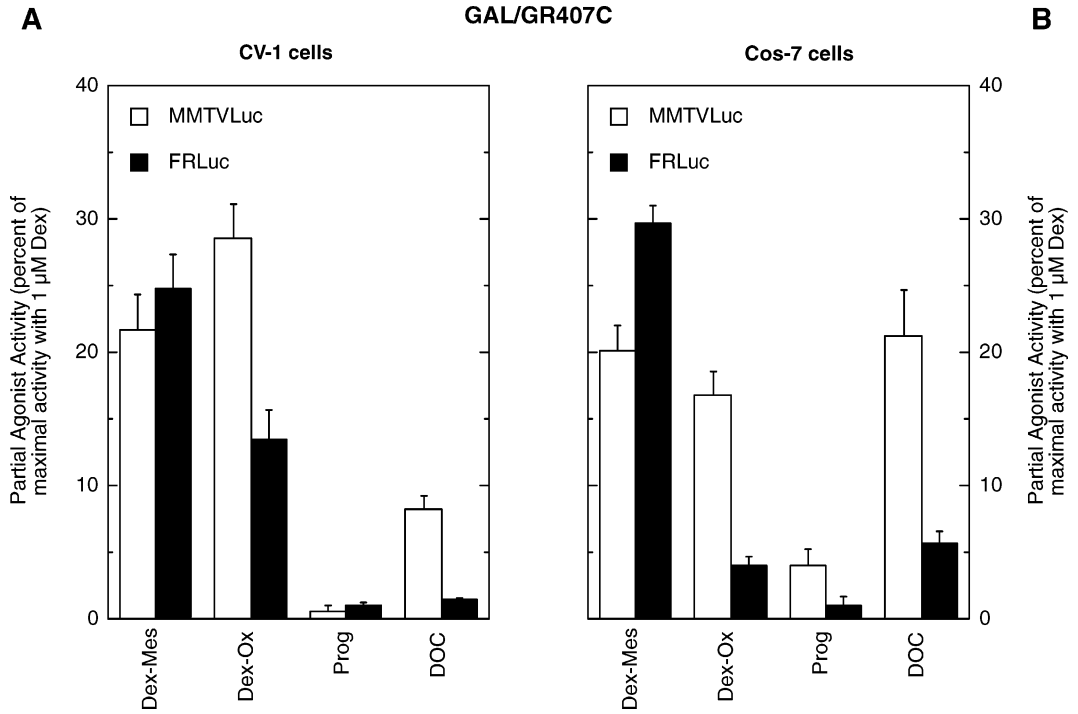

FIGURE 3: Effect of GR binding to DNA vs recruiting to DNA on the amount of partial agonist activity. (A) CV-1 or (B) Cos-7 cells were transiently transfected with 0.63 ng of GAL/GR407C, 100 ng of MMTVLuc or FRLuc reporter, and Renilla control plasmid, and induced with EtOH ± 1 μM Dex or 1 μM antisteroid (Dex-Mes, Dex-Ox, Prog, or DOC). The partial agonist activities were determined and plotted as for Figure 2A. The plotted values represent the average ± SEM of 4−7 independent experiments.

of partial agonist activities in Cos-7 vs CV-1 cells suggests that cell-specific factors can influence the net activity. Nevertheless, the present data clearly demonstrate that the amino terminal 406 amino acids, including the AF-1 domain, are unnecessary for the expression of partial agonist activity by a variety of antiglucocorticoids under several conditions.

Role of Receptor−DNA Contacts in the Expression of Partial Agonist Activity. We next asked whether the physical contact of GR with the specific DNA sequence of a glucocorticoid response element (GRE) in DNA-bound GRs is important in the expression of partial agonist activity. If specific DNA-induced conformational changes are required to elicit the partial agonist activity of a GR−antagonist complex, we should see marked differences between GR complexes bound directly to a GRE and complexes that are localized to DNA by virtue of the association of GRs with some other DNA binding molecule. To answer this question, we looked at the activity of steroids bound to GR407C fused to the DNA binding domain (DBD) of the GAL4 protein (GAL/GR407C in Figure 1A). GAL/GR407C retains the GR DBD and would be expected to afford transactivation when bound either to the upstream GREs of the MMTVLuc reporter via the GR LBD or to the GAL4 upstream activating sequences (UAS) of the FRLuc reporter via the GAL DBD. Thus, GR receptor sequences make direct DNA contacts with MMTVLuc while GR is recruited to the DNA but does not contact the DNA or a GRE with FRLuc. Again, control experiments confirmed the absence of any inducible Luciferase activity from the FRLuc reporter in both cell lines with any of the steroids when GAL/GR407C is omitted (Figure 1B). As shown in Figure 3, appreciable amounts of partial agonist activity are displayed by GAL/GR407C on the MMTVLuc reporter in both CV-1 and Cos-7 cells. Unexpectedly, the partial agonist activity of Dex-Ox and DOC is markedly decreased with the FRLuc vs MMTVLuc reporter in both cell lines. This suggests that, for some GR complexes, the contact of the GR protein with GRE DNA sequences is a contributing factor, possibly due to DNA-induced conformational changes for the GRE-bound chimera.

Role of GR LBD in the Expression of Partial Agonist Activity. The data of Figure 3 support the conclusions from Figure 2 that the AF-1 domain is not essential to elicit the partial agonist activity of many antiglucocorticoids. Because the other major transactivation domain, AF-2, is fully contained within the GR LBD, we asked whether there would be any further change in the production of partial agonist activity for a DNA-localized complex upon deletion of the GR DBD. This question was addressed by contrasting the activities of the GAL/GR525C chimera (see Figure 1A) with those of GAL/GR407C in Figure 3. Surprisingly, the only complex of GAL/GR525C that displays large amounts of partial agonist activity in either CV-1 or Cos-7 cells is that with Dex-Mes (Figure 4). This result does not depend on the receptor concentration. It is known that elevating the concentration of full length GR does increase the amount of partial agonist activity of antiglucocorticoids (*9*−*11*); and a 20-fold increase in the amount of transfected GAL/GR525 plasmid causes a 1.4-fold increase in the partial agonist activity of Dex-Mes from 42% to 57% (data not shown; $n = 4$). However, no increase is seen in the partial agonist activities of Dex-Ox or Prog when a 20-fold increase in GAL/GR525C (2 vs 0.1 ng of plasmid) is used (data not shown). Thus, we can conclude that features between amino acids 407 and 524 contribute to the expression of partial agonist activity for GR complexes of some (i.e., Dex-Ox and DOC), but not all (i.e., Dex-Mes), antiglucocorticoids (cf. Figures 3 vs 4).

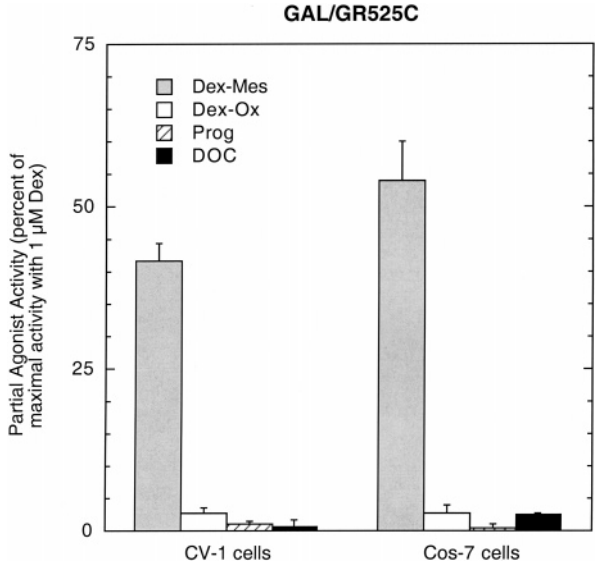

FIGURE 4: Partial agonist activity of antiglucocorticoids with GAL/GR525C. CV-1 or Cos-7 cells were transiently transfected with 0.1 ng of GAL/GR525C, 100 ng of FRLuc reporter, and Renilla control plasmid, and induced with EtOH ± 1 μM Dex or 1 μM antisteroid (Dex-Mes, Dex-Ox, Prog, or DOC). The partial agonist activities were determined and plotted as for Figure 2A. The plotted values represent the average ± SEM of 3−7 independent experiments.

Ability of Coactivator To Increase the Partial Agonist Activity of Antiglucocorticoids. We have previously reported that the p160 coactivators (SRC-1, GRIP1/TIF2, AIB1) and CBP and PCAF increase the amount of partial agonist activity of antiglucocorticoids (*9*, *10*, *13*) in a manner that involves the binding of coactivator to the GR−antagonist complex (*13*). We recently found that the GR LBD is a sufficient target for GRIP1 to increase the partial agonist activity of the truncated GRs bound by Dex-Mes (*61*). However, in view of the reduced ability of GR LBD chimeras to express partial agonist activity when bound by other antiglucocorticoids (Figure 4), it is important to know if coactivators are able to increase the partial agonist activity of other GR−antagonist complexes or whether some defect in coactivator binding to these other complexes inhibits the expression of partial agonist activity. For these studies, we substituted the antagonist RU486 for DOC so that we could simultaneously examine the effects of added coactivator on an antiglucocorticoid that usually shows negligible amounts of partial agonist activity (*9*, *11*, *12*). We also used higher, but still subsaturating, concentrations of GAL/GR525C (*61*) compared to Figure 4 (5−10 ng vs 0.1 ng) because higher receptor concentrations often increase the partial agonist activity of antisteroids (*9*, *11*, *61*), thereby making any increased partial agonist activity with added coactivator easier to see.

The addition of exogenous GRIP1 increases the partial agonist activity in CV-1 cells for each antisteroid, except RU486, when bound to all GR constructs: full length GR, GR407C, and GAL/GR525C **(**Figure 5). In each case, this increase in partial agonist activity is relative to the increased activity of 1 μM Dex that is seen with GRIP1 under identical conditions. Thus the ability of GRIP1 to increase the partial agonist activity of Dex-Mes is not unique because the same behavior is seen with Dex-Ox and Prog. Furthermore, there does not appear to be a major difference in the ability of GRIP1 to interact with the GR-LBD whether present in the full length GR or by itself when fused to the GAL-DBD. However, these data suggest that the low amount of partial agonist activity of some antisteroids, like Prog and RU486, could be due to less efficient interactions with coactivators. Together, the data of Figures 4 and 5 indicate that the GR LBD is sufficient for the expression of partial agonist activity with a variety of antiglucocorticoids, although the DBD-containing sequence of 407−524 can facilitate the expression of partial agonist activity.

Titration of Increased Partial Agonist Activities by Exogenous GRIP1. To determine the efficiency of coactivator-induced increases in partial agonist activity of Dex-Mes vs Prog complexes of GR in Figure 5, we compared the ability of these complexes to respond to a range of GRIP1 concentrations that are lower than the 50 ng used in Figure 5. Differences in the efficiency of coactivator actions will be more noticeable when using subsaturating concentrations of GRIP1. As in Figure 5, the activity of 1 μM Dex in the presence of the same amount of transfected GRIP1 is used to calculate the percent agonist activity at each concentration of exogenous GRIP1. For induction of the GREtkLUC reporter by the full length GR in CV-1 cells, the concentration of GRIP1 required for half-maximal increase in the Dex-Mes partial agonist activity is about 0.2 ng of GRIP1 (Figure 6A). The comparable value to augment Prog partial agonist activity is difficult to calculate as the total increase was much less. However, the much greater additional partial agonist activity for Dex-Mes than Prog complexes with exogenous GRIP1 suggests that there is more binding of GRIP1 to the Dex-Mes complexes of full length GR.

The main binding site of coactivators to GRs is in the LBD (*71*, *72*) and GRIP1 increases the partial agonist activity of many antisteroids bound to GAL/GR525C (Figure 5). Therefore, we next determined the concentration of GRIP1 needed to increase the partial agonist activities of antiglucocorticoids inducing the FRLuc reporter with GAL/GR525C. As shown in Figure 6B, 0.2 ng of GRIP1 is again half-maximal for GAL/GR525C complexes with Dex-Mes while much less of an effect is seen with Prog complexes. These results are consistent with the greater partial agonist activity of GR complexes of Dex-Mes vs Prog being due to GRIP1 having a greater affinity for GR bound with Dex-Mes.

Binding of GRIP1/TIF2 to DNA-Bound GR Complexes of Antiglucocorticoids. While the results of Figure 6 suggest that coactivators have different binding affinities to various GR−antagonist complexes, it is also possible that coactivators have similar affinities for the assorted complexes but different efficacies for augmenting the partial agonist activity of each GR−steroid−coactivator complex. To resolve these questions, we employed a modified ABCD assay (*73*), in which receptor−steroid complexes are first activated and then bound to biotinylated DNA containing a single GRE that has been immobilized on Streptavidin-agarose beads. Coactivator is then added, and its binding to the different DNA-bound GR-steroid complexes is determined by extraction followed by Western blotting. The coactivators that we used are the full length GRIP1 and TIF2.4 (Figure 7A), which is a fragment of TIF2 that contains the receptor interaction domains needed for TIF2 binding to GR and that is known to bind to GR (*13*, *74*, *75*). All proteins were overexpressed

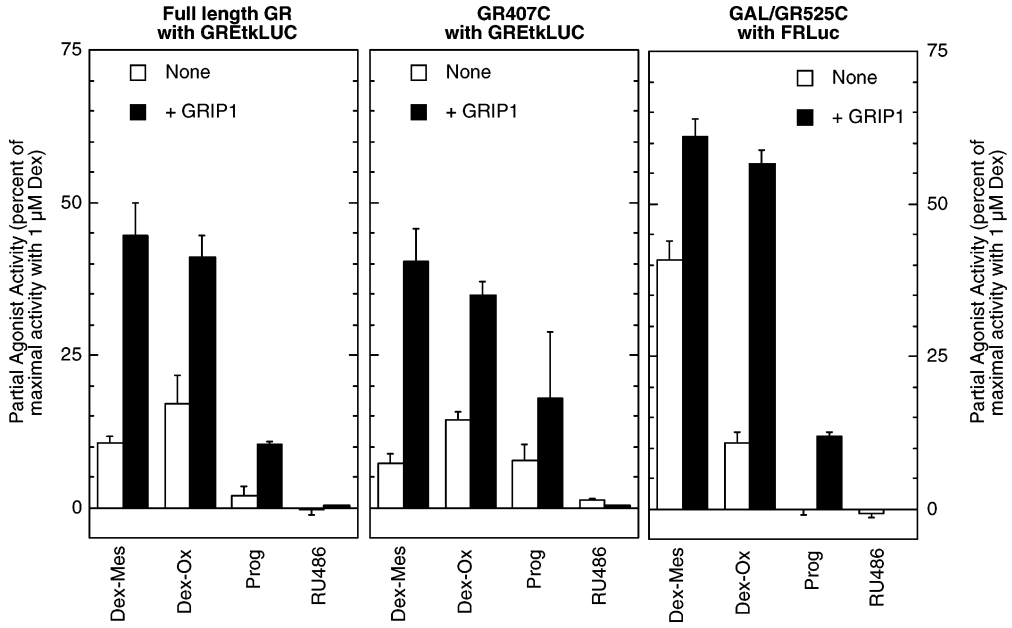

FIGURE 5: Increase by added coactivator GRIP1 in partial agonist activity with different GR constructs. CV-1 cells were transiently transfected with 10 ng of GR, GR407C, or GAL/GR525C, 100 ng of GREtkLUC or FRLuc reporter, and Renilla control plasmid plus 50 ng of HA/GRIP or an equimolar amount of human serum albumin (hSA) in the same vector, and induced with EtOH ± 1 μM Dex or 1 μM antisteroid (Dex-Mes, Dex-Ox, Prog, or RU486). The partial agonist activities were determined as a percent of the maximal activity with 1 μM Dex in the presence of the same amount of transfected GRIP1 and plotted as for Figure 2A. The plotted values represent the average ± SEM of 3−7 independent experiments.

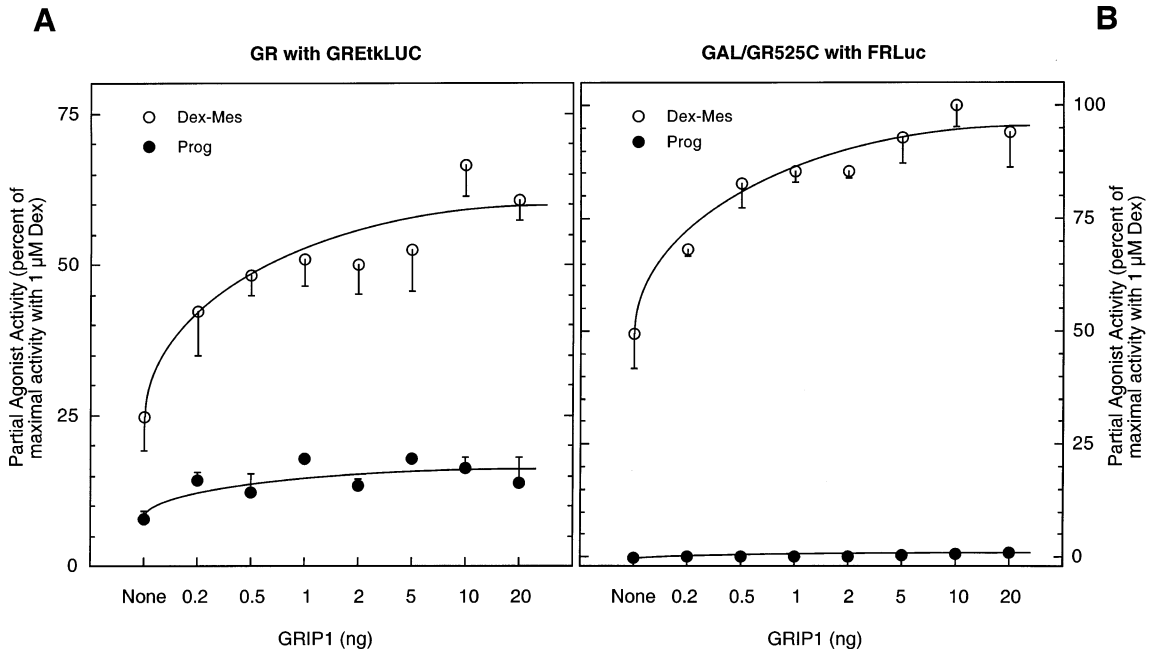

FIGURE 6: Titration of GRIP1 ability to increase the partial agonist activity of antiglucocorticoids with (A) full length GR and (B) GAL/GR525C. CV-1 cells were transiently transfected with 2.5 ng full length GR (or 0.25 ng of GAL/GR525C), 100 ng GREtkLUC (or 100 ng of FRLuc) reporter, and Renilla control plasmid plus the indicated amounts of HA/GRIP1 (with the total amount of vector kept constant by the addition of hSA in the same vector), and induced with EtOH ± 1 μM Dex or 1 μM antisteroid (Dex-Mes or Prog). The partial agonist activities were determined as a percent of the maximal activity with 1 μM Dex in the presence of the same amount of transfected GRIP1 and plotted as for Figure 2A. The plotted values represent the average ± SD of the triplicate wells. Similar results were obtained in two additional experiments.

in Cos-7 cells due to the higher levels of expression compared to CV-1 cells (data not shown).

As shown in the Western blot of Figure 7B, no GR binding to the GRE oligonucleotide is seen in the absence of overexpressed GR (lanes 3−5). The addition of GR affords DNA-bound GR independent of the steroid in the presence of mock cytosol (M), GRIP1 (G), or TIF2.4 (2.4). The amount of DNA-bound GRs in two experiments was quantitated by laser densitometry and plotted in the lower panel of Figure 7B, where each bar is aligned with the corresponding lane of the Western blot. These results are consistent with the observation that ligand binding is not essential for the DNA binding of activated steroid-free GRs (*76*). It is important to note that the subsequent addition of

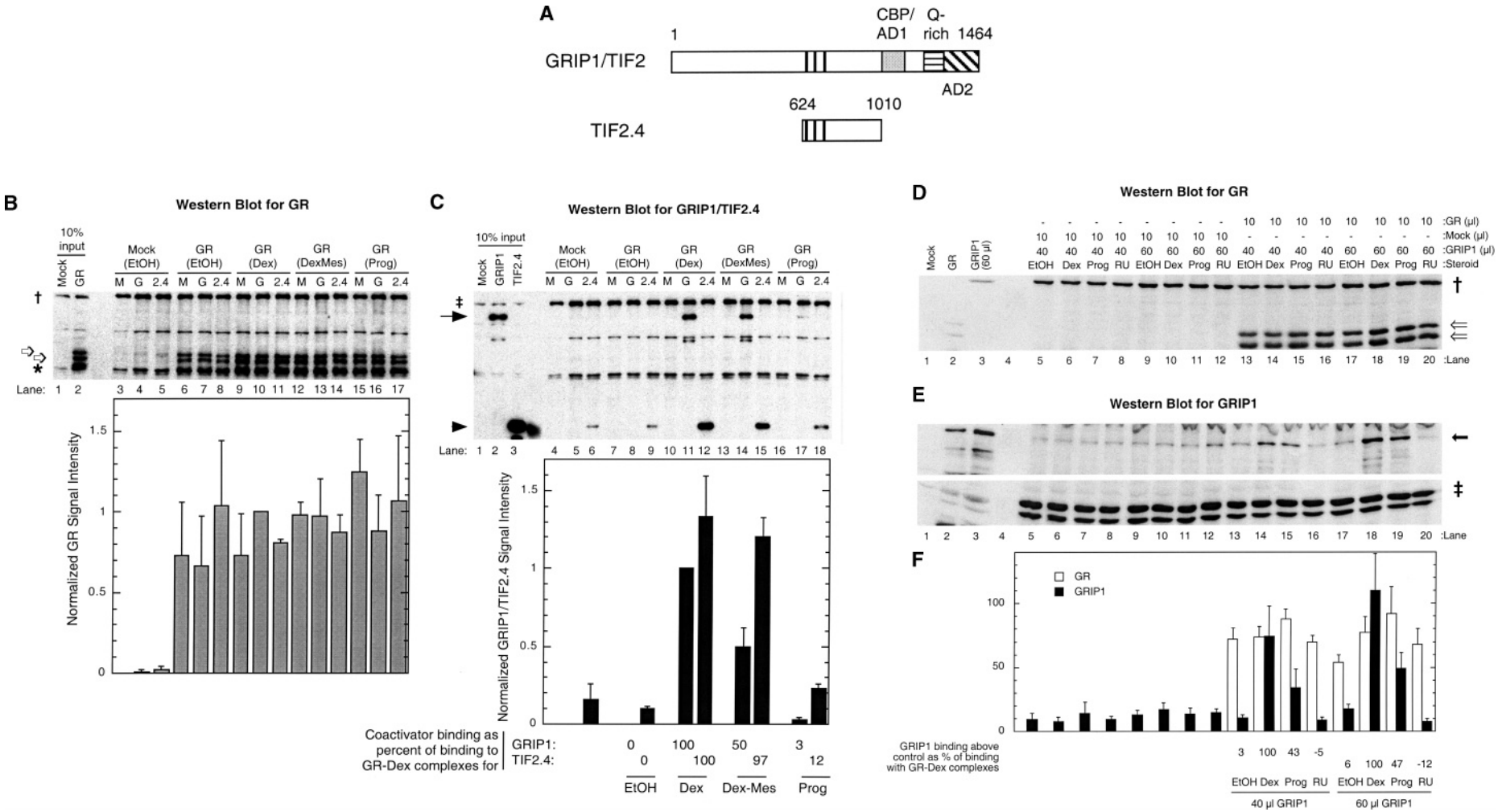

FIGURE 7: Steroid-dependent binding of coactivator to DNA-bound GR. (A) Cartoon of the various domains of TIF2/GRIP1. The amino acid positions from the literature (*74*, *83*) are given above the figure of each protein (RID, receptor interaction domain; AD1 and AD2, activation domain 1 and 2; Q-rich, glutamine rich). (B and C) Coactivator binding in ABCD assays to DNA-bound GR complexes in the presence of Dex, Dex-Mes, and Prog. Cos-7 cell cytosols without (Mock) or with overexpressed GR that had been prebound with EtOH ± 1 μM steroid were incubated with biotinylated GRE oligonucleotides attached to Steptavidin beads followed by Cos-7 cytosols (30 μL) without (M, for Mock) or with overexpressed HA/GRIP1 (G), or HA/TIF2.4 (2.4). DNA-bound GR and HA/GRIP1, or HA/TIF2.4, were separated on SDS-gels and visualized by Western blotting with anti-GR (panel B) or anti-HA (panel C) antibodies. Lane 2 (panel B) shows position of full length GR and two species due to down stream start sites (open arrows, lower species overlaps with nonspecifically detected species [*]) (*62*, *97*). Coactivator locations are shown in lane 2 of panel C and are marked by arrow or arrowhead. The amount of GR or coactivator protein was quantitated by laser densitometry, normalized to the levels of nonspecifically detected proteins († in panel B, ‡ in panel C) to correct for loading differences, and plotted (average value ± range) in the lower panels directly below the corresponding Western blot signal. (D and E) GRIP1 binding in ABCD assays to DNA-bound GR complexes in the presence of EtOH, Dex, Prog, or RU486. Cos-7 cell cytosols without (lanes 5−12) or with (lanes 13−20) overexpressed GR that had been pre-bound with EtOH ± 1 μM steroid were bound to immobilized, biotinylated GRE oligonucleotides. Two different amounts (40 and 60 μL) of Cos-7 cytosol containing overexpressed HA/GRIP1 were added and the amount of DNA-bound GR (⇐, panel D) and GRIP1 (←, panel E) was then visualized, normalized to the levels of nonspecifically detected proteins († in panel D, ‡ in panel E) to correct for loading differences, and plotted (average value ± SEM, $n = 3$) in panel F, with each bar being directly below the corresponding Western blot signal. The amount of specific GRIP1 binding above controls is calculated as a percent of that for GR-Dex complexes and listed at the bottom of panel F.

Cos-7 cell cytosol ± overexpressed GRIP1 or TIF2.4 does not alter the amount of GRE-bound receptor for each GR-steroid complex. The amount of coactivator that associates with the different GRE-bound GR complexes is shown by the Western blot of Figure 7C (upper panel), with the quantitative amount of bound GRIP1 or TIF2.4 being plotted in the lower panel of Figure 7C as for Figure 7B. The amount of bound coactivator with each ligand, expressed as the percent of the binding seen to GR−Dex complexes, is presented at the bottom of the lower panel. GRIP1 binding to ligand-free GRE-bound GRs is negligible (lanes 8 vs 7) but is robust for GRE-bound GR−Dex complexes (lanes 11 vs 10 and 8). By comparison, the binding of GRIP1 to GRE-bound GR−Dex-Mes and GR−Prog complexes is progressively less (lanes 11 vs 14 and 17). Likewise, the binding of TIF2.4 (Figure 7A) to GRE-bound GR complexes depends on the nature of the ligand and decreases in the order of Dex > Dex-Mes > Prog > EtOH (lanes 12 vs 15 vs 18 vs 9 vs 6 = nonspecific binding, lanes 9 vs 6) (Figure 7C). Similar results are obtained for GRIP1 binding to different GR complexes prebound to an oligonucleotide containing a MMTV GRE (data not shown).

To obtain a clearer relationship between the coactivator binding to DNA-bound GR−antagonist complexes and the amount of partial agonist activity of the antagonist in assays such as in Figure 5, we compared the binding of different amounts of GRIP1 to GRs bound by Dex, Prog, or RU486, which is a pure antagonist in the system of Figure 5. As shown in Figure 7D,E and quantitated in Figure 7F, GR binding is quite constant (open bars in Figure 7F). In contrast, GRIP1 binding (filled bars) increases with higher amounts of added GRIP1. The binding of GRIP1 in lanes 13−20 that is above the controls lacking GR (lanes 5−12) is expressed as the percent of that with GR−Dex complexes and listed at the bottom of Figure 7F. For both amounts of added GRIP1, the amount of GRIP1 binding to GRE-bound GR−Prog complexes is intermediate between that with Dex and RU486 (lanes 15 vs 14 and 16 and lanes 19 vs 18 and 20). The data of Figure 7F again illustrate that minimal amounts of GRIP1 bind to GRs in the absence of Dex. More importantly, in the presence of constant amounts of GRE-bound GRs (Figure 7B,D,F), the amount of GRIP1 binding decreases with the GR ligand in the order of Dex > Dex-Mes > Prog > RU486 (Figure 7C,E,F), which is the same order as the partial agonist activity displayed by these steroids with exogenous GRIP1 in Figure 5. Collectively, these data support the hypothesis that the differences in partial agonist activity of various GR−antagonist complexes derive in part from unequal amounts of associated coactivator as opposed to variations in the biological activities of equal amounts of GR−steroid−coactivator complexes.

DISCUSSION

We have quantitated the activities of four different antiglucocorticoids with a variety of GR constructs on two reporters in two different cell lines to examine the role of GR domains, DNA sequence, and cell-specific factors. The summary of these data (Figure 8) shows how the activity of each steroid varies with GR deletion and reporter in the two cell lines. The data points for each steroid are connected by a line in order to more easily track the effects of changing the various parameters. The data for GR407C clearly show that the amino terminal half of GR, and the AF-1 domain, are unnecessary for the expression of the full amount of partial agonist activity seen with the wild type receptor. The role of the DNA sequence is evident under two distinct circumstances. When GR binds directly to DNA, such as the induction of MMTV- vs simple GRE-regulated reporters by wt GR and GR407C, both the rank order and the absolute amount of partial agonist activity change with the DNA sequence (Figure 8A vs Figure 8B, and Figure 8C vs Figure 8D). Further alterations are noted for Dex-Ox and DOC activities when GR is recruited to DNA as part of a chimera in which non-GR sequences contact the enhancer DNA sequences (i.e., GAL/GR407C induction of FRLuc) vs the same molecule inducing gene expression via the GR portion of the chimera making contact with specific GRE DNA sequences (i.e., GAL/GR407C induction of MMTVLuc) (Figure 8A,C). The amount of partial agonist activity with Dex-Ox and DOC decreases when the GR protein does not directly contact GRE sequences. Together, these two lines of evidence support the interpretation that specific DNA-induced conformational changes can influence the amount of partial agonist activity of antiglucocorticoids. Finally, cell-specific factors can contribute to the final activity, as most clearly seen by the greater amount of partial agonist activity of Prog with the full length GR in Cos-7 vs CV-1 cells. For the other antagonists, these putative cell-specific factors are relatively unimportant. However, greater differences may be seen between cells that are not as closely related as Cos-7 and CV-1. Thus, the AF-1 domain and the N-terminal half of GR do not contribute to the expression of partial agonist activity of a selection of antiglucocorticoids while several parameters including steroid structure, DNA sequence, GRE-induced conformational changes, and cell-specific factors are of varying importance.

In contrast to the above results for GR, the AF-1 domain has been found to be very important for the expression of partial agonist activity of other steroid receptors. The AF-1 domain is required for partial agonist activity of antiestrogens (*52*−*57*, *59*). A GAL chimera with the C-terminal half of ER (DBD to C-terminus) was inactive ± steroid in yeast cells (*77*). Amino terminal sequences of AR are needed for expression of the partial agonist activity of antiandrogens (*51*, *78*). Results with PR are less clear and depend on the reporter examined (*50*, *79*). Thus, the importance of the AF-1 domain in the expression of partial agonist activity for antisteroids is not constant but varies with the receptor.

The effects of added coactivator GRIP1 are particularly informative. The amount of partial agonist activity displayed by Dex-Mes, Dex-Ox, or Prog plus the coactivator GRIP1 with GR LBD in the context of GAL/GR525C induction of the FRLuc reporter is very similar to that for induction of the GREtkLUC reporter by the same steroids with full length (Figure 5). Therefore, under these conditions, the GR LBD is sufficient for the expression of high levels of partial agonist activity with a variety of antagonists. We therefore conclude that GR sequences outside of the LBD are not essential for the expression of partial agonist activity with many antiglucocorticoids.

The N-terminal AF-1 domain is a much stronger inducer of gene expression than is the AF-2 domain of the LBD for most steroid receptors, including GRs (*11*, *70*). Coactivators such as GRIP1/TIF2 are thought to preferentially augment

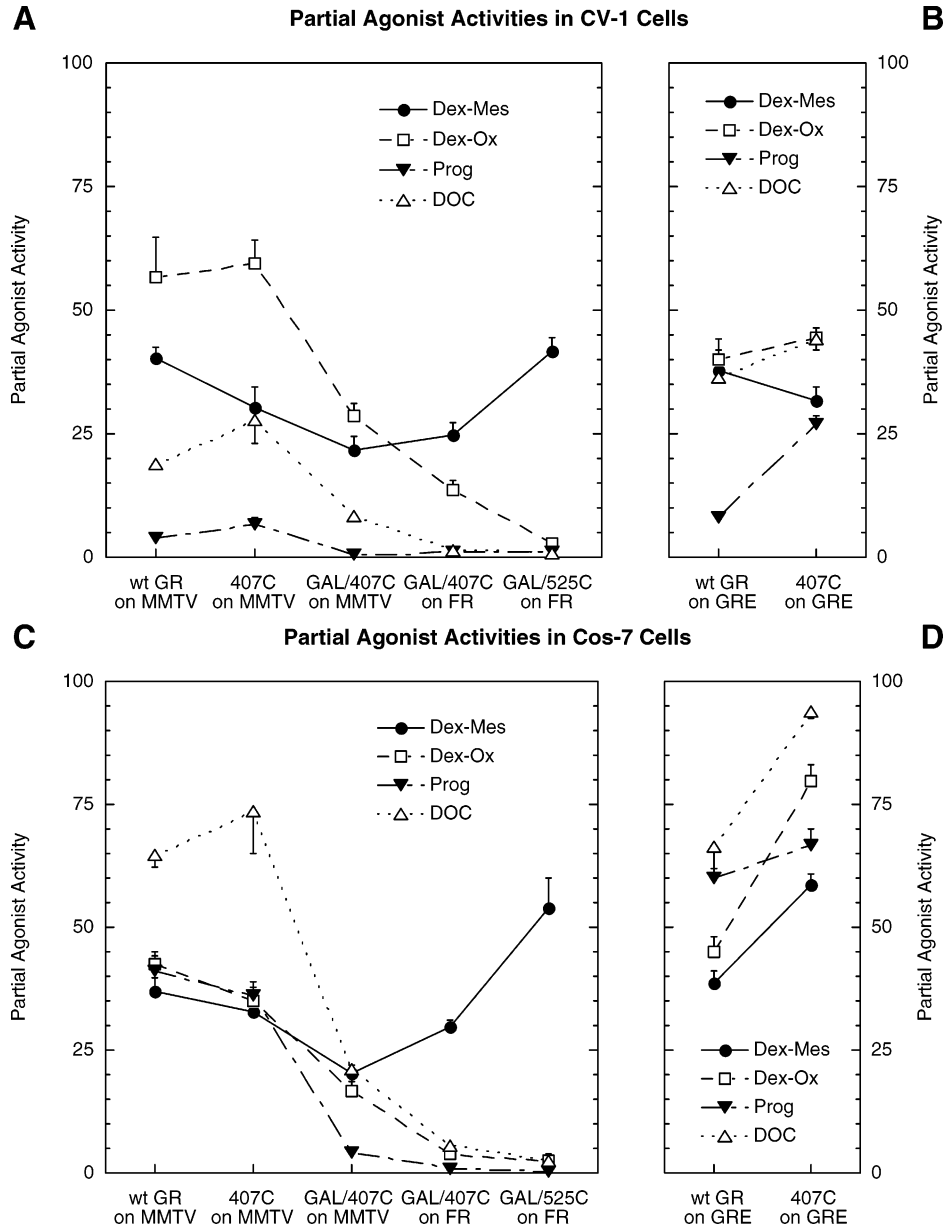

FIGURE 8: Summary of changes in partial agonist activity with different steroids, reporters, and GR constructs in (A, B) CV-1 and (C, D) Cos-7 cells. Data for the four antiglucocorticoids in Figures 1−3 are combined and plotted to allow a direct comparison of the effect of progressive deletions of GR on the partial agonist activity of each antisteroid in CV-1 and Cos-7 cells. The data with MMTVLuc and FRLuc reporters form a continuous series and are plotted in one graph (A and C). The data with GREtkLUC constitute a smaller series and are plotted separately (B and D).

the activity of the AF-1 domain (*51*, *59*, *60*, *80*−*84*). However, we find that added GRIP1 causes an 11 ± 2-fold (SEM, $n = 6$) increase in the total amount of transactivation with GR407C vs a 4.4 ± 0.5-fold (SEM, $n = 7$) increase with the full length GR (data not shown). This suggests that the coactivator GRIP1 has a much greater effect on the intrinsic transcriptional activity of the AF-2 domain than the AF-1 domain of GR. Alternatively, these data may indicate that the ability of GRIP1/TIF2 to augment AF-2 activity is blunted when the AF-1 domain is present. Interestingly, although the fold increase in total activity with added GRIP1 is about 2.5-fold higher for GR407C than wild type GR (11 vs 4.4), the partial agonist activities for various antisteroids are essentially unchanged with GR407C ± GRIP1 and full length GR ± GRIP1 (Figure 5). These data support our earlier conclusion that the effects of coactivator on the total level of transactivation are separable from those on the partial agonist activity of GR complexes (*13*).

GRIP1/TIF2 and the TIF2 fragment (TIF2.4) each display more binding to ligand-bound GRs than to ligand-free GRs (except with RU486) both for GRs specifically bound to DNA (Figure 7) and for GRs immobilized on a matrix in a GST-pulldown assay (*13*). Therefore, while the affinity of coactivator binding to DNA-bound GRs may increase, as has been reported for retinoic acid receptors (*85*), the specificity of GRIP1/TIF2 binding to different GR−steroid complexes appears to be unaffected by DNA binding. We note that ligand-free GRs can be activated to bind to DNA (Figure 7B,D). However, activation per se is not sufficient for robust coactivator GRIP1/TIF2 binding to GRs; an additional process, most likely a steroid-induced conformational change that varies with the steroid, is required.

Small differences in ligand structure can make major differences in the biological activity of receptor−steroid complexes (*12*, *30*, *36*, *86*−*88*). Recently, these differences have been proposed to cause unequal binding affinities of cofactors (*27*, *89*, *90*). Our data support this hypothesis. The partial agonist activities of GR complexes of four antiglucocorticoids (Dex-Mes, Dex-Ox, Prog, and RU486) are differentially enhanced in intact CV-1 cells by GRIP1 (Figure 5), with low concentrations of GRIP1 preferentially augmenting the activity of Dex-Mes vs Prog-bound GRs (Figure 6). Similarly, the binding in the ABCD assay of both full length GRIP1 and the truncated TIF2.4 (Figure 7A) decreases with the GR ligand in the order of Dex > Dex-Mes > Prog > RU486 (Figure 7C,E,F). This nicely recapitulates the observed order of decreasing partial agonist activity of these ligands in CV-1 cells. Thus, the changes in partial agonist activity among various antiglucocorticoids may reflect differences in the binding affinity of the GR complexes for coactivators. This order of partial agonist activity is not the same in Cos-7 cells (Figure 8C,D), which is the source of the cytosolic extracts used in Figure 7. However, recent results indicate that coactivators and corepressors can each bind to both GR−agonist and −antagonist complexes and that the amount of binding of each factor to GR complexes is partially determined by a dynamic competitive equilibrium of coactivator vs corepressor binding (*9*, *18*, *91*). Therefore, the equal levels of partial agonist activity of Dex-Mes and Prog complexes in Cos-7 (Figure 8C,D) cells may reflect higher levels of coactivators, lower levels of corepressors, or cell-specific differences in some other factor, present in the nuclei of Cos-7 cells but absent in the cytosolic extracts used in the ABCD assays of Figure 7.

The behavior of Dex-Mes-bound GRs is unique among the antiglucocorticoids examined. Shifting the DNA-associated GR from directly contacting DNA to being localized to DNA via the DNA binding of the GAL4 DBD (GAL/407C on MMTV vs FR) and removing the GR DBD (GAL/525C vs GAL/407C on FR) combine to reduce the partial agonist activity of GRs bound by Dex-Ox, Prog, and DOC (compare GAL/407C on MMTV vs GAL/GR525C on FR in Figure 8). The loss of partial agonist activity of some steroids when removing the DBD (GAL/407C vs GAL/525C), though small, is interesting especially when contrasted with the high partial agonist activity of Dex-Mes. These responses may reflect the presence of a weak transactivation domain (enh1) that has been described in the second zinc finger of the GR DBD (*92*). In contrast, the partial agonist activity of receptors bound by Dex-Mes is relatively unaffected by these modifications (Figure 8). This would not be expected to result from the ability of Dex-Mes to covalently label GRs to give nondissociable receptor−steroid complexes (*68*, *69*) because only a fraction of the receptors are labeled at the nearly neutral intracellular pH (*93*, *94*). Also, mutation of Cys-656, which is affinity labeled by Dex-Mes (*69*), eliminates the covalent attachment of Dex-Mes (*95*) with little change in the amount of partial agonist activity for Dex-Mes (*12*). Therefore, the constant partial agonist activity of Dex-Mes-bound GRs in the current study presumably reflects a unique tertiary structure of the receptor−steroid complex that is generated by Dex-Mes vs the other steroids, even though the size differences among the various steroids is small (*29*). Unfortunately, the technique of trypsin digestion of Dex-Mes vs Dex-Ox bound complexes (*96*) of each of the four GR constructs of this study in the unactivated and activated states, and ± DNA in the activated state, failed to detect any differences in tertiary structure (data not shown).

The current observations indicate that the partial agonist activity of a given receptor−steroid complex can vary depending upon the steroid, the target gene, and/or cellular factors such as coactivators, each of which can change among cells and tissues of organisms. This, therefore, suggests that it may be possible in endocrine therapies to find a combination of steroid, gene, cell, and growth conditions that allow for the preferential inhibition of a subset of genes in a few cells as opposed to uniformly suppressing all glucocorticoid-inducible genes. While collecting this information will take time, it would allow a rational approach to the selective blockage of clinically relevant target genes, which in turn should reduce the number of undesirable side effects of antisteroid therapies that usually result from the indiscriminant suppression of all responsive genes.

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