Mutations of glucocorticoid receptor differentially affect AF2 domain activity in a steroid-selective manner to alter the potency and efficacy of gene induction and repression[†]

Yong-guang Tao[‡], **Yong Xu**[§], **H. Eric Xu**[§], and **S. Stoney Simons Jr.**[‡,*]

[‡] *Steroid Hormones Section, NIDDK/CEB, National Institutes of Health, Bethesda, MD*

[§] *Laboratory of Structural Sciences, Van Andel Research Institute, Grand Rapids, Michigan 49503*

Abstract

The transcriptional activity of steroid hormones is intimately associated with their structure. Deacylcortivazol (DAC) contains several features that were predicted to make it an inactive glucocorticoid. Nevertheless, gene induction and repression by complexes of glucocorticoid receptor (GR) with DAC occurs with greater potency (lower EC_{50}) than, and equal efficacy (maximal activity, or A_{max}) to, the very active and smaller synthetic glucocorticoid dexamethasone (Dex). Guided by a recent x-ray structure of DAC bound to the GR ligand binding domain (LBD), we now report that several point mutants in the LBD have little effect on the binding of either agonist steroid. However, these same mutations dramatically alter the A_{max} and/or EC_{50} of exogenous and endogenous genes in a manner that depends on steroid structure. In some cases, Dex is no longer a full agonist. These properties appear to result from a preferential inactivation of the AF2 activation domain in the GR LBD of Dex-, but not DAC-, bound receptors. The Dex-bound receptors display normal binding to, but greatly reduced response to, the coactivator TIF2, thus indicating a defect in the transmission efficiency of GR-steroid complex information to the coactivator TIF2. In addition, all GR mutants that are active in gene induction with either Dex or DAC have greatly reduced activity in gene repression. This contrasts with the reports of GR mutations preferentially suppressing GR-mediated induction. The properties of these GR mutants in gene induction support the hypothesis that the A_{max} and EC_{50} of GR-controlled gene expression can be independently modified, indicate that the receptor can be modified to favor activity with a specific agonist steroid, and suggest that new ligands with suitable substituents may be able to affect the same LBD conformational changes and thereby broaden the therapeutic applications of glucocorticoid steroids

Keywords

mutant glucocorticoid receptors; potency; efficacy; partial agonist activity; transactivation; transrepression

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Steroid binding to its cognate receptor protein is the obligate first step in steroid-regulated gene transcription. Specificity in steroid action is obtained by virtue of dissociation constants for steroid binding to receptors being in the nanomolar range or below. Not surprisingly, x-ray structures of steroids bound in the ligand binding domain (LBD) of receptors reveal a good fit with several amino acid side chains of the LBD cavity contacting the ligand (1;2). For steroids

[*]Address correspondence to Dr. S. Stoney Simons, Jr., Bldg. 10, Room 8N-307B, NIDDK/CEB, NIH, Bethesda, MD 20892-1772 (Phone: 301-496-6796; FAX: 301-402-3572; e-mail: E-mail: steroids@helix.nih.gov).

with different structures, additional x-ray studies have documented ligand-induced conformational changes that were difficult to predict (3–5).

Early structure-activity relationships for glucocorticoid receptors (GRs) state that high affinity binding of steroid to GRs requires a C-3 ketone and no steric bulk on the Aring (6;7). Shortly thereafter, it was reported that deacylcortivazol (DAC), which lacks a C-3 ketone and has a bulky phenylpyrazole substituent on the A-ring (Fig. 1), was about the highest affinity binding glucocorticoid steroid known and with the greatest potency for gene transcription (8). Because "efficacy" and "potency" are not uniquely described, we define greater potency in this report to mean a lower concentration of steroid required for half-maximal induction, or EC_{50}. We use greater efficacy to mean a higher total amount of the maximal activity, or A_{max}, of the gene being expressed[1]. Thus, the EC_{50} for DAC is 10–40 fold lower than that for the very active synthetic glucocorticoid, dexamethasone (Dex), in both gene induction (8) and gene repression (9). The affinity of DAC for GRs is at least 10-fold higher than that of Dex (8). Thus, the higher affinity of DAC for GR can account for most of its lower EC_{50}.

GRs can effect both gene induction and gene repression but their mechanisms are often different (10;11). Furthermore, most reported mutations and/or deletions in the amino terminal half of GR (12), the DNA binding domain (13–15), and the LBD (16–18) reduce GR-mediated induction more than repression. Nevertheless, DAC is more potent than Dex in both gene induction and repression. This suggests that DAC binding to GR does not dramatically alter the ability of GR to regulate gene transcription in either of two mechanistically different modes.

So how does DAC bind in the GR LBD cavity that is too small (2) without causing ligand-induced rearrangements of the LBD that would disrupt normal GR actions? The recent x-ray structure of a human GR-DAC complex indicates an almost identical binding as Dex except for the opening of an additional cavity in the LBD to accommodate the bulky A-ring substituents of DAC (19). Thus, most of the contacts of GR protein with Dex and DAC are the same. Human and rat GRs have been examined interchangeably with no apparent differences, as might be expected from the 96.4% identity in their LBDs and 90.8% overall identity (20; 21). Therefore, we used rat GRs in this study when making selected point mutations of the GR LBD to uncover possible differences between Dex- and DAC-bound GRs and thereby advance our understanding of the molecular determinants of steroid activity in two different GR conformations. We find that some mutations eliminate all detectable steroid binding. However, other novel mutations selectively alter not only the absolute A_{max} vs. EC_{50} of GR-mediated gene induction, but also the relative A_{max} of induction vs. repression, in an agonist steroid-specific manner. These results demonstrate that it is possible to separate the control of A_{max} and EC_{50}, and to preferentially inhibit repression vs. induction. This ability to dissociate these parameters opens new avenues for the selective control of gene expression and possible therapeutic applications of glucocorticoids.

Materials and Methods

Unless otherwise indicated, all operations were performed at 0 °C.

Chemicals

Dexamethasone (Dex) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Dex-21-mesylate (Dex-Mes) was synthesized as previously described (22). Cortivazol (gift from Roussel UCLAF) was converted to Deacylcortivazol (DAC) by Dr. Craig Thomas (NIDDK/NIH, Bethesda, MD). RU486 was a gift from Etienne Baulieu (Paris, France).

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[1]In previous reports, we used the term V_{max} for the maximal amount of activity. To avoid misinterpretation of our intent, and inappropriate association with the V_{max} of enzyme kinetics, we here introduce the term A_{max}.

[1,2,4,6,7-^{3}H] Dexamethasone (Dex, 90.0 Ci/mmol) was purchased from Amersham Pharmacia Biotech, restriction enzymes and T4 DNA ligase from New England Biolabs, Fermentas, or Promega, and the dual-luciferase reporter assay from Promega (Madison, WI).

Plasmids

Renilla TS was a gift from Nasreldin M. Ibrahim, Otto Frohlich, and S. Russ Price (Emory University School of Medicine). pM vector and GAL/VP16 were purchased from CLONTECH (Palo Alto, CA), and pFR-LUC reporter from Stratagene (La Jolla, CA). Rat GR (pSG5-GR), GREtkLUC, TIF2/GRIP1, and GAL/GRIP1 (23), GAL/GR and VP16/GR (24), and GAL/GR525C (25) have been previously described. GAL/NCoR-RID [amino acids 1944-2453] was a gift from Mitch Lazar (University of Pennsylvania School of Medicine). MMTVLuc (pLTRLUC) was donated by Gordon Hager (NCI/NIH).

The site-directed mutagenesis kit (Stratagene) was used with the following primers (modified nucleotides of codon [bold} are underlined) to make mutant GRs. E558A, 5′ CTG GAG GTG ATT **GCA** CCC GAG GTG TTG 3′; L584D, 5′ C ACA CTC AAC ATG **GAC** GGT GGG CGT CAA GTG 3′; Q588K, 5′ TG TTA GGT GGG CGT **AAA** GTG ATT GCA GCA GTG AAA TGG 3′; A625I, 5′ GG ATG TTT CTC ATG GCA TTT **ATC** CTG GGT TGG AGA TC 3′; L626I, 5′ CTC ATG GCA TTT GCC **ATC** GGT TGG AGA TCA TAC 3′; R629Y, 5′ G GCA TTT GCC CTG GGT TGG **TAC** TCA TAC AGA CAA TC 3′. After verifying the sequences of all mutant DNAs, Smal I/Sap I fragments replaced the corresponding segment of wild type GR plasmid, pSG5-GR. For mutant VP/GR plasmids, Sal I/Xba I fragments replaced the wild type region. Pst I/Bsp119 I (Asu II) fragments replaced the wild type sequence of GAL/GR525C.

Cell culture, transient transfection, and reporter analysis

Triplicate samples of CV-1 or U2OS cells were transiently transfected with FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) in 24-well plates with either GRE-regulated luciferase reporter plasmid or the GAL-regulated reporter plasmid, pFRLuc (Stratagene; for two-hybrid assays), plus other plasmids (total DNA = 300 ng/well), induced with steroid for 20 hr, and assayed for Luciferase activity as described (26;27). For gene repression, cells were treated with steroid for 16 hr ± PMA for the last 2 hr in serum-free media as this treatment gives greater fold repression than with 2 hr of steroid ± PMA (27). The maximal induced activity (A_{max}) was obtained with saturating concentrations of agonist steroid, which was either ≥ 100-fold higher than the EC_{50}, or 10 μM, whichever was lower. In all cases, the data were normalized for Renilla TS activity. In two-hybrid assays, the A_{max} values are normalized to that of one condition. For dose-response curves, the data are expressed as a percentage of the maximal response and then plotted to determine the EC_{50} and percent partial agonist activity. In gene repression, the maximal response is set as the activity without agonist steroid and the basal activity is the lower plateau activity seen with saturating concentrations of agonist steroid. For gene induction, the basal activity is that without steroid and maximal activity is that produced by saturating agonist steroid concentrations. The fold induction is (induced value)/ (basal activity). Fold repression is similarly calculated as (basal activity)/(repressed value). The percent partial agonist activity of each antisteroid was calculated by expressing the activity of a saturating concentration of antagonist (1 or 10 μM) as percent of maximal activity of a saturating concentration of agonist (see above) under the same conditions. For dose-response curves, each point is the average of triplicate samples ± S.D. One curve of average points yields one value of EC_{50} (the concentration of agonist required for 50% of maximal response) via curve-fitting programs (see "Statistical analysis" below). For bar graphs giving average values of A_{max}, EC_{50}, and percent partial agonist activity, the average of n replicates (each in triplicate but considered, statistically, as one observation) was plotted ± S.E.M. (n observations) unless otherwise noted.

Steroid binding and Scatchard assays

Cytosols from Cos-7 cells that had been transiently transfected with 5 μg/100-mm dish of GR plasmids were processed and analyzed for the binding of 50 nM [^{3}H]Dex ± 100-fold excess of non-radioactive Dex in the presence of 20 mM sodium molybdate as described previously (28). For the competition assays, the only changes were to use increasing concentrations of non-radioactive Dex or DAC at 22 °C for 16 hr. For Scatchard assays, 5–6 concentrations of [^{3}H]Dex (≤50 nM) ± 100-fold excess of non-radioactive Dex in the presence of 20 mM sodium molybdate was employed.

Total RNA extraction and quantitative real time-PCR (qRT-PCR)

U2OS cells (100,000 cells/well in 6-well plates) were treated as described for the analysis of induction of ladinin 1 (27) or the repression of collagenase 3 (29). The relative levels of target mRNAs were quantitated using SyberGreen and the ABI 7900HT real-time PCR system for ladinin 1. Collagenase 3 and glyceraldehyde-3-phosphate dehydrogenase (primer from ABI, 4310884E) were quantitated by Taqman.

Western blotting

Western blots were prepared, probed with rabbit anti-GR antibodies (PA1-511A and PA1-516, ABR), rabbit VP16 polyclonal antibody (Santa Cruz), or mouse GAL4 DBD monoclonal antibody (Santa Cruz), and visualized by ECL detection reagents as described by the manufacturer (Amersham Biosciences).

Statistical analysis

Unless otherwise noted, the values of n independent experiments, performed in triplicate, were analyzed for statistical significance by the two-tailed Student's t test using InStat 2.03 (GraphPad Software, San Diego, CA). In every case, each average of triplicates was treated as one value of the n experiments. Paired t-test is often used when n=3. When the difference between the SDs of two populations was significantly different, the Mann-Whitney or Alternate Welch t test was used. A nonparametric test was used if the distribution of values was non-Gaussian. Best-fit curves (R^2 almost always ≥ 0.95) following Michaelis-Menten kinetics were obtained for the dose-response experiments with KaleidaGraph (Synergy Software, Reading, PA).

Results

Effect of selected GR LBD mutations on steroid binding affinity

To probe the binding and activation properties of DAC and Dex, we performed detail structural comparisons between the DAC- and Dex-bound GR x-ray structures (2;19). Superposition of these two structures reveals several major structural differences among the amino acids surrounding the different A-ring substitutes (Figure 1A, a phenylpyrazole group in DAC vs. a C3-ketone in Dex). Guided by these analyses, we prepared seven mutations in the GR pocket residues that are near the different substitutes of DAC and Dex (Fig. 2A). The rationale for these mutations is as follows. The first three mutations (E558A, L584D, and L584D/Q588K) are designed to change the charge properties of the ligand-binding pocket. The Q588K mutation is intended to mimic the wild type receptor as the mutated lysine can serve as a hydrogen bond donor, like the glutamine side chain. The A625I and L626I mutations are calculated to change the shape but not the charge properties of the pocket. The R629Y mutation is planned to disrupt the binding of Dex but not DAC to GR. In the GR/DAC structure, R629 forms packing interactions with DAC whereas in the GR/DEX structure R629 forms hydrogen bonds with the C-3 ketone of the steroid. The R629Y mutation is expected to disrupt the H-bond interaction with Dex but not the packing interactions with DAC.

Ligand binding assays reveal that these seven mutations fall into four classes. The Class I (E558A, L584D, and L584D/Q588K) mutants no longer bind Dex, indicating that changing the hydrophobicity of the GR pocket has a deadly detrimental effect on ligand binding (Fig. 2A and Table 1). The Class II (Q588K, where Q588 moves dramatically upon DAC binding) and Class III (A625I and L626I, which involve small alterations) mutants bind Dex with only ~2.6- (Q588K and L626I) or 5.9-fold (A625I) reduced affinity. The Class IV mutant (R629Y) also displays negligible Dex binding, due to the disruption of the hydrogen bonds to the C-3 ketone of Dex, but it may retain DAC binding as R629Y is expected to form the same packing interactions with the phenylpyrazole ring of DAC. Interestingly, the binding affinity of DAC is largely unaffected in the mutants that still bind Dex, as determined by cell-free competition assays with [^{3}H]Dex (Table 1). The different binding affinity in the GR mutants does not result from changes in protein abundance as Western blots show that each mutant GR is expressed at the same level (data not shown). To further assess changes in DAC- and Dex-mediated properties of the mutants, functional cell-based assays were employed below.

Effect of selected GR LBD mutations on A_{max} of GR-regulated gene induction

Three important properties of GR-mediated gene transcription (A_{max}, EC_{50}, and the percent partial agonist activity of antiglucocorticoids) (30;31) were measured for induction of two exogenous genes (GREtkLUC with a simple tandem repeat of GREs from the tyrosine aminotransferase gene (32) and MMTVLuc with a more complex enhancer from the mouse mammary tumor virus (MMTV) (33)). Initial experiments with varying amounts of receptor plasmid revealed that the 0.5 ng of each mutant receptor plasmid provides about 70% of the activity seen with up to 100-fold higher levels of receptor plasmid (data not shown). Thus, we conclude that our below described assays with 0.5 ng of receptor plasmid involve approximately equal, and non-limiting, amounts of each mutant receptor. Figs. 2B&C show that maximal activity (A_{max}) of Class I mutants with saturating concentrations (1–10 μM) of agonist steroid (Dex or DAC) in CV-1 cells is barely above basal activity with both transfected reporters (= 5.6% for GREtkLUC), consistent with their lack of steroid binding activity (Table 1). The A_{max} of the Class II mutant (Q588K) appears to be reduced more for DAC induction (by ~55%) than for Dex induction (~15% reduction). The Class III mutants (A625I and L626I) retain ≤ 50% of the wild type activity when bound by Dex. In contrast, the same mutant receptors maintain >80% of the activity of wild type GR when bound by DAC. Thus, Dex shows much less activity than DAC and is no longer a full agonist with Class III mutants. This phenomenon is especially dramatic with the Class IV mutant, R629Y, where Dex has negligible activity (consistent with its undetectable binding of Dex) while DAC can be more active than with the wt GR. Therefore, the ability of the mutant receptors to induce gene expression is now very sensitive to the structure of "agonist" steroids.

The A_{max} for DAC induction of the endogenous tyrosine aminotransferase gene in rat hepatoma tissue culture (HTC) cells is about 10% greater than for Dex induction (8). To examine the effect of the above mutations on the induction of an endogenous gene in greater detail, we selected the GR-responsive ladinin 1 (LAD1) gene in human U2OS cells (27;34). The A_{max} for DAC induction of LAD1, as measured by quantitative RT-PCR, is again slightly higher than that for Dex (Fig. 2D). Likewise, the changes in A_{max} for induction of LAD1 by each mutant receptor relative to wild type GR show similarities to those seen for the exogenous reporters: negligible activity with the Class I mutations and much greater activity of DAC than Dex with R629Y. The higher activity of Class II and III mutants with Dex and DAC for LAD1 than the transfected reporters (Figs. 2B&C) is notable.

Effect of selected GR LBD mutations on EC_{50} of GR-regulated gene induction

Dose-response experiments were performed with the above exogenous genes to confirm the affinity of each steroid for those receptors that bound steroid (Table 1) and afforded appreciable

induced gene expression (Figs. 2B&C). Representative results are shown in Fig. 3A. In each experiment, the EC_{50} for gene induction was determined as described in Material and Methods and the average values plotted in Figs. 3B&C. Surprisingly, the EC_{50} for Dex induction of both genes induced by each mutant GR is increased 50–700 times that of the wild type GR (note logarithmic scale of y-axis), or 16-to 91-fold more than predicted from the affinity of Dex for each GR. The discrepancies were largest (66- to 91-fold) for induction of MMTVLuc. Similar, unexpectedly large right-shifts in the dose-response curves are observed for induction by DAC, especially the $\geq$ 300-fold difference with the Q588K mutant, despite marginal changes in steroid affinity. Selected mutants with significant levels of induction were chosen for studies with the endogenous LAD1 gene and displayed similar results (Figs. 3D&E). As noted above, the amount of biologically active receptor present is about the same for each mutant receptor. Thus, a change other than steroid binding affinity or amount of receptor protein must be responsible for these large increases in EC_{50}.

There is no established relationship between A_{max} and EC_{50}. It is often anticipated that the A_{max} will decrease as the EC_{50} increases and this is seen when comparing the properties of Dex induction via the wild type vs. Class II and III mutants (Figs. 2B–D vs. 3B,C&E). However, the present data provide several exceptions to this expected behavior. The A_{max} values for Dex induction of the LAD1 gene by these mutants are not significantly different despite 75- to 190-fold increases in EC_{50} (Figs. 2D vs. 3E; note log scale of Y-axis in Fig. 3E). Similarly, the A_{max} for DAC induction of GREtkLUC or MMTVLUC reporters is unchanged (Class III) or increases (Class IV) while the EC_{50} values for gene induction increase by 8- to 19-fold (Figs. 2B&C vs. 3B&C). With DAC and the endogenous LAD1 gene, the differences are even more pronounced: less than 2-fold rises in A_{max} values are associated with 25- and 300-fold increases in EC_{50} (Fig. 3E; note log scale of Y-axis in Fig. 3E). These data clearly show that the A_{max} and EC_{50} can be independently modified and suggest that separate pathways or factors are involved.

Effect of selected GR LBD mutations on percent partial agonist activity of antiglucocorticoids in GR-regulated gene induction

We have reported that an increase in EC_{50} is associated with a decrease in percent partial agonist activity of antiglucocorticoids and *vice versa* (26;27;30;35–38). This is an empirical observation with no theoretical explanation so far. With the present mutant GRs, an increase in EC_{50} for Dex induction is always coupled with a decrease in the percent partial agonist activity of the antiglucocorticoid Dex-Mes (Table 2). Thus, the data from our mutants further support a linkage of these two parameters.

Effect of selected GR LBD mutations on GR-regulated gene repression

We next examined the ability of our mutants to repress the induction of an endogenous gene, collagenase 3 (Coll3), again in U2OS cells (27;39). Most reported GR mutations reduce the A_{max} of induction much more than that of repression (12–16;40;41). Therefore, the ability of each mutant that bound steroid (i.e., Classes II-IV) to selectively suppress A_{max} for repression of the endogenous Coll3 gene (Fig. 4A) with little effect on A_{max} for induction of the endogenous LAD1 gene (Fig. 2D) in the same cells was unexpected. The A_{max} values of the various mutants (with significant steroid binding activity) for LAD1 induction by Dex or DAC are between 73 and 180% of that with wild type GR but only 1–23% of wild type GR for Coll3 repression. This behavior is opposite to the commonly observed response and suggests a new approach for preferential inactivation of GR-mediated gene repression. The dose-response curves for GR-mediated repression of the Coll3 gene by Dex and DAC with the same mutants as used with LAD1 in Fig. 3E were then determined by qRT-PCR. Representative experiments are shown in Fig. 4B. Interestingly, each mutation shifts the dose-response curve for Coll3 gene repression to higher steroid concentrations (Fig. 4C) by about the same amount as for

induction of the LAD1 gene (Fig. 3E). Therefore, the effects of these mutations on endogenous gene induction and repression are the same for the determinants of the EC_{50} and different for the A_{max}. This is additional evidence that the pathways controlling the EC_{50} and A_{max} can be separated.

Interaction of coactivators and corepressors with mutant GRs

Changes in the ratio of receptor-associated coactivators *vs.* corepressors is one mechanism by which the EC_{50} for gene induction can be raised or lowered (26;30;31;35–37;42;43). To determine whether the EC_{50} differences observed in Figs. 2–4 are due to alterations in coactivator and/or corepressor binding affinity to the mutant GRs, we used mammalian two-hybrid assays of interactions between full-length GR fused to the VP16 transactivation domain and chimeras of the GAL4 DBD with either full-length p160 coactivator TIF2 or the receptor interaction domain of the corepressor NCoR. In this assay, the total amount of product (A_{max}) reflects the strength or affinity of cofactor binding to GR. TIF2 is the human homolog of, is 94% identical to, and is biologically indistinguishable from, the mouse protein GRIP1. Therefore, although we use different constructs of each (designated in the figure legends), we will refer to both proteins as TIF2. Western blots indicate that each VP16/GR construct is expressed at similar levels (data not shown). The wt GR and most of the mutant GRs yield almost the same A_{max} with GAL/TIF2 chimera in the presence of excess Dex or DAC (Fig. 5A). The exception is R629Y, which displays no Dex-induced interaction with coactivator because this mutant has no appreciable Dex binding. Thus, these mutations do not significantly alter the amount or affinity of receptor-coactivator interactions at saturating concentrations of ligand. However, the EC_{50} of steroid-induced binding of receptor-agonist complexes with TIF2 is increased by at least a factor of 10 with each mutation (Fig. 5B), which is much more than expected from the changes in steroid binding affinity (Table 1). Also, the amount of GR-antagonist (RU486) binding to coactivator relative to that for DAC-bound complexes is reduced by $\geq 85\%$ (Table 3). Therefore, the changes in EC_{50} in Fig. 5B and in percent partial agonist activity reflect different properties of the mutant GRs than their affinity for coactivators.

In two-hybrid interactions of GR with the corepressor NCoR, there is also very little change in the A_{max} with any mutant (Fig. 5C), except for Dex with the R629Y mutant that minimally binds Dex. This similarity in the total luciferase activity produced with Dex or DAC in the interaction of each mutant receptor, except R629Y, with either coactivator (Fig. 5A) or corepressor (Fig. 5C) suggests that the capacity of both receptor-Dex and receptor-DAC complexes to interact with these cofactors, which is dictated by GR-cofactor affinity, is independent of most of the mutations examined. At the same time, there are dramatic and steroid-specific increases in the EC_{50} for steroid-induced reporter gene induction by the corepressor/mutant receptor interactions (Fig. 5D) that are nearly identical to those observed with coactivator (Fig. 5B). Each receptor mutation also eliminates most of the ability of the antagonist RU486 to promote a productive interaction between GR and corepressor, just as is seen for GR-coactivator interactions (Table 3). Collectively, these results suggest that the mutation-induced changes in the EC_{50} of GR-mediated induction (Figs. 3B, C, E, and 4D) are independent of coactivator or corepressor affinity and, instead, that some other property of receptor-cofactor interaction is involved.

Steroid-specific inactivation of AF2 domain by mutations in truncated GRs

The above observations that mutations in GR affect GR-steroid complexes to separately alter their A_{max} and EC_{50} for gene induction is reminiscent of the ability of coactivators and corepressors to independently influence the A_{max} and EC_{50} of gene expression (23;25;30;31; 36;38). Coactivators bind more extensively to agonist-bound GRs than do corepressors in a competitive equilibrium manner (26;42), suggesting that coactivator functions may also have a more prominent role in the current responses. This possibility was examined by looking at

the induction properties of the GR LBD (amino acids 525 to the C-terminus). Because the GR LBD does not associate with DNA, it was fused to the GAL4 DNA binding domain (DBD) to give GAL/GR525C, which binds to and induces gene transactivation from a GAL4 regulated gene such as FRLuc. The GAL/GR525C construct has two especially useful properties for the present study. First, it contains only one activation function, AF2, so that significant effects of added TIF2 can be unambiguously assigned. Second, it is known that TIF2 causes a larger increase in A_{max} for GAL/GR525C than for full-length GR with activation functions AF1 and AF2 (37). Therefore, if our mutations are affecting coactivator function as opposed to binding, we predict that the changes in A_{max} with and without exogenous coactivator will be magnified when these mutations are in the context of the GAL/GR525C.

All of the mutant GAL/GR525C plasmids expressed equal levels of protein (data not shown). With Dex, most mutants display marginal activity (A_{max}) while that for the Class II mutant Q588K is reduced by ~65% (Fig. 6A; note split Y-axis and that † = no significant difference). For DAC-bound receptors, only the Q588K mutation causes a >65% decrease in A_{max} (Fig. 6A). Thus the AF2 domain, the only activation domain in the GAL/GR525C constructs and a domain known to interact with coactivators, has been largely inactivated by these mutations in Dex-bound receptors. In contrast, much more AF2 transactivation activity remains with all of the DAC-bound mutants except for Q588K. When TIF2 is cotransfected, the A_{max} increases for every receptor-steroid complex other than Dex-bound R629Y. However, those receptor-steroid combinations with low A_{max} in the absence of TIF2 (Dex with Class III [A625I and L626I] and Class IV [R629Y] mutants and DAC with the Class II mutant [Q588K]) still display a dramatically lower A_{max} compared to wild type controls with added TIF2 (Fig. 6A). This argues that the Class III mutations greatly reduce the ability of the AF2 domain of Dex-, but not DAC-, bound receptors to increase the A_{max} in response to both the endogenous coactivators in CV-1 cells and to the elevated levels of exogenous TIF2. The Class II and IV mutations also reduce this response of the AF2 domain to coactivators in DAC-and Dex-bound receptors respectively but are less inhibitory.

As expected (37), cotransfected TIF2 also reduces the EC_{50} (increases the potency) both of Dex- and DAC-bound wild type GR525C. A reduction similar to that for the wild type chimera is seen for DAC-bound A625I and L626I mutant receptors; but, TIF2 is much less effective with the Dex-bound mutant receptors (Fig. 6B; note logarithmic Y-axis). The ability of TIF2 to increase the percent partial agonist activity of an antisteroid (37) is likewise lost with the mutant receptors of GAL/GR525C (Table 4). These results suggest that the various mutations in GR have not caused the loss of TIF2 binding, because TIF2 still produces increases in A_{max}. However, the effectiveness of TIF2 activity has been diminished for most of the Dex-bound mutants and for DAC-bound Class II and IV mutants. This is seen by the inability of added TIF2 to restore the A_{max}, EC_{50}, and the percent partial agonist activity values of the affected mutants to wild type GR values. Importantly, this ineffectiveness of TIF2 is determined by the steroid bound to the mutant GR and not the mutation itself (e.g., compare responses of the A625I mutant receptor with Dex or Dex-Mes vs. DAC).

Steroid-specific inactivation of AF2 domain by mutations in full-length GRs

In order to determine whether the various mutations can also disable the AF2 domain in the context of full-length GRs, we next examined the effect of added coactivator on the properties of full-length receptors containing both activation functions: the C-terminal AF2 domain of GAL/GR525C and the N-terminal AF1 domain. We previously documented reduced responses of full-length GR vs. GAL/GR525C with added coactivator on the A_{max}, but approximately equal effects on the EC_{50}, of induction by receptors containing the AF1 domain (37), which can interact directly with TIF2 (43). Therefore, we predicted that the differences ± TIF2 with

full-length GR mutants would be less for the A_{max} than those with the GAL/GR525 constructs and more similar for the changes in EC_{50}.

As shown in Fig. 7, the predicted behavior of full-length GRs is precisely what is observed. In the absence of exogenous TIF2, the differences between the A_{max}s of Dex- and DAC-bound wild type and mutant GRs is much less than with the GAL/GR525 constructs (Fig. 6A), except for the non-Dex binding R629Y, due to the presence of the AF1 domain. Added TIF2 elevates the A_{max} in all cases; however, those receptor-steroid combinations with a defective AF2 domain in the context of GAL/GR525 (Classes III and IV with Dex and Class II with DAC) display lower A_{max} values (Figs. 7A vs. 6A). For the EC_{50} values of gene induction, TIF2 lowers the EC_{50} of almost all mutant GRs, but wild type GR values are more closely approximated by receptor-steroid combinations with a functional AF2 domain in this assay (e.g, DAC-bound Class III and IV mutants). It should be noted that the ability of added TIF2 to almost completely restore wild type properties to the mutant GRs for A_{max} but not for EC_{50} is yet another example of the ability of these mutations to differentially affect these two important properties of transactivation. Furthermore, the efficiency of TIF2 in the EC_{50} assay is sensitive to steroid structure (e.g., values for A625I are much closer to wild type GR when DAC is the ligand).

Finally, because the AF2 domain is largely responsible for the percent partial agonist activity of Dex-Mes (37), Dex-Mes displays the predicted minimal percent partial agonist activity with the mutant full-length GRs containing a partially (Class II) or more completely (Classes III and IV) inactivated AF2, even in the presence of a functional AF1 domain (Table 4). Furthermore, added TIF2 only minimally increases the partial agonist activity of Dex-Mes with the mutant full-length GRs to a value that is dramatically lower than that for the wild type GR with TIF2. Collectively, these results show that selected mutations in the GR LBD can unequally affect several properties of GR induction (EC_{50} and A_{max} of agonist steroids and the percent partial agonist activity of antiglucocorticoids) in a manner that introduces previously unobserved differences among potent agonist glucocorticoids. In several cases, the mechanism of this new, steroid-selective behavior involves a steroid-specific inactivation of the AF2 domain and its ability to communicate with coactivators. Several of these mutations are also able to preferentially inhibit the repression vs. induction of responsive genes, although the mechanism of this response has not yet been elucidated.

Discussion

DAC is 25% larger than Dex (44) but displays a 10- to 40-fold lower EC_{50} (or potency) for GR-mediated gene induction and repression while producing the same A_{max} (= maximal activity or efficacy). The recent x-ray structure of DAC bound to the GR LBD (19) reveals significant reorganization of several residues to accommodate the bulky steroid but an otherwise similar binding in the LBD. Here we report that mutations of some amino acids near the A-ring of the bound steroid eliminate steroid binding. However, other mutations unexpectedly have little effect on the affinity of either Dex or DAC compared to the large changes in A_{max} and EC_{50} of agonists and in percent partial agonist activity of antiglucocorticoids. Interestingly, the effects of these latter mutations depend upon steroid structure and the type response (induction or repression) to give a spectrum of activities that has not been previously described.

Class I mutations (E558A, L584D, and L584D/A588K) eliminate Dex binding as predicted. Because DAC displays no biological activity with these mutants, we presume that DAC binding is also destroyed. In contrast, Class II and III mutations produce relatively small reductions in Dex binding affinity (≤ 6-fold) and activity (A_{max}) and almost negligible decreases in DAC binding or A_{max}. Surprisingly, the Class IV mutation (R629Y) affords barely detectable Dex

binding in Scatchard assays and Dex activity (A_{max}) in bioassays but a large A_{max} with DAC in the same bioactivity assays. We conclude that this mutant displays significant binding affinity for DAC but not Dex. This suggests that both the binding affinity and transactivation activity can vary not only with receptor mutation but also with steroid structure. With GREtkLUC reporter, the A_{max} of DAC with the R629Y mutant is also significantly greater than that with the wild type GR (Fig. 2B). Even more unexpected is the much larger, steroid-dependent effects of Class II-IV mutations on the EC_{50} (Fig. 3) and percent partial agonist activity (Table 2) of gene induction than on steroid binding affinity. The selectivity is greatest with Q588K and R629Y, both for exogenous and endogenous genes, and thus is insensitive of promoter sequence and DNA architecture. There are few precedents for this behavior (45) and no mechanistic studies of which we are aware.

The mutations cause the EC_{50}s for Dex and DAC to vary in unpredictable ways. The EC_{50} of DAC is 8–12 fold lower than Dex with the wt GR, 20–90 times lower with R629Y, and 2–6 times higher with Q588K. More remarkable is that Class II–IV mutations reduce the A_{max} of GR-mediated repression (Fig. 4A) more than that for induction (Fig. 2D), which is opposite to most reports and rarely observed (40) in a manner that can depend upon the cell line (41). This cell-specificity explanation does not appear to be a factor here, though, as the same U2OS cells display preferential inhibition of induction over repression with other GR mutants (12;15). The variations in EC_{50} and A_{max} cannot be accounted for by effects on steroid binding affinity or receptor abundance. Therefore other phenomena must be involved.

The greatly reduced A_{max}s for induction of FRLuc by Dex bound to chimeras of GAL4 DBD fused to mutant GR LBDs indicate that Class II, III, and IV mutations disrupt the activity of the AF2 domain, which is the only transactivation domain present (Figs. 2A and 6A). Unexpectedly, this nearly complete inactivation of the AF2 domain depends upon steroid structure. The AF2 activity of mutants binding the bulkier DAC is largely intact for Class III and IV mutations and is dramatically reduced only for the Class II mutant, Q588K. The biological activities of full-length GR (Fig. 7) are consistent with the predicted properties of Dex complexes being without AF2 activity while DAC complexes (except with Q588K) enjoy contributions of both AF1 and AF2 transactivation domains. A particularly clear demonstration is the minimal percent partial agonist activity of Dex-Mes ± TIF2 with both GAL/GR525C and full-length GR mutants (Table 4), which is predicted from our demonstration that the AF2 domain is necessary and sufficient for the expression of Dex-Mes percent partial agonist activity ± TIF2 (46). Thus, we have identified several amino acid mutations in the GR LBD that inactivate the AF2 domain in a manner that depends upon both the structure of the bound ligand and the nature of gene expression (induction vs. repression), thereby imparting major differences in biological activity to various steroid-receptor complexes.

The underlying molecular explanations for the observed mutation-induced changes in AF2 and coactivator activity in both GR-mediated transactivation and repression are not yet clear. Our mutations of residues in the ligand-binding pocket do not appear to alter the binding affinity of coactivators and corepressors (Figs. 5A&B). Computer modeling predicts no dramatic mutation-induced changes in the peptide backbone of the coactivator binding pocket of GR with Dex or DAC (Fig. 8). However, the effects of mutations can be propagated throughout the protein even when the backbone conformation is preserved and there is no clear path of contacts (47; 48). Thus, it is likely that the small conformational changes of Fig. 8 resulting from Class II–IV mutations of Dex-bound receptors are transmitted to the amino acid side-chains at the surface of the LBD and may even be magnified, as seen for GR DBD mutations that are amplified at a distant location in the DBD (49). Similarly, small changes in ligand binding position can yield much larger effects on LBD structure (50). These surface topology changes could affect the nature of protein-induced conformational changes in bound coactivators and thus influence the efficacy of cofactor-protein interactions and eventually the

EC_{50} (38; 51). This proposed mechanism is thus similar to the recent report that phosphorylation of the human estrogen receptor alpha at Ser305, which is just before helix 1, alters the orientation (and presumably activity) of bound coactivator SRC-1 without affecting the affinity of SRC-1 binding (52). This mechanism is in contrast to the ability of Y753 mutations in rat GR to reduce the binding of SRC-1 coactivator and preferentially reduce transactivation as opposed to transrepression (53), which is inhibited in our study (Figs. 4A vs. 2D).

The reported ability of coactivators to selectively modify A_{max} *vs*. EC_{50} of agonists, and the percent partial agonist activity of antagonists, in gene induction by GRs (23;25;30;31;36;38) suggests that these biological properties are uniquely sensitive to structural changes. The current results support this conclusion. For example, the A_{max} of full-length Q588K, either with or without TIF2, is similar to that of the wild type receptor with both Dex and DAC while the EC_{50} of Q558K $\pm$ TIF2 with either steroid is orders of magnitude higher than that of the wild type GR (Fig. 7). Furthermore, TIF2 increases the A_{max} of all GR mutants but has greatly reduced ability both to lower the EC_{50} of most mutant GAL/GR525C complexes and to increase the percent partial agonist activity of Dex-Mes (Fig. 6 and Table 4). The generally greater fold decrease in EC_{50} with added TIF2 for the full-length vs. truncated mutant GR-Dex complexes (Fig. 7B vs. Fig. 6B) is consistent with our recent finding that an amino terminal sequence of TIF2 interacts with the N-terminal region of full-length GRs (43). Nevertheless, even with full-length GRs, added TIF2 is less able to reverse the effects of each mutation on the EC_{50} of Dex than of DAC gene induction, presumably due to the presence of the inactivated AF2 domain in the Dex-bound receptors. We have not examined other coactivators under similar situations. However, the fact that the same increases in EC_{50} are seen in cells containing other coactivators (Fig. 3) suggests that the behavior with TIF2 is shared by the other coactivators. Thus, the mutational inactivation of the steroid-bound AF2 domain appears to unequally impact the ability of TIF2, and probably all p160 coactivators, to modulate the EC_{50} vs. A_{max} of GR-regulated gene expression. These differential effects on two aspects of regulated gene transcription are conceptually similar to the emerging hypothesis that steroid hormone induced changes in gene transcription and alternative splicing of transcripts are controlled by different processes (54;55).

In summary, this study demonstrates that mutations within the GR steroid-binding cavity can selectively affect AF2 activity, and thus the EC_{50} and/or A_{max} of GR-regulated gene induction and repression, in a manner that is further regulated by steroid structure. Such discrimination is unique among glucocorticoid agonists. These results also suggest that, in a variation of reverse chemical genetics (56;57), modifications of steroid structure could mimic the effects of the current receptor mutations to reproduce the separation of GR induction and repression properties, thereby generating new steroids with greater therapeutic selectivity. Some examples of such steroid derivatives appear to exist (58–62). However, our current x-ray structure based approach offers a more structurally directed avenue by which to design new derivatives. A similar method is being used to develop ligands that will induce the orphan receptor ERR to accept a molecule in the normally unoccupied LBD and induce desirable changes in receptor activity (63). The present data also suggest that the determinants of EC_{50} are more responsive than those of A_{max} to changes in the GR binding cavity, possibly due to more efficient transmission of critical structural changes in steroid-bound GRs through the receptor surface to the coupled target proteins. The fact that several mutations in helices 3 and 5 can preferentially reduce GR repression of at least one endogenous gene offers hope that this will prove to be more common. Together, these structural modifications suggest new avenues for the differential control of gene expression by glucocorticoids, and probably other steroid/nuclear receptor ligands, during development, differentiation, homeostasis, and endocrine therapies.

Acknowledgements

Abbreviations

A_{max}

        maximal activity

AF1 and AF2

        activation function 1 and 2

coll3

        collagenase 3

DAC

        deacylcortivazol

Dex

        dexamethasone

Dex-Mes

        Dex-21-mesylate

DBD

        DNA binding domain

GR

        glucocorticoid receptor

LAD1

        ladinin 1

LBD

        ligand binding domain

MMTV

        mouse mammary tumor virus

PMA

        phorbol 12-myristate 13-acetate

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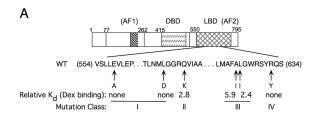
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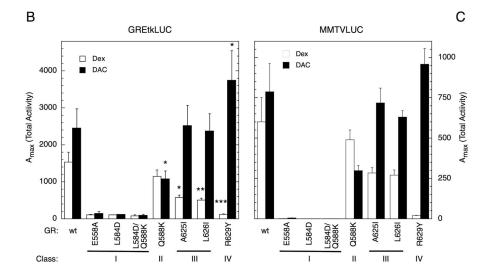
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Fig. 1.
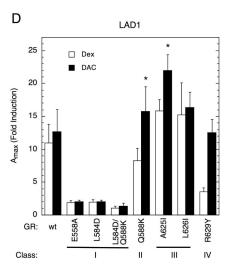Structures of agonist glucocorticoids Dex and DAC.

A

B   GREtkLUC

C   MMTVLUC

D   LAD1

Fig. 2.
Effects of GR mutations on level of gene induction. A. Schematic of position of point mutations in LBD with numbers above the drawing indicating the amino acids of the LBD with AF2 (cross hatched), AF1 (core is striped), and DBD (dashes) domains. The affinity of Dex binding (relative to wt GR) was determined by Scatchard assays (see Materials and Methods). B-D. The A_{max}, or total amounts of reporter gene induction (GREtkLUC in B, MMTVLUC in C), by EtOH ± saturating concentrations of Dex or DAC was determined in CV-1 cells as described in Materials and Methods. For the endogenous gene LAD1, the fold induction by 1 μM Dex or DAC in U2OS cells was determined by qRT-PCR as presented in Materials and Methods. The average values ± S.E.M. from 7 (GREtkLUC; 2 for Class I mutants), 2 (MMTVLUC) and

4 (LAD1) independent experiments are plotted. ND = not determined. Relative to wild type GR, * $P < 0.050$, ** $P = 0.0070$, *** $P = 0.0006$.
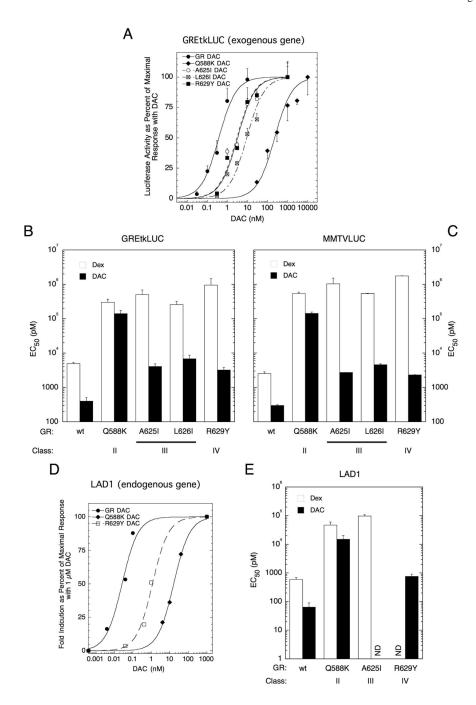
Fig. 3.
Dose-response curves for mutant GR induction of reporter genes. A. Dose-response curves for DAC induction of exogenous GREtkLUC in CV-1 cells. For each receptor, the data of one experiment are plotted as percent of the maximal level of induction by DAC, as described in Materials and Methods. B-C. Change in EC_{50} with receptor mutation for induction of exogenous reporter genes by Dex or DAC. D. Dose-response curves for DAC induction of endogenous LAD1 gene in U2OS cells. A representative experiment is shown. E. Change in EC_{50} with receptor mutation for induction of LAD1 gene by Dex or DAC. Note that the average EC_{50} values (bar graphs) $\pm$ S.E.M. are plotted on a logarithmic y-axis for n = 5–8 (GREtkLUC; relative to wt GR, P = 0.0016 for all Dex-bound receptors and P = 0.0025 for all DAC-bound

receptors), 2 (MMTVLUC) and 4 (LAD1; relative to wt GR, P = 0.029 for all Dex- and DAC-bound receptors) independent experiments. ND = not determined.
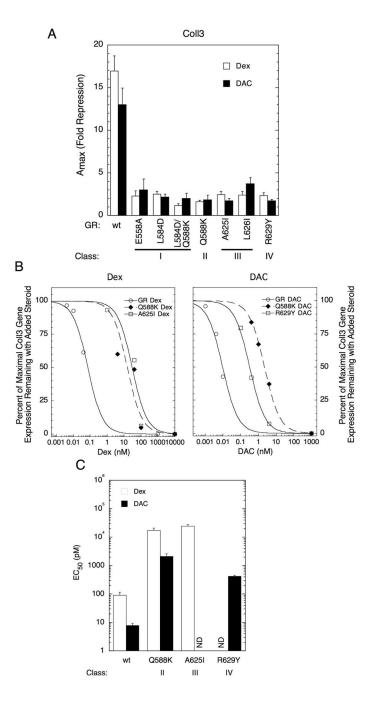
Fig. 4.
Repression of the endogenous Coll3 gene by mutant GRs in U2OS cells. A. Effect of mutations on fold repression of Coll3 mRNA by Dex and DAC. Data represent average of 4 independent experiments ± S.E.M. (relative to wt GR, $P < 0.05$ for all except DAC with E558A and L626I). B. Dose-response curves for Dex or DAC repression of endogenous Coll3 by indicated mutant GRs. The Coll3 mRNA obtained with increasing steroid, expressed as percent of Coll3 mRNA remaining with a saturating concentration of steroid, is plotted for each mutant receptor in one representative experiment. C. Change in EC_{50} for repression of Coll3 mRNA expression with receptor mutation in presence of Dex or DAC. See Materials and Methods for experimental

details. The average values ± S.E.M. of 4 independent experiments are plotted on a logarithmic y-axis (relative to wt GR, P <0.05 for all). ND = not determined.
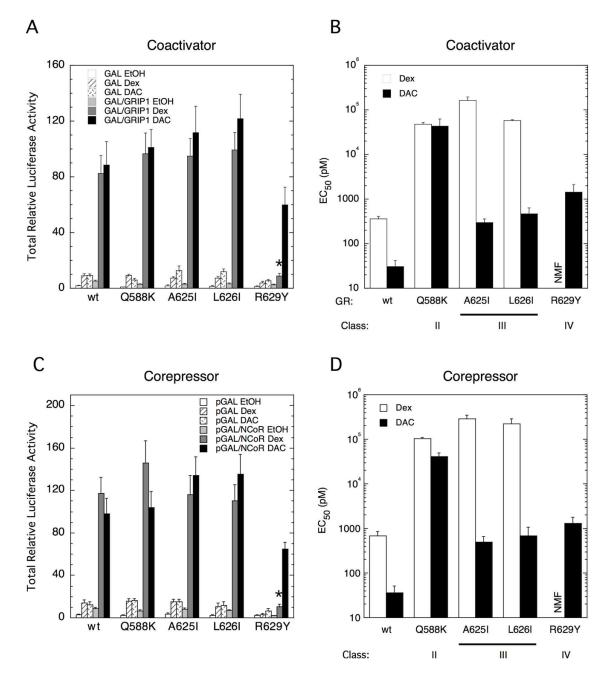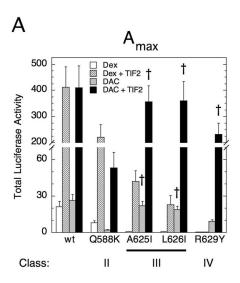
Fig. 5.
Two-hybrid assays for VP16/GR chimeras with GAL/coactivator or GAL/corepressor in CV-1 cells. A&C. Total Luciferase activity induced from FRLuc reporter by GAL ± GRIP1 (A) or NCoR-RID (C) with indicated mutant VP16/GRs plus EtOH, 1 μM Dex, or 0.1 μM DAC was determined, normalized to the value for GAL/EtOH with the Q588K mutant, and plotted as described in Materials and Methods. *P < 0.05 for mutant vs. wt GR with GAL/GRIP1 or GAL/NCoR-RID and steroid. B&D. EC$_{50}$ values for Dex or DAC induction of FRLuc reporter by GAL/GRIP1 (B) or NCoR-RID (D) with indicated mutant VP16/GRs was determined and plotted as described in Materials and Methods. Error bars for all graphs are S.E.M of 5 independent experiments (4 experiments for pGAL controls for NCoR-RID; 3 experiments for

VP16/GRR629Y). NMF = no meaningful figure because the fold induction approached the usual error bars in the data points, thereby precluding a meaningful dose-response curve. In all cases, P = 0.029 for mutant vs. wt GR with GAL/GRIP1 or GAL/NCoR-RID and steroid.
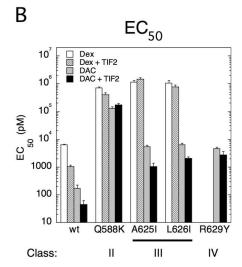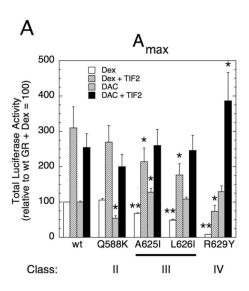
Fig. 6.
Transcriptional properties of GAL/GR525C mutants ± coactivator TIF2 in CV-1 cells. Total Luciferase activity (A_{max}) (A) and EC_{50} (B) for induction by GAL/GR525C alone from the FRLuc reporter with Dex or DAC ± TIF2 was determined and plotted as described in Materials and Methods. Error bars are S.E.M of 4 (A) or 5 (B) independent experiments. For all cases, † = not significant ($P > 0.05$) for mutant vs. wt GR ± GRIP1 and steroid. No symbol above bar indicates $P \leq 0.029$.
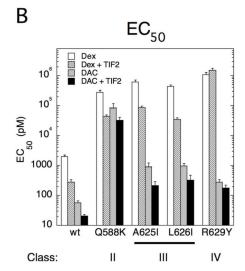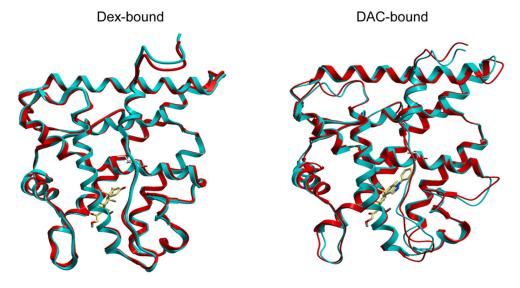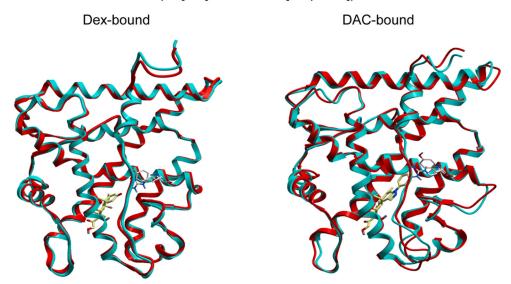
Fig. 7.
Transcriptional properties of full-length GR mutants ± coactivator TIF2 in CV-1 cells. Total Luciferase activity (A_{max}) (A) and EC_{50} (B) for induction of GREtkLUC reporter by Dex or DAC ± TIF2 was determined and plotted as described in Materials and Methods. Error bars are S.E.M of 5 independent experiments. In all cases either with or without TIF2, $*P < 0.05$, $**P < 0.0005$ for mutant vs. wt GR in (A). In (B), $P = 0.0079$ for all comparisons of mutant vs. wt GR.

GR LBD (wt [red] and A625I [turquoise])

Dex-bound   DAC-bound

GR LBD (wt [red] and R629Y [turquoise])

Dex-bound   DAC-bound

Fig. 8.
Predicted structures of mutant GR LBDs. Computer modeling of Dex and DAC (both in yellow) binding to GR LBD of wt vs. A625I (top) and wt vs. R629Y (bottom) mutants. See Discussion for further details.

Table 1

Affinity of mutant GRs for Dex and DAC

| Mutation | Class | DEX binding K_d | | Relative K_d from Competition binding | |
		Scatchard (nM)	Relative to wt	Dex	DAC
Wt		1.93 ± 0.56	1	1	0.32 ± 0.07
E558A	I	NSB			
L584D	I	NSB			
L584D/Q588K	I	NSB			
Q588K	II	5.95 ± 1.64	2.78 ± 1.05	1.67 ± 0.49	0.35 ± 0.06
A625I	III	13.64 ± 2.89	5.86 ± 1.07	3.24 ± 1.15	0.35 ± 0.03
L626I	III	6.18 ± 2.04	2.37 ± 0.30	2.14 ± 0.62	0.32 ± 0.05
R629Y	IV	NSB			

Scatchard assay values were determined from binding assays with [^{3}H]Dex, while competition binding assays involved displacement of [^{3}H]Dex binding by non-labeled Dex or DAC, as described in Materials and Methods. Scatchard assay values are absolute. All other values are relative to Dex binding. Errors are S.E.M. of n = 5 (Scatchard plots for Dex binding Kd) and n = 4 (Competition binding) independent experiments. NSB, no significant binding detected above background. Blanks indicate no measurement.

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Table 2

Correlation of decreased percent partial agonist activity of Dex-Mes and increased EC_{50} for mutant GRs with different reporter genes

Mutation	Class	GREtkLUC		MMTVLuc		LAD1		LAD1 (re DAC)	
		% Activ	Rel EC_{50}	% Activ	Rel EC_{50}	% Activ	Rel EC_{50}	% Activ	Rel EC_{50}
Wt		32.8 ± 3.4	1	36.1 ± 0.1	1	29 ± 10	1	25 ± 6	1
E558A	I	NMF		NMF					
L584D	I	NMF		NMF					
L584D/Q588K	I	NMF		NMF					
Q588K	II	2.5 ± 0.7	57	1.5 ± 0.1	215	17.2 ± 10.9	75	6.1 ± 1.5	319
A625I	III	5.6 ± 2.7	95	1.6 ± 0.0	391	10.2 ± 6.2	191		
L626I	III	5.2 ± 1.9	50	1.9 ± 0.1	215				
R629Y	IV	NMF		-0.6 ± 0.1	708			6.9 ± 4.2	26

The percent partial agonist activity of Dex-Mes, expressed as the percent of maximal activity with Dex for each gene, was determined as described in Materials and Methods. To obtain the percent agonist activity of Dex-Mes with R629Y (which has minimal binding of Dex) and the LAD1 gene, the activity of Dex-Mes is expressed as the percent of maximal activity with DAC and listed in the column labeled "LAD1 (re DAC)". NMF, no meaningful figure. Blanks indicate no measurements. Averages are ± S.E.M. of n = 4–8 (GREtkLUC), n = 2 (MMTVLuc), and n = 4 (LAD1) independent experiments. For ease of comparison, the average EC50 values from Fig. 2 (without error bars) are listed.

Table 3

Interaction of RU486-bound GRs with cofactors in two-hybrid assays as percent of maximal activity with DAC (= 100)

Mutation	Class	GAL/TIF2	GAL/NCoR-RID
Wt		66 ± 4	590 ± 16
Q588K	II	1.9 ± 0.4	32 ± 2
A625I	III	6.9 ± 0.6	63 ± 7
L626I	III	9.2 ± 2.5	64 ± 3
R629Y	IV	0.6 ± 0.2	7.1 ± 1.8

The percent partial agonist activity of RU486, expressed as the percent of maximal activity with DAC, in the two-hybrid assays with VP16/GR chimeras and GAL/TIF2 (= full-length GRIP) or GAL/NCoR-RID was determined as described in Materials and Methods. The percent partial agonist activity of RU486 with GAL/NCoR-RID and wt GR is >100 because interaction of DAC-bound GR is weaker than with RU486-bound GR. Averages are ± S.E.M. of n = 3 independent experiments.

Table 4

Percent partial agonist activity of Dex-Mes with mutant GAL/GR525C or full-length GR $\pm$ TIF2

Mutation	Class	GAL/GR525C			GR	
		w/o TIF2	with TIF2	w/o TIF2	with TIF2
Wt		27 ± 1	57 ± 2	21 ± 3	45 ± 4
Q588K	II	2.0 ± 0.9	1.9 ± 0.1	0.6 ± 0.7	9.9 ± 0.6
A625I	III	NMF	1.8 ± 0.3	0.3 ± 0.9	8.6 ± 1.0
L626I	III	NMF	2.9 ± 0.9	-0.2 ± 0.5	7.6 ± 2.2
R629Y	IV	NMF	NMF	-9.7 ± 2.9	-0.4 ± 0.1

The percent partial agonist activity of Dex-Mes, expressed as the percent of maximal activity with Dex, in assays with GAL/GR525C chimera and FRLuc, or full-length GR (GR) and GREtkLUC, $\pm$ the coactivator TIF2 was determined as described in Materials and Methods. NMF, no meaningful figure. Averages are $\pm$ S.E.M. of n = 4–5 independent experiments.