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Ligand Binding Domain Mutations of the Glucocorticoid Receptor Selectively Modify the Effects with, but Not Binding of, Cofactors†

Geun-Shik Lee and S. Stoney Simons Jr.*

Steroid Hormones Section, National Institute of Diabetes and Digestive and Kidney Diseases/Clinical Endocrinology Branch, National Institutes of Health, Bethesda, Maryland 20892-1772, United States

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ABSTRACT: We previously reported that several point mutations in the ligand binding domain (LBD) of glucocorticoid receptors (GRs) marginally affect the binding affinity of the synthetic glucocorticoids dexamethasone (Dex) and deacylcortivazol (DAC). However, these mutations dramatically alter the efficacy (A_{max}) and potency (EC_{50}) of agonists, along with the partial agonist activity (PAA) of the antisteroid Dex-mesylate (DM), for gene induction and repression in a steroid-dependent manner. This was proposed to result, in part, from altered protein−protein interactions in the complex of GR with the coactivator TIF2 despite normal TIF2 binding. To explore the generality of this phenomenon, we now ask whether these mutations also affect the transactivation properties, but not binding, of other GR-bound factors. We find that an elevated concentration of GR, to probe unidentified cofactors, or of the comodulator Ubc9 does not reverse the effects of GR LBD mutations that increase the EC_{50} and lower the PAA with the GREtkLUC reporter in both CV-1 and U2OS cells. This behavior is more dramatic with Ubc9 and the isolated GR LBD fused to the GAL4 DNA binding domain, despite normal binding of Ubc9 to the mutant GRs. Similar effects, albeit gene, steroid, and transcriptional property-specific, are seen with full-length GRs and three endogenous genes in U2OS cells. Thus, changes in simple steady-state binding capacities of mutant receptors for factors cannot account for the modified transcriptional properties. In all cases, the nuclear translocation of Dex- and DAC-bound wild-type and mutant receptors is the same. These results are consistent with the earlier results with TIF2 and support the hypothesis that small changes in the GR LBD can alter the activities of the bound cofactor without modifying cofactor binding. We propose that this separation of binding and the modulation of transactivation parameters occurs for a wide variety of GR-associated cofactors.

The ability of steroid receptors to regulate gene transcription lies at the heart of their roles in differential gene expression during development, differentiation, and homeostasis. Glucocorticoids are particularly effective because they affect virtually every cell in the body and are used to treat a variety of human pathologies, including asthma, autoimmune diseases, cancer, and the induction of surfactant in the lungs of premature infants (*1*, *2*). These effects of glucocorticoid steroids are thought to occur via the classical mechanism of passive diffusion into the cell and binding to the intracellular receptor protein. The resulting glucocorticoid receptor−steroid complex binds with high affinity to biologically active DNA sequences to recruit other transcriptional cofactors, thereby increasing or decreasing the rates of transcription of gene expression.

In addition to modifying the rates of transcription to increase or decrease the maximal levels of gene product (A_{max}),[1] many transcriptional cofactors also alter both the concentration of agonist steroid required for half-maximal induction (EC_{50}) and the partial agonist activity of antisteroids (PAA) (reviewed in refs *3* and *4*). The EC_{50} is a measure of steroid potency, but it is not the same for all genes regulated by that steroid. Thus, the endogenous subsaturating amount of steroid will cause a different percentage of full induction (or repression) for most responsive genes. The PAA is expressed as the percent of maximal activity of an agonist under the same conditions. Therefore, the PAA is a measure of the amount of residual agonist activity displayed by an antisteroid. This parameter is critical when antisteroids are used in endocrine therapies to block the action of endogenous agonist steroids. The PAA also varies with the gene examined. For this reason, it is theoretically possible during endocrine therapy to target the inhibitory action of antisteroids to a subset of the regulated genes, thereby reducing the number of unwanted side effects from that which occurs when all regulated genes are repressed. For all three parameters of GR-regulated gene expression (A_{max}, EC_{50}, and PAA), changing the level of factor acts as a rheostat to give a continuum of responses for GRs and the other classical steroid receptors (*3*, *4*). These two effects of changing cofactor concentration provide attractive additional mechanisms for achieving differential control of gene expression during development, differentiation, and homeostasis, i.e., by altering the responses in a gene-selective manner to subsaturating, physiological concentrations of agonist steroid and to pharmacologically administered doses of antisteroid.

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*To whom correspondence should be addressed: Bldg. 10, Room 8N-307B, NIDDK/CEB, NIH, Bethesda, MD 20892-1772. E-mail: steroids@helix.nih.gov. Phone: (301) 496-6796. Fax: (301) 402-3572.

[1]Abbreviations: A_{max}, maximal activity; AF1 and AF2, activation functions 1 and 2, respectively; DAC, deacylcortivazol; Dex, dexamethasone; DM, Dex-21-mesylate; DBD, DNA binding domain; GR, glucocorticoid receptor; IGFBP1, insulin-like growth factor binding protein 1; I6PK, inositol hexaphosphate kinase 3; LAD1, ladinin 1; LBD, ligand binding domain; PAA, partial agonist activity.

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One frequently studied modulatory factor of GR transcriptional properties is TIF2/GRIP1. TIF2 (the name for the human coactivator) is a member of the p160 family of coactivators that includes SRC-1 and AIB1/pCIP/ACTR/TRAP1/RAC3. A central region of TIF2 containing three receptor interacting domains (RIDs) binds to a pocket in the AF2 activating function domain of the GR ligand binding domain (LBD), which is formed by helices 3−5 and 12 (*5*). More recently, a second, amino-terminal region of TIF2 has been found to interact with the amino-terminal domain of GR and inhibit the binding of the corepressor to GR (*6*). Even with two GR interaction domains for TIF2, there appears to be a limit to how much the A_{max}, EC_{50}, and PAA of GR-mediated transactivation can be altered with increasing amounts of TIF2 (*7*). Of further interest is the recent report that several GR LBD mutations in the steroid binding pocket (*8*) selectively alter both the absolute A_{max} and EC_{50} of GR-mediated gene induction in a steroid-specific manner (*9*). These GR mutations were chosen following a comparison of the X-ray structures of GR LBD bound by two very differently shaped steroids: dexamethasone (Dex) and deacylcortivazol (DAC) (*8*). It was predicted that these mutations would selectively affect Dex versus DAC binding. Unexpectedly, the changes in steroid binding of many mutants were minor compared to their effects on A_{max}, EC_{50}, and PAA (*9*). This was proposed to result in part from alterations in specific GR surfaces. These perturbed surfaces did not affect the binding of TIF2 to GR but did modify, via protein−protein induced changes, some TIF2 domains interacting with downstream transcription factors (*9*). This observation led us to ask if such changes in the efficiency of information transmission through a GR-bound cofactor to the transcriptional machinery was unique to TIF2 or also occurred with other factors known to influence GR transactivation properties. The phenomenon would be of even greater interest if it also occurred for a cofactor that bound to a different region of GR (*10*). Such a result would suggest that subtle changes in LBD structure can have more widespread topological consequences (*11*, *12*) and are not limited to the GR−TIF2 contact surface(s).

A particularly attractive cofactor with which to address this question is Ubc9, which is the human homologue of a yeast E2-ubiquitin conjugating enzyme. Ubc9 mediates the attachment of a small ubiquitin-like molecule (SUMO) to proteins. However, Ubc9 also alters the A_{max}, EC_{50}, and PAA of GR-regulated transcription in a manner that is independent of its sumoylation activity (*13*−*15*). Like TIF2, Ubc9 binds to the GR LBD (*10*, *14*). Like TIF2, Ubc9 action depends upon domains in both the N-terminal half and LBD of GR, although the LBD interactions are the strongest (*14*). However, there are two important differences between Ubc9 and TIF2. First, the modulatory effects of TIF2 are most dramatic at low and disappear at high GR concentrations (*7*). In contrast, Ubc9 increases the A_{max} at low and high concentrations of GR but reduces the EC_{50}, and enhances the PAA, only with high GR concentrations (*13*−*16*). Second, the effects of Ubc9 appear to be manifested at a step downstream of TIF2 action (*13*, *16*). This argues that TIF2 and Ubc9 do not interact with the same factors to modulate A_{max}, EC_{50}, or PAA and could contact different surfaces of the GR LBD.

The purpose of this study was to determine whether the receptor mutations described above that alter GR activity with TIF2 also affect the activity of two other cofactors that are known to modulate the transactivation properties of GR but bind to different regions of the GR LBD and/or interact with cofactors other than TIF2. The first factor is Ubc9. The second is GR itself. It is well documented that increasing concentrations of GR elevate the PAA and decrease the EC_{50} of GR gene transactivation (*3*, *7*, *17*). GR also forms dimers using surfaces removed from that for binding TIF2 (*18*). We now find that, when the concentration of GR or Ubc9 is increased, there are minimal consequences of these LBD mutations on A_{max} but much larger reductions in PAA and EC_{50}. Under some circumstances, changes in EC_{50} depend upon the structure of the bound agonist steroid. These results, in combination with our previous data with TIF2, led us to conclude that minor changes in GR LBD structure can influence the activity, but not the binding, of a variety of associated transcriptional cofactors in a manner that is sometimes further influenced by the nature of the agonist steroid.

MATERIALS AND METHODS

Unless otherwise indicated, all cell growth occurred at 37 °C and all other operations were performed at room temperature or as recommended by the supplier.

Chemicals. Dexamethasone (Dex) was purchased from Sigma (St. Louis, MO). Dex-21-mesylate (DM) was synthesized as previously described (*19*). Cortivazol (gift from Roussel UCLAF) was converted to deacylcortivazol (DAC) by C. Thomas (National Institute of Diabetes and Digestive and Kidney Diseases) via hydrolysis of the C21-acetyl group. Renilla TS was a gift from N. M. Ibrahim, O. Fröhlich, and S. R. Price (Emory University School of Medicine, Atlanta, GA).

Transient Transfection and Reporter Analysis. CV-1 or U2OS cells were grown and assayed as previously described (*20*, *21*). Briefly, cells were seeded (20000 cells per well) one day before transfection in 24-well plates. GRE-tk-luciferase reporter plasmid or pFRLuc (Stratagene-Agilent Technologies, Inc., Santa Clara, CA; for the GAL-regulated reporter assay) and other plasmids (total DNA of 300 ng/well) were transiently transfected with FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The mutant GRs were described in a previous study (*9*). The cells were induced by steroids (dissolved in absolute EtOH; final EtOH concentration in assays with or without steroid is constant and ≤1%) for 16 h and assayed for lucifierase activity using the dual-luciferase reporter assay (Promega, Madison, WI). The luciferase activity was normalized with Renilla TS activity.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). U2OS cells (200000 cells per well in six-well plates) were treated with various concentrations of Dex, Dex-mesylate (DM), or DAC for 16 h. Total RNA was extracted using TriZol reagent (Invitrogen), and cDNA was synthesized using SuperScript III, First-Strand Synthesis (Invitrogen). The relative expression levels of insulin-like growth factor binding protein 1 (IGFBP1), inositol hexakisphosphate kinase 3 (I6PK), or ladinin 1 (LAD1) were quantitated with SYBR Green in an ABI 7900HT real-time PCR system (*21*). The quantitation was normalized with glyceraldehyde-3-phosphate dehydrogenase (catalog no. 4310884E, Applied Biosystems, Carlsbad, CA).

Co-Immunoprecipitation Assay and Western Blotting. Cos-7 cells were transiently transfected with plasmids for the wild-type GR and its mutants without or with Flag-tagged Ubc9 using FuGENE6 (Roche). After 48 h, cells were induced with Dex for 1 h and then cross-linked at 37 °C with 1 mM dimethyl 3,3′-dithiobispropionimidate (DTBP) (Thermo Fisher Scientific

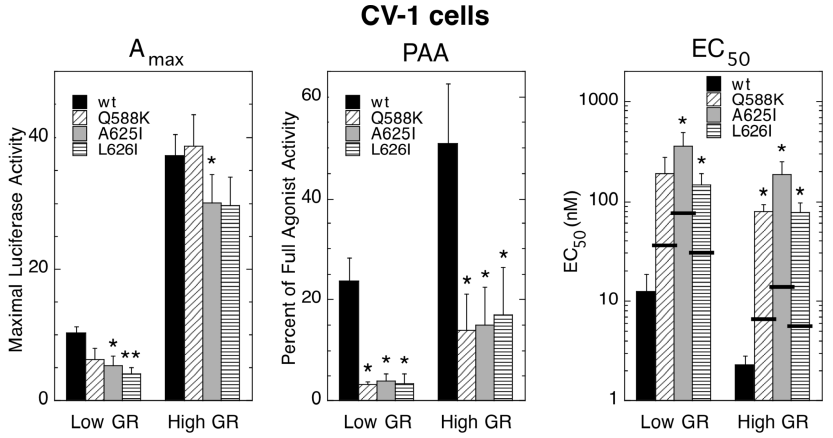

FIGURE 1: Variation in induction parameters of mutant GRs in CV-1 cells with more GR. Triplicate data points with or without a transiently transfected GR plasmid (low GR = 0.05 ng/well; high GR = 0.5−1 ng/well) were analyzed to determine the A_{max}, PAA, and EC_{50} as described in Materials and Methods [error bars denote the standard error of the mean (SEM) from four independent experiments]. *P* values for mutant vs wild-type GR under the same conditions are as follows: < 0.05 for one asterisk and < 0.005 for two asterisks. Under conditions such as ours where very few differences exist between the conditions with different receptors, the prevailing model of steroid hormone action is one in which the EC_{50} of gene induction is directly proportional to the affinity of steroid binding to its cognate receptor. Therefore, the predicted EC_{50} of Dex with the mutant GRs, indicated by the thick horizontal lines, can be calculated by multiplying the EC_{50} of Dex with the wild-type GR by the relative increase in the K_d for cell-free Dex binding to the mutant receptors. This relative increase in K_d was previously determined (*9*) by Scatchard analysis to be 1 for the wild-type GR, 2.8 for Q588K, 5.9 for A625I, and 2.4 for L626I.

Inc., Rockford, IL) as described by Vicent et al. (*22*). The cell lysates were immunoprecipitated with anti-Flag M2-agarose beads (Sigma) at 0 °C and eluted for immunoblotting by being boiled for 10 min in 2× SDS sample buffer (NuPAGE LDS Sample Buffer + NuPAGE Sample Reducing Agent). Western blots were prepared, probed at room temperature with mouse anti-Flag monoclonal antibody (Sigma), rabbit anti-β-actin polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-GR antibody (PA1-511A, Affinity BioReagents, Dublin, OH), or mouse anti-GAL4 DBD monoclonal antibody (Santa Cruz), and visualized with ECL detection reagents as described by the manufacturer (GE Healthcare, Piscataway, NJ).

Immunocytochemistry. U2OS cells (10000 cells per well) in eight-well chamber slides (BD Biosciences, San Jose, CA) were transiently transfected with the GR or its mutants with Lipofectamine 2000 (Invitrogen). After 48 h, cells were treated with 1 μM Dex, 100 nM DAC, or ethanol for 1 h and fixed with 4% paraformaldehyde at room temperature. The fixed cells were incubated with rabbit anti-GR antibody and further probed with anti-rabbit IgG-Cy3 (Sigma) at room temperature. The slides were mounted using VECTASHIELD with DAPI (Vector Laboratories, Inc., Burlingame, CA) and analyzed by fluorescence microscopy.

Data Analysis. The maximum induced activity (A_{max}) in cells transiently transfected with GR plasmids was obtained with saturating concentrations of agonist steroid, which was either $\geq$100-fold higher than the EC_{50} or 10 μM, whichever was lower. For gene induction, the basal activity is that without steroid and the maximal activity is that produced by saturating agonist steroid concentrations. The fold induction equals (induced value)/(basal activity). The partial agonist activity (PAA) of the antagonist (DM) was calculated by expressing the activity of a saturating concentration of DM (10 μM) as the percent of maximal activity of a saturating concentration of agonist under the same conditions. For dose−response curves, seven concentrations of Dex were used, with each point being the average of triplicate samples ± the standard deviation. One curve of average points yields one value of EC_{50} (the concentration of agonist required for 50% of the maximal response) via best-fit curve fitting programs with KaleidaGraph (Synergy Software, Reading, PA) following a first-order Hill plot (R^2 almost always $\geq$0.95). For bar graphs giving average values of A_{max}, EC_{50}, and PAA, the average of *n* replicates (each in triplicate but considered, statistically, as one observation) was plotted ± the standard error of the mean (*n* observations) unless otherwise noted. Statistical significance was assessed 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. 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.

RESULTS

Activities of Mutant GRs with Higher GR Concentrations. One possible reason for the generally lower activities of the previously reported mutant receptors (*9*) is that the steady-state interactions of the GR with unidentified factors are weakened by the LBD mutations. This hypothesis can be tested by increasing the concentration of GR in cells under conditions where GR is a limiting component. It is well-known that gene transactivation with elevated levels of the wild-type GR gives rise to an increased A_{max} and PAA, along with a lower EC_{50} (*4*, *9*, *17*). This indicates that the GR is limiting in these reactions. Mass-action considerations predict that augmenting the levels of GR will force the steady-state reaction toward formation of the GR complex with normally binding cofactors. Thus, increasing the concentration of subsaturating amounts of presumed low-avidity binders (e.g., mutant GRs) will increase the level of formation of the complex with a cofactor much more than increasing the amount of strong binders (e.g., wild-type GRs). In this case, elevated levels of the weakly binding mutant GRs would drive the A_{max}, PAA, and EC_{50} back to wild-type levels if our hypothesis is correct.

Four rat GR LBD mutations (Q588K, A625I, L626I, and R629Y) were selected for this study. The R629Y mutant, though, has negligible transactivation activity under many conditions (*9*)

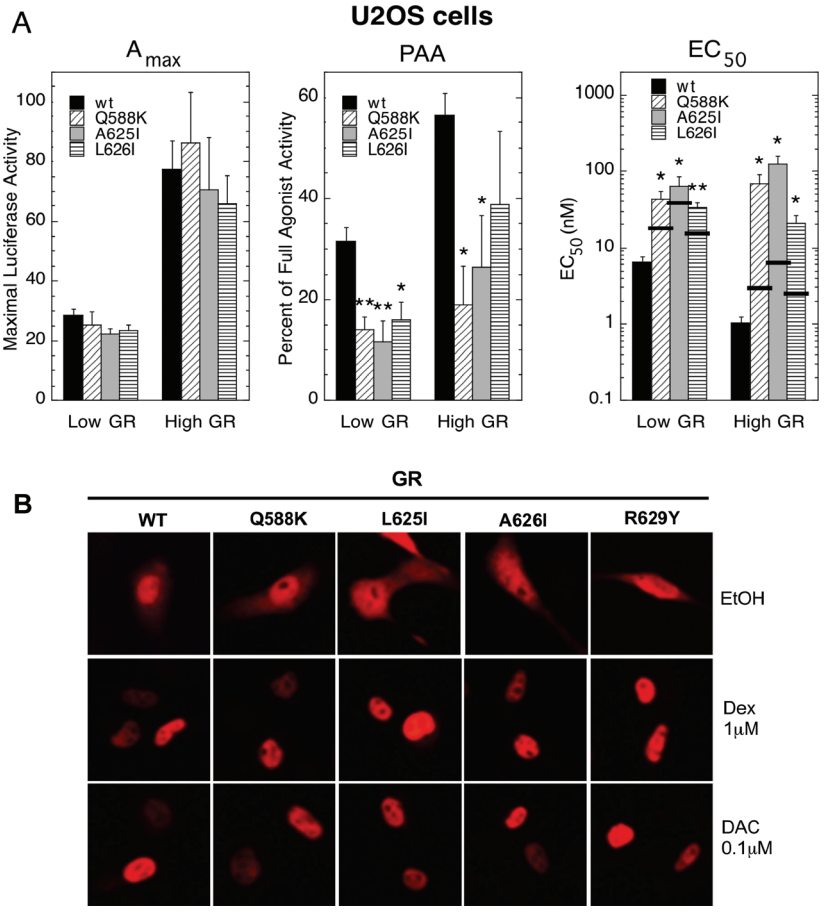

FIGURE 2: Properties of mutant GRs in U2OS cells. (A) Variation in induction parameters of mutant GRs in U2OS cells with an elevated GR level. Data from triplicate transiently transfected cells were generated, analyzed, and plotted along with predicted EC_{50} values as in Figure 1 (error bars denote the SEM from five independent experiments). *P* values for mutant vs wild-type GR under the same conditions are as follows: < 0.05 for one asterisk and < 0.005 for two asterisks. (B) Whole-cell localization of wild-type and mutant GRs following treatment with Dex and DAC. Cells were treated as described in Materials and Methods with the indicated steroids. GR was identified by immunofluorescence.

and thus was not included in the experiments in the first phase of this study. The three other mutants, and the wild-type receptor, were transiently transfected into CV-1 cells at low and high concentrations of the receptor plasmid. The effect of varying receptor concentrations on the induction properties of a cotransfected, synthetic reporter gene (GREtkLUC) was then determined. The properties examined were the A_{max} and EC_{50} for the agonist dexamethasone (Dex) and the PAA for the antiglucocorticoid Dex-21-mesylate (DM) (Figure 1). Most values of A_{max} and PAA with low levels of mutant receptors (0.1 ng of receptor plasmid) in CV-1 cells are significantly lower than those for the wild-type GR, while the EC_{50} values are higher. Transfections with 10−20-fold higher concentrations of each receptor plasmid cause the expected (*9*, *17*) increases in A_{max} and PAA and decreases in EC_{50}. It should be noted that the 4-fold increase in A_{max} is smaller than the 10−20-fold increase in the level of plasmid for the wild-type GR. This suggests that the GR levels are approaching saturation under the high-GR conditions (*7*). The A_{max} and PAA of the mutants increase more than for the wild-type GR. Thus, the A_{max} of each mutant GRs is now closer to wild-type levels. This is what might be expected if the mutant GRs have weakened binding of a factor(s) required for A_{max}. However, the PAA with the antiglucocorticoid DM is still significantly lower than that of the wild-type GR. More receptor is actually less effective in reducing the EC_{50} of the mutant GRs compared to the wild-type receptor (note the log scale). Thus, the ratio of EC_{50} values for mutant and wild-type receptors increases from 12−29 with small amounts of receptor to 33−81 with high receptor concentrations. Importantly, Western blots indicate that all mutants are expressed in Cos-7 cells at approximately the same level as the wild-type GR (see Figure 6 below). Therefore, increasing the level of mutant GRs cannot overcome the effects of the LBD mutations in decreasing the PAA and increasing the EC_{50} for GR-mediated gene induction. We conclude that these reduced transcriptional activities of the mutant receptors are not attributable to lower binding capacities of important endogenous cofactors.

The generality of the behavior described above was addressed by using a different cell line, i.e., U2OS cells. As seen in Figure 1 for CV-1 cells, the PAA was again lower, and the EC_{50} higher, for induction of the GREtkLUC reporter by the mutant GRs in U2OS cells (Figure 2A). An increased GR concentration affected the PAA of wild-type and mutant GRs approximately equally, so that the mutant GRs still display substantially less activity than the wild-type GR in U2OS cells. The EC_{50} values (note the log scale) of mutant receptors generally went from 5−10-fold greater than the EC_{50} for the wild-type GR at low receptor concentrations to 20−120-fold greater at high receptor levels.

In both CV-1 and U2OS cells, the EC_{50} for gene induction at elevated levels of receptor is 20−120-fold higher for the mutant GRs than for the wild-type GR. These differences are much greater than what can be explained by the 2−6-fold increase in K_d

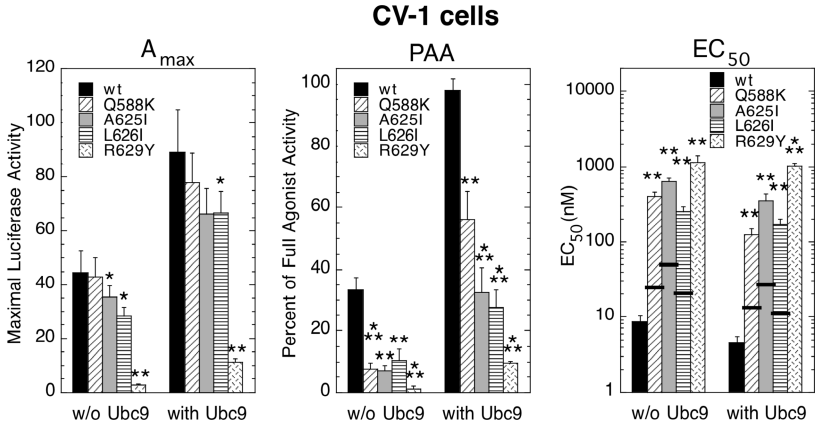

FIGURE 3: Variation in induction parameters of mutant GRs in CV-1 cells with an increased level of Ubc9. Data from triplicate transiently transfected cells (1 ng of GR with or without 150 ng of Ubc9 plasmid) were generated, analyzed, and plotted along with predicted EC_{50} values as in Figure 1 (error bars denote the SEM from six or seven independent experiments). The absence of any measurable affinity of Dex for R629Y (*9*) precluded the calculation of a predicted EC_{50}, so no thick horizontal bar is included. *P* values for mutant vs wild-type GR under the same conditions are as follows: < 0.05 for one asterisk, < 0.005 for two asterisks, and < 0.0005 for three asterisks.

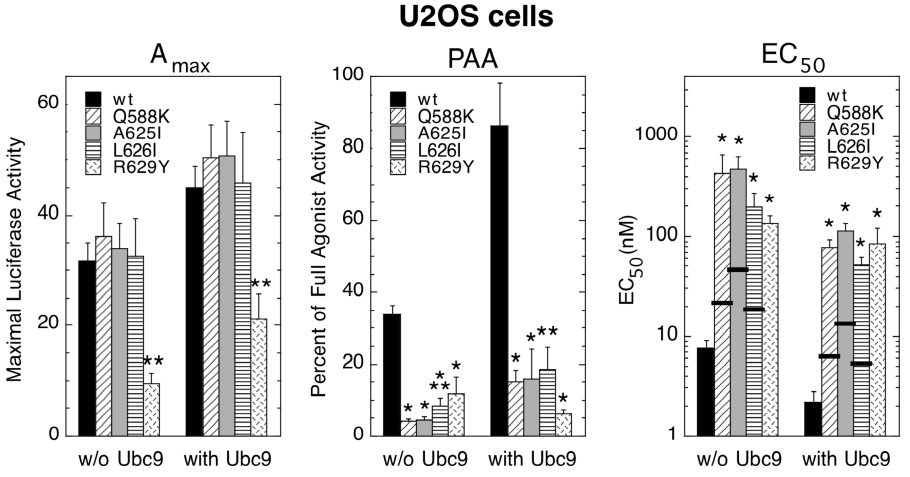

FIGURE 4: Variation in induction parameters of mutant GRs in U2OS cells with a larger amount of Ubc9. Data from triplicate transiently transfected cells were generated, analyzed, and plotted along with predicted EC_{50} values as in Figure 3 (error bars denote the SEM from five independent experiments). *P* values for mutant vs wild-type GR under the same conditions are as follows: < 0.05 for one asterisk, < 0.005 for two asterisks, and < 0.0005 for three asterisks.

for steroid binding to the mutant GRs (*9*). The expected value of the EC_{50}, if it reflected only the reduced affinity of Dex for each mutant receptor, is indicated by the thick horizontal line through each bar of the EC_{50} graphs. Furthermore, these differences are exacerbated by high receptor concentrations. These differences would be diminished if they were simply caused by an inhibited ability of mutant GRs to form dimers or interact with other required factors. Thus, some other explanation is required.

This inability of an increased GR concentration to counter the effects of the LBD mutations on PAA and EC_{50} could be due to unequal nuclear binding of GR complexes. However, immunocytochemistry shows equal nuclear localization by 1 μM Dex and by 100 nM DAC (discussed below) in U2OS cells under the bioassay conditions described in the legend of Figure 2 (Figure 2B). Thus, the altered transactivation properties of the mutant GRs, which are disproportionate to changes in steroid binding affinity, are not due to altered nuclear binding of GR complexes.

Activities of Mutant GRs with Added Ubc9. Ubc9 binds to the GR and has been identified as acting downstream of the GR (*13*−*16*). For studies with Ubc9, we have included the fourth mutant, R629Y. Ubc9 is known to increase the A_{max} at high GR concentrations (*13*−*16*). Thus, the marginal activity at high levels of the R629Y mutant might also be augmented by added Ubc9. As shown in Figure 3 for CV-1 cells, the ability of added Ubc9 to increase the A_{max} of mutant and wild-type GRs is approximately the same. This is true for both those mutants that have similar levels of activity and, as for R629Y, dramatically reduced activity. The PAA of the antiglucocorticoid DM with the four mutant GRs is well below that for wild-type GRs. An elevated level of Ubc9 does produce a larger increase with mutant GRs than the wild-type GR. However, this comparison is theoretically flawed. The scale for PAA is nonlinear and cannot exceed 100% of a full agonist. Clearly, factors causing a 10-fold increase in the PAA of one antagonist (e.g., from 5 to 50%) cannot produce the same 10-fold increase of another antagonist that displays 50% PAA before the addition of factors. Thus, the increase in the PAA of DM with the wild-type GR to ~100% in Figure 3 is as large as possible even if the fold increase is smaller than that seen with most of the mutant receptors. In contrast, the EC_{50} values for Dex induction of GREtkLUC by the wild-type and mutant GRs are all decreased by ~2-fold with added Ubc9. It should be noted that no thick horizontal bar is present for the R629Y mutant because its affinity for Dex was below the level of

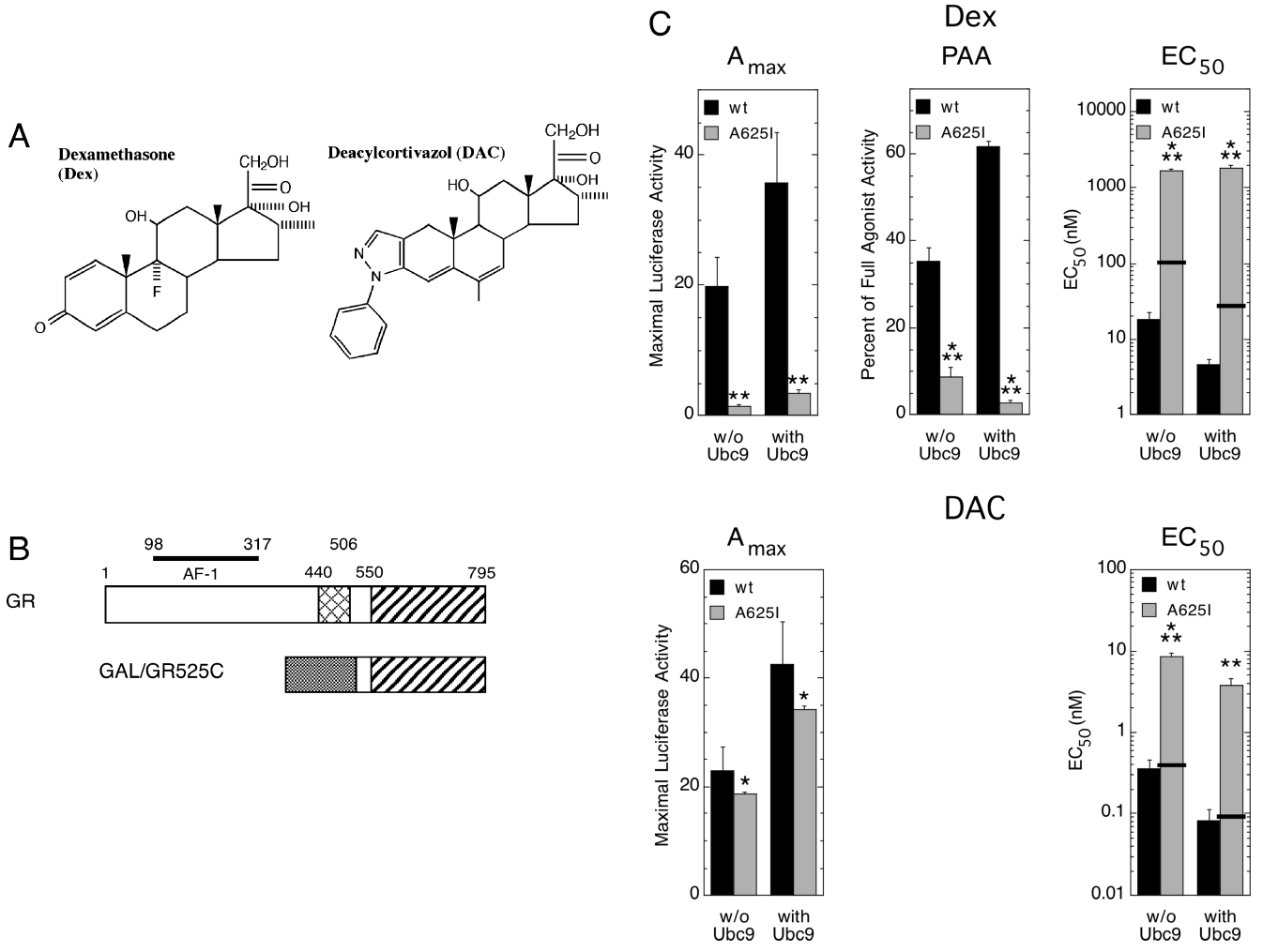

FIGURE 5: Role of the GR LBD in determining the induction properties of Dex- vs DAC-bound mutant vs wild-type GRs with added Ubc9. (A) Structures of Dex and DAC. (B) Organization of chimeric truncated GRs (GAL/GR525C). Residues 1−524 of the wild-type GR, including AF1 and DNA binding domains, are replaced with the GAL4 DNA binding domain (shaded box) while the GR LBD (diagonal striping) remains. (C) Variation in induction parameters of GAL/GR525C chimeric receptors in U2OS cells with elevated Ubc9 levels. Data from triplicate transiently transfected cells (1 ng of GAL/GR525C with the wild-type or A625I GR LBD with or without 150 ng of Ubc9 plasmid) were generated, analyzed, and plotted along with predicted EC_{50} values as in Figure 4 (error bars denote the SEM from seven independent experiments). *P* values for mutant vs wild-type GR under the same conditions are as follows: < 0.05 for one asterisk, < 0.005 for two asterisks, and < 0.0005 for three asterisks. The predicted EC_{50} of Dex with each mutant GR was calculated as described in the legend of Figure 1. For DAC, the predicted EC_{50} is based upon the relative increase in K_d for DAC binding to the A625I mutant vs wild-type GR being 1.1 (*9*).

detection (*9*), thus making the calculation of a theoretical EC_{50} impossible.

The modulatory activity of additional Ubc9 in CV-1 cells (Figure 3) is recapitulated in U2OS cells (Figure 4). The changes with exogenous Ubc9 in A_{max}, PAA (given the constraints mentioned above), and EC_{50} are approximately the same for wild-type and mutant GRs. It should be noted that the EC_{50} for gene induction in both cell lines by each of the mutant receptors except R629Y, which does not have a measurable affinity for Dex (*9*), is still 9−16-fold higher than that predicted from the Dex binding affinity of each receptor (indicated by a thick horizontal line in each bar graph). This difference persists in the presence of exogenous Ubc9. Thus, the increased EC_{50}, decreased PAA, and often decreased A_{max} of the mutant receptors cannot be rectified simply by a higher concentration of Ubc9, and these behaviors are not unique to a single cell line.

Role of AF Domains and Steroid Structure in Determining Mutant GR Properties. The data described above establish that more of either GR or Ubc9 is active in altering several induction parameters (A_{max}, PAA, and EC_{50}) of both mutant and wild-type GRs. However, we remain confronted with the conundrum of why the LBD mutations have a much stronger effect on PAA and EC_{50} than A_{max}. One possible explanation derives from the fact that the major determinant of A_{max} is the activation function domain in the amino-terminal half of the GR (i.e., AF1) while the C-terminal AF2 domain is sufficient to regulate the PAA and EC_{50} with added GR, Ubc9, or TIF2 (*14*). Furthermore, as reported with TIF2, the effects of these mutations are greatly reduced when GR is bound by the bulky glucocorticoid, deacylcortivazol (DAC) (*9*). DAC looks (Figure 5A) and binds to GR in a manner very different from that of Dex (*8*, *23*). Therefore, we predicted both that the ability of the mutant receptors to respond to added Ubc9 would be weakened upon removal of the AF1 domain and that the A_{max} and EC_{50} of such truncated GRs would be less affected by the mutations when bound by DAC than by Dex. To address this question, we used the chimeric receptor GAL/GR525C, which involves the GAL4 DNA binding domain fused to the GR LBD [from amino acid 525 to the C-terminus (Figure 5B)]. We then determined the transactivation properties of GAL/GR525C with a GAL-regulated reporter,

FRLuc. We limited our comparisons to a single, representative mutant (A625I). While the other mutants may display different properties, this has generally not been the case so far except for R629Y (see Figures 1−4 and ref *9*).

As previously reported (*9*), the A625I mutation in the context of the GAL/GR525C construct greatly decreases the A_{max} with Dex but not with DAC in U2OS cells (Figure 5C). This disparity is maintained after the addition of excess Ubc9. The PAA of DM, relative to that of Dex, is also lower here for the A625I mutant than for the wild-type GR in the GAL/GR525C construct. The addition of Ubc9 does not increase the PAA, as might be expected from Figure 3, but rather lowers it even further. Finally, in the presence of Dex, the addition of Ubc9 has no effect on the EC_{50} of the mutant GR while that of the wild-type GR is lowered. In contrast, with DAC, exogenous Ubc9 lowers the EC_{50} of both wild-type and mutant GRs. Thus, the properties of the isolated AF2, with or without Ubc9, are more severely diminished by the mutation with Dex as the bound steroid than with DAC. We conclude that the biological responses, and thus the governing biochemical interactions, of Ubc9 with the DAC-bound mutant GR LBD are much more like those of the wild-type GR LBD than are those of the Dex-bound mutant GR LBD.

Binding of Ubc9 to Wild-Type and Mutant GRs. The data with elevated concentrations of Ubc9 suggest that the defects of the mutant GRs are more evident in the absence of the AF1 domain (Figure 5C), are sensitive to the structure of the bound steroid (Figure 5C), and are not due to altered binding capacities of GR for Ubc9 (Figures 3 and 4). To obtain more direct evidence of this last conclusion, we used a co-IP assay with GR and Flag-tagged Ubc9. Flag-tagged Ubc9 was used because the available antibodies to Ubc9 are not very sensitive (see Figure 6A) and because the biological activities of Flag-Ubc9 and Ubc9 are the same (data not shown and ref *24*).

Full-length wild-type and mutant receptors, along with Flag/Ubc9, were overexpressed in transiently transfected Cos-7 cells. The cells were then treated with different concentrations of Dex for 1 h, followed by mild cross-linking with DTBP to freeze the weakly associated complexes (Y.-G. Tao and S. S. Simons, Jr., unpublished results). Cytosolic extracts were prepared, and anti-Flag antibody was added to immunoprecipitate Flag/Ubc9 and any associated GR. Figure 6A shows that the amount of GR that was co-immunoprecipitated (co-IP'd) with Flag/Ubc9 is the same for EtOH-treated mutant GRs and wild-type GRs. Likewise, the amount of wild-type GR co-IP'd in the presence of 1 μM Dex is very similar to that of the mutant GRs treated with a larger amount of Dex (10 μM) to compensate for their lower steroid binding affinity. At the same time, the binding of Ubc9 to wild-type and mutant (A625I) LBDs fused to the GAL DNA binding domain was examined (Figure 6B). This shows that the GR LBD is sufficient for Ubc9 binding and that there is no significant difference between the wild-type and mutant GR binding of Ubc9. We conclude that the deficiencies in Ubc9 modulation of the A_{max}, PAA, and EC_{50} of the mutant GRs are not due to any alterations in the ability to bind Ubc9.

Induction of Endogenous Genes by the Wild-Type GR versus the A625I Mutant GR Bound by Dex versus DAC. These data suggest that the PAA and EC_{50} for gene induction by full-length GR, with and without exogenous Ubc9, are much more sensitive than A_{max} to mutations in the GR LBD. These changes are influenced by steroid structure but dissociated from the GR binding affinity of steroid and the GR binding capacity of Ubc9. To determine whether these phenomena with an exogenous,

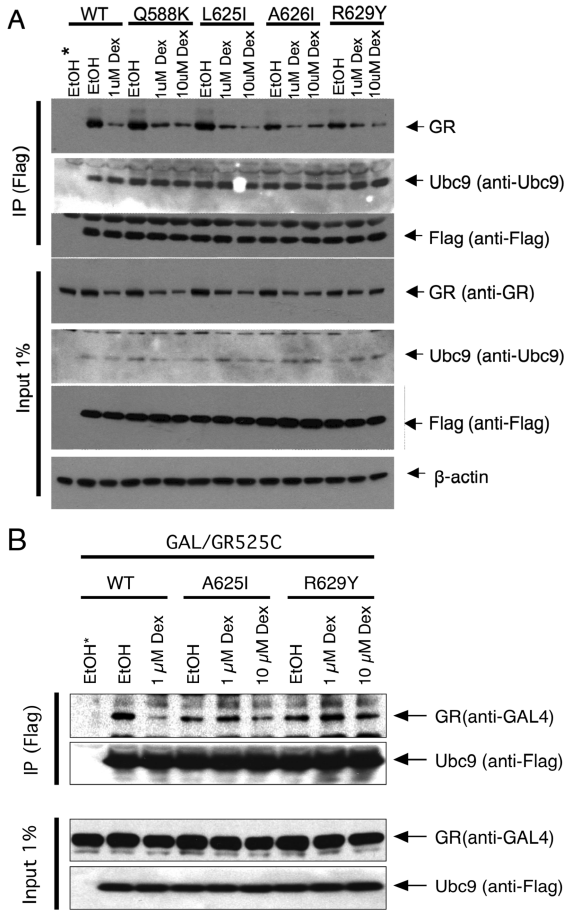

FIGURE 6: Binding of Ubc9 to wild-type and mutant GRs. Co-immunoprecipitation of full-length (A) and truncated (B) wild-type and mutant GRs with Ubc9. Extracts were prepared from Cos-7 cells, which were transiently transfected with GR and Flag/Ubc9 (or empty Flag vector, indicated with an asterisk) and subsequently incubated with the indicated steroids, treated with anti-Flag, and subjected to Western blotting as described in Materials and Methods.

synthetic reporter gene also occur under more physiologically relevant conditions, we examined the behavior of three endogenous genes with the full-length wild-type GR and mutant GR (A625I) treated with Dex or DAC in the presence or absence of exogenous Ubc9. The three genes selected were IGFBP1, I6PK, and LAD1. IGFBP1 is a member of the family of structurally homologous proteins that circulates in the plasma and specifically binds and modulates the half-life of IGF-1 and IGF-2 and their interaction with cell surface receptors. Very little is known about I6PK except that it is glucocorticoid inducible (*25*). LAD1 (ladinin) is a novel component of the basement membranes and may function in contributing to the stability of the association of the epithelial layers with the underlying mesenchyme (*26*).

In these studies, fold induction, as opposed to the often closely related A_{max}, was assessed because the qRT-PCR determinations with SYBR Green cannot give quantitative measurements of cDNAs. In all cases with Dex, the fold induction is somewhat less with the mutant GR (Figure 7). In contrast, the fold induction by the mutant GR with DAC is always the same or more than that with the wild-type GR. Furthermore, added Ubc9 affords a greater increase in fold induction with the DAC-bound mutant receptor. Large differences in the PAA of the antiglucocorticoid DM are seen between mutant and wild-type receptors, both without and with added Ubc9. The PAA, whether without or with added Ubc9, is always between 11 and 21% of the value for

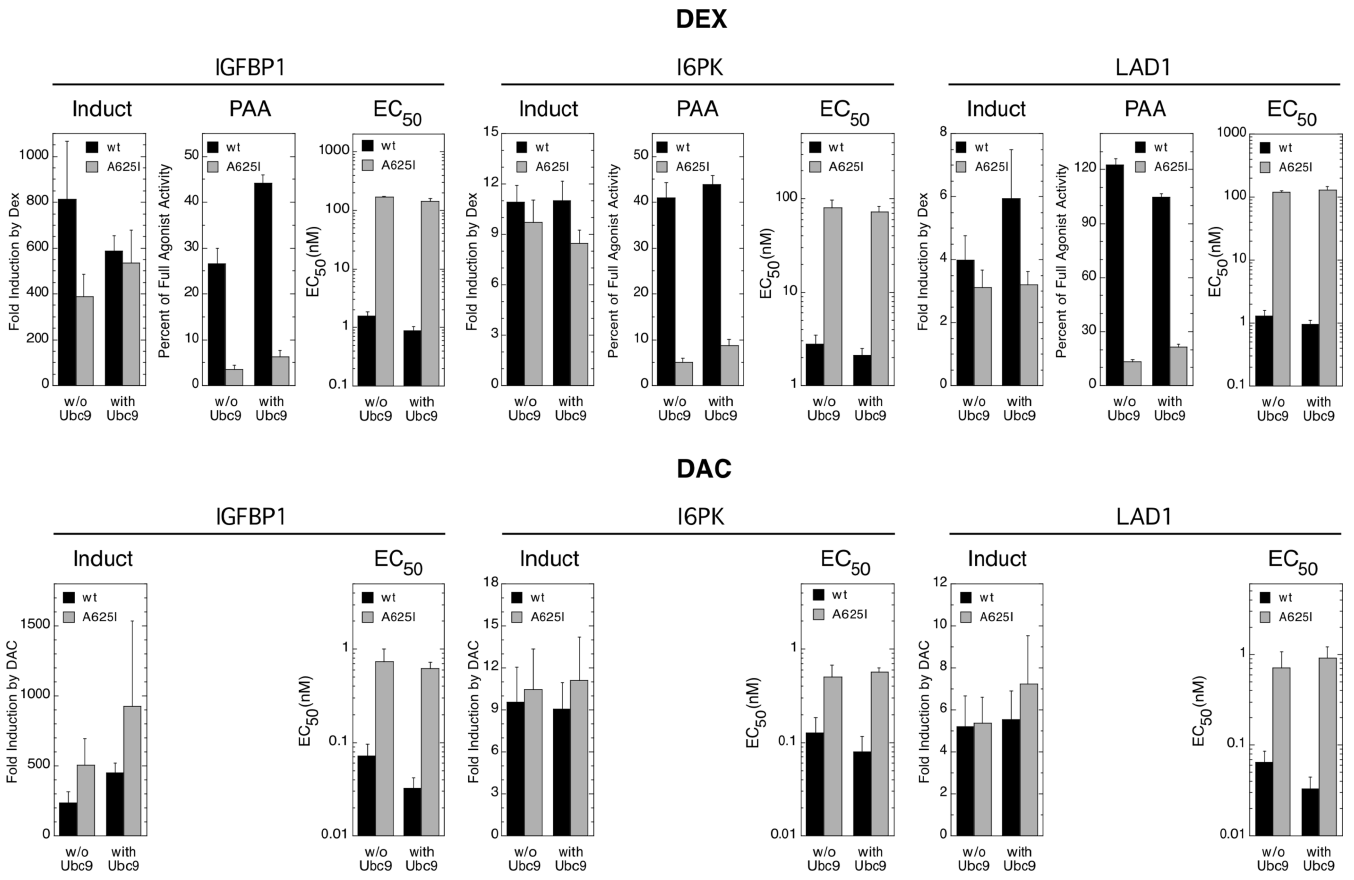

FIGURE 7: Variation in induction parameters of endogenous genes by mutant GR in U2OS cells with more Ubc9. Data from triplicate transiently transfected cells (50 ng of GR and 200 ng of Ubc9) that had been treated with Dex, DM, or DAC were generated by a qRT-PCR assay of mRNA preparations as described in Materials and Methods. The data were then analyzed and plotted as in Figure 4 (error bars denote the SEM from five to seven independent experiments with Dex and four with DAC).

wild-type GRs. There is no significant difference between the Ubc9-induced changes in EC_{50} for Dex- versus DAC-bound receptors. Interestingly, excess Ubc9 only exacerbates the difference between wild-type and mutant GR EC_{50} values, which are 4−30-fold higher than those predicted in the absence of exogenous Ubc9. Thus, the consequences of the A625I mutation vary in severity with the parameter examined in a manner that is sensitive to steroid structure and endogenous gene. Similarly, the effect of an elevated Ubc9 level can be steroid- and gene-dependent. The PAA of the antiglucocorticoid DM is much lower with the mutant than with the wild-type GR, with or without exogenous Ubc9. The fold induction by mutant versus wild-type GR is always greater for DAC- than Dex-bound receptors, but what happens with added Ubc9 is gene- and steroid-dependent. Finally, the EC_{50} of mutant receptors is always lower than that predicted from the binding affinity of the inducing steroid and relatively insensitive to added Ubc9.

DISCUSSION

Selected point mutations in the LBD pocket of GR are shown to alter the three major parameters of gene induction (A_{max}, PAA, and EC_{50}) by glucocorticoid receptors in a manner that depends upon numerous factors: the concentration of GR itself, the amount of the comodulator Ubc9, the structure of the bound steroid, and the induced gene. These changes cannot be explained by mutation-induced differences in either steroid binding affinity of or factor binding capacity with the GR. Several lines of evidence suggest that the altered transcriptional parameters are derived from minor conformational changes in the GR LBD that modify the activities of the bound cofactor without affecting binding of the cofactor to the GR.

The point mutations examined here were among those predicted from X-ray studies (*8*) to have the greatest effect on the binding of two differently sized agonists, Dex and DAC (*9*). We previously found that these mutations do slightly reduce the affinity of each steroid for GR but that the greatest effect was on the gene induction parameters of GR with and without the added coactivator TIF2, which were affected much more than predicted from the changes in steroid binding affinity. The earlier studies with TIF2 led to the conclusion that the activity of GR-bound TIF2, but not the binding of TIF2 to GR, was altered in the mutant GRs in a manner that was much greater for Dex- than for DAC-bound receptors (*9*). This work confirms and extends these conclusions to other GR-binding factors (i.e., GR and Ubc9) in two different cell lines and for selected endogenous genes. The added receptor decreases the EC_{50} of the mutant GRs much less than for the wild-type GR in two cell lines (Figures 1 and 2). This suggests that the mutant GRs are desensitized in a manner other than a reduced avidity for some factor, which would be expected to be counterbalanced by the increase in receptor concentration. With Ubc9 and the exogenous GREtkLUC reporter, the effects are somewhat masked by the presence of the amino-terminal AF1 domain. We have previously documented that the GR LBD in GAL/GR525C is sufficient to mediate the modulatory activity of Ubc9 (*14*). Therefore, the isolated GR LBD in GAL/GR525C should be more responsive to added Ubc9 than the full-length GR. In fact, experiments with the mutant GAL/GR525C chimera

clearly show that none of the parameters (A_{max}, PAA, and EC_{50}) with the Dex-bound mutant receptor are appreciably modulated by added Ubc9 (Figure 5C) even though Ubc9 binding is unchanged (Figure 6). In contrast, the induction parameters of DAC-bound mutant GAL/GR525C remain responsive to Ubc9 (Figure 5C).

These experiments also support and expand our earlier observations with TIF2 siRNA, where the induction properties of endogenous genes in human peripheral blood mononuclear cells (PBMCs) were selectively affected in a gene-specific manner upon reduction of the intracellular levels of TIF2 protein (*27*). In the current studies with three different endogenous genes in U2OS cells, the ability of added Ubc9 to modify the parameters of the mutant A625I receptor is again gene-selective. Furthermore, in contrast to the Dex-bound mutant receptor, the fold induction of the DAC-bound mutant receptor is greater than that of the wild-type GR without or with added Ubc9 (Figure 7). This selective modulation of individual induction parameters has frequently been documented for synthetic reporter genes with mutant receptors (estrogen, progesterone, and glucocorticoid) and with chemicals such as TSA and valproic acid (reviewed in ref *4*). Our results with GR-binding proteins led us to conclude that the ability to separate both factor binding to and factor activities with GRs appears to be a general feature of GR-regulated gene induction. This modular control of gene induction properties greatly expands the possible mechanisms for differential control of gene expression, particularly if it is found to be a property of steroid receptors in general.

Several alternative explanations could be eliminated as possible causes for the changes in the induction parameters. While the affinity of Dex (and to a lesser extent of DAC) was reduced by the mutations examined, steroid affinity differences per se cannot account for changes in A_{max}. The affinity of DAC for wild-type GRs is ~20-fold higher than that of Dex, yet the A_{max} is usually very similar (Figures 5 and 7 and refs *9* and *23*). For the same reason, the reduced affinity of the mutant GRs for steroid would not be expected to account for the greatly diminished PAA of the antiglucocorticoid DM. The EC_{50} is also increased in every instance much more than expected from the small reduction in steroid binding affinity. Nonetheless, the nuclear translocation of mutant and wild-type receptors is approximately the same (Figure 2B). Possible effects on GR dimerization are unlikely for two reasons. First, a decrease in GR avidity for dimer formation on DNA should be countered by an elevated GR concentration, which was not observed, especially when looking at EC_{50} values (Figures 1 and 2). Second, the mutations are not in a region known to affect GR dimerization (*18*). Unequal effects on DNA binding can be ruled out by the observations with GAL/GR525C. None of the GR sequences in GAL/GR525C contact DNA. The only DNA−protein interactions are those of the GAL4 DBD. Nonetheless, the changes in A_{max}, PAA, and EC_{50} for mutant versus wild-type GAL/GR525C chimeras with a GAL-regulated reporter (Figure 5C) are very similar to those seen with the full-length mutant versus wild-type GR that binds directly to a GRE-controlled reporter (Figures 3 and 4). Thus, the GR LBD appears to be the dominant domain for mediating the consequences of the LBD mutations in a manner independent of whether GR sequences have any direct contact with DNA. Furthermore, the A_{max} value, which is indicative of the total DNA-bound GR, is relatively constant with the various LBD mutations compared to the larger changes in PAA or EC_{50} (Figures 1−4 and 7). This separate modulation of transcription parameters has also been seen for three other endogenous genes in human PBMCs with reduced levels of TIF2 (*27*). Collectively, these data illustrate how increased mechanistic information is accessible when EC_{50} and PAA are determined in addition to the conventional A_{max}.

Two results discount the explanation that the LBD mutations reduce the level of binding of the cofactor to the GR LBD. First, co-IP experiments did not reveal any less binding of Ubc9 to full-length or truncated (GAL/GR525C) receptors, especially when adjusted for the higher levels of Dex needed to achieve similar levels of GR−steroid complexes (Figure 6). Second, excess exogenous Ubc9 or GR cannot reverse the changes in A_{max}, PAA, or EC_{50} of the mutant GRs (Figures 1−5 and 7). Simple mass-action considerations (*16*) indicate that increasing the concentration of one component of a complex in the entire steroid-regulated transcription scheme will increase the amount of that complex. Overexpression of the GR or Ubc9 does have some effect in two cell lines and on exogenous and endogenous reporters. However, the minimal reversals of the consequences of each mutation with an added factor argue that less binding of the GR to itself, of the GR to Ubc9, or of the GR to any cofactor is not the explanation. These conclusions are identical to those reached previously with TIF2 (*9*).

The results of exogenous Ubc9 binding to GAL/GR525C are particularly instructive. Ubc9 binds to the GR LBD (*14*). The GR LBD with the AF2 domain is sufficient to mediate the Ubc9-induced changes in induction parameters displayed by the full-length GR, and complications caused by the stronger AF1 domain are eliminated (Figure 5 and ref *14*). Thus, GAL/GR525C is ideally constructed to reveal the maximal effects of Ubc9 binding. Figure 5C shows that the mutant GAL/GR525C has more nearly wild-type properties when bound by DAC than by Dex. The A_{max} of the DAC-bound mutant GR, with or without added Ubc9, and the ability of exogenous Ubc9 to decrease the EC_{50} of the DAC-bound mutant receptor are much closer to those of wild-type GRs. Similar more nearly wild-type properties of DAC- versus Dex-bound mutant receptors and the greater sensitivity of GAL/GR525C versus full-length GR induction parameters to added cofactor were seen in the activities of these mutant GRs with TIF2 (*9*).

It is difficult to quantitatively compare the effects of an increased level of Ubc9 or GR to the effects of an increased level of TIF2 because the mechanisms of action appear to be quite different (*16*) (C. Chow et al., manuscript submitted for publication). The inability of Ubc9 and TIF2 to decrease the EC_{50}, or increase the PAA, of the A625I mutation in the context of GAL/GR525C is comparable. In contrast, the changes in A_{max} are 30-fold with added TIF2 versus 2-fold with exogenous Ubc9 (Figure 5 and ref *9*). These differences further document the ability to separately modify selected induction parameters and emphasize the mechanistic benefits of examining more parameters for steroid receptor-regulated gene transcription than just A_{max}. Because the site for binding of TIF2 to GR appears to be different from those for Ubc9 association and GR dimerization (*10*), qualitative and quantitative differences might be expected. Conversely, these results argue for the generality of small changes in the tertiary structure of the receptor LBD having stronger effects on the biological activity of the receptor-bound cofactor than on the binding of the factor. As these same mutations disproportionately modified the A_{max}, PAA, and EC_{50} of GR-repressed genes (*9*), we speculate that the dissociation of factor binding and of the three transcription parameters

occurs both in GR-regulated induction and in GR-mediated repression.

The ability of changes in factor activity in the presence of unaltered factor binding to a mutant receptor appears to involve action-at-a-distance effects. However, the mounting number of reports of changes that are not immediately recognizable as sequential modifications in contacting amino acids suggest that this may be a more prevalent phenomenon that hitherto considered (*11*, *28*), particularly for DNA-bound proteins (*29*). The DNA binding of GRs is known to stabilize the GR AF1 domain (*30*). Reminiscent of what we report, DNA sequence can also act as an allosteric ligand of DNA-bound GRs, with mutant GRs having different fold induction patterns with assorted glucocorticoid response elements (GREs) that are unrelated to the DNA binding affinity of GR (*31*). Also, modification of ligand structure can cause a decreased level of transactivation by GR with no change in the binding of TIF2 peptides (*32*). Jun dimerization protein 2 (JDP2) binds to the progesterone receptor LBD but affects the activity of the progesterone receptor AF1 domain (*33*). Finally, an elegant compilation of three-dimensional structures shows different binding sites on TIFIID of p53, Sp1, and c-Jun with action-at-a-distance changes in structure in the absence of any modifications in the intervening structures (*12*). Collectively, the observations described above provide several plausible mechanisms for how the A_{max}, PAA, and EC_{50} could vary among endogenous genes with different receptor mutations and agonist steroid structures.

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REFERENCES

1. Haas, D. M., McCullough, W., Olsen, C. H., Shiau, D. T., Richard, J., Fry, E. A., and McNamara, M. F. (2005) Neonatal outcomes with different betamethasone dosing regimens: A comparison. *J. Reprod. Med. 50*, 915–922.
2. Stahn, C., Lowenberg, M., Hommes, D. W., and Buttgereit, F. (2007) Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. *Mol. Cell. Endocrinol. 275*, 71–78.
3. Simons, S. S., Jr. (2003) The importance of being varied in steroid receptor transactivation. *Trends Pharmacol. Sci. 24*, 253–259.
4. Simons, S. S., Jr. (2008) What goes on behind closed doors: Physiological versus pharmacological steroid hormone actions. *BioEssays 30*, 744–756.
5. Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) Structure and specificity of nuclear receptor-coactivator interactions. *Genes Dev. 12*, 3343–3356.
6. Wang, D., Wang, Q., Awasthi, S., and Simons, S. S., Jr. (2007) Amino-terminal domain of TIF2 is involved in competing for corepressor binding to glucocorticoid and progesterone receptors. *Biochemistry 46*, 8036–8049.
7. Chen, S., Sarlis, N. J., and Simons, S. S., Jr. (2000) Evidence for a common step in three different processes for modulating the kinetic properties of glucocorticoid receptor-induced gene transcription. *J. Biol. Chem. 275*, 30106–30117.
8. Suino-Powell, K., Xu, Y., Zhang, C., Tao, Y. G., Tolbert, W. D., Simons, S. S., Jr., and Xu, H. E. (2008) Doubling the size of the glucocorticoid receptor ligand binding pocket by deacylcortivazol. *Mol. Cell. Biol. 28*, 1915–1923.
9. Tao, Y.-G., Xu, Y., Xu, H. E., and Simons, S. S., Jr. (2008) 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. *Biochemistry 47*, 7648–7662.
10. Szapary, D., Song, L.-N., He, Y., and Simons, S. S., Jr. (2008) Differential modulation of glucocorticoid and progesterone receptor transactivation. *Mol. Cell. Endocrinol. 283*, 114–126.
11. Hilser, V. J., and Thompson, E. B. (2007) Intrinsic disorder as a mechanism to optimize allosteric coupling in proteins. *Proc. Natl. Acad. Sci. U.S.A. 104*, 8311–8315.
12. Liu, W. L., Coleman, R. A., Ma, E., Grob, P., Yang, J. L., Zhang, Y., Dailey, G., Nogales, E., and Tjian, R. (2009) Structures of three distinct activator-TFIID complexes. *Genes Dev. 23*, 1510–1521.
13. Kaul, S., Blackford, J. A., Jr., Cho, S., and Simons, S. S., Jr. (2002) Ubc9 is a novel modulator of the induction properties of glucocorticoid receptors. *J. Biol. Chem. 277*, 12541–12549.
14. Cho, S., Kagan, B. L., Blackford, J. A., Jr., Szapary, D., and Simons, S. S., Jr. (2005) Glucocorticoid receptor ligand binding domain is sufficient for the modulation of glucocorticoid induction properties by homologous receptors, coactivator transcription intermediary factor 2, and Ubc9. *Mol. Endocrinol. 19*, 290–311.
15. Kim, Y., Sun, Y., Chow, C., Pommier, Y. G., and Simons, S. S., Jr. (2006) Effects of acetylation, polymerase phosphorylation, and DNA unwinding in glucocorticoid receptor transactivation. *J. Steroid Biochem. Mol. Biol. 100*, 3–17.
16. Ong, K. M., Blackford, J. A., Jr., Kagan, B. L., Simons, S. S., Jr., and Chow, C. C. (2010) A new theoretical framework for gene induction and experimental comparisons. *Proc. Natl. Acad. Sci. U.S.A. 107*, 7107–7112.
17. Szapary, D., Xu, M., and Simons, S. S., Jr. (1996) Induction properties of a transiently transfected glucocorticoid-responsive gene vary with glucocorticoid receptor concentration. *J. Biol. Chem. 271*, 30576–30582.
18. Bledsoe, R. K., Montana, V. G., Stanley, T. B., Delves, C. J., Apolito, C. J., McKee, D. D., Consler, T. G., Parks, D. J., Stewart, E. L., Willson, T. M., Lambert, M. H., Moore, J. T., Pearce, K. H., and Xu, H. E. (2002) Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell 110*, 93–105.
19. Simons, S. S., Jr., Pons, M., and Johnson, D. F. (1980) α-Keto mesylate: A reactive thiol-specific functional group. *J. Org. Chem. 45*, 3084–3088.
20. Wang, Q., Blackford, J. A., Jr., Song, L.-N., Huang, Y., and Simons, S. S., Jr. (2004) Equilibrium interactions of corepressors and coactivators modulate the properties of agonist and antagonist complexes of glucocorticoid receptors. *Mol. Endocrinol. 18*, 1376–1395.
21. He, Y., and Simons, S. S., Jr. (2007) STAMP: A novel predicted factor assisting TIF2 actions in glucocorticoid receptor-mediated induction and repression. *Mol. Cell. Biol. 27*, 1467–1485.
22. Vicent, G. P., Ballare, C., Nacht, A. S., Clausell, J., Subtil-Rodriguez, A., Quiles, I., Jordan, A., and Beato, M. (2006) Induction of progesterone target genes requires activation of Erk and Msk kinases and phosphorylation of histone H3. *Mol. Cell 24*, 367–381.
23. Simons, S. S., Jr., Thompson, E. B., and Johnson, D. F. (1979) Anti-inflammatory pyrazolo-steroids: Potent glucocorticoids containing bulky A-ring substituents and no C3-carbonyl. *Biochem. Biophys. Res. Commun. 86*, 793–800.
24. Poukka, H., Karvonen, U., Janne, O. A., and Palvimo, J. J. (2000) Covalent modification of the androgen receptor by small ubiquitin-like modifier 1 (SUMO-1). *Proc. Natl. Acad. Sci. U.S.A. 97*, 14145–14150.
25. Rogatsky, I., Wang, J. C., Derynck, M. K., Nonaka, D. F., Khodabakhsh, D. B., Haqq, C. M., Darimont, B. D., Garabedian, M. J., and Yamamoto, K. R. (2003) Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. *Proc. Natl. Acad. Sci. U.S.A. 100*, 13845–13850.
26. Motoki, K., Megahed, M., LaForgia, S., and Uitto, J. (1997) Cloning and chromosomal mapping of mouse ladinin, a novel basement membrane zone component. *Genomics 39*, 323–330.
27. Luo, M., and Simons, S. S., Jr. (2009) Modulation of glucocorticoid receptor induction properties by cofactors in peripheral blood mononuclear cells. *Hum. Immunol. 70*, 785–789.
28. Clarkson, M. W., Gilmore, S. A., Edgell, M. H., and Lee, A. L. (2006) Dynamic coupling and allosteric behavior in a nonallosteric protein. *Biochemistry 45*, 7693–7699.
29. Leatherwood, J., and Futcher, B. (2010) King of the castle: Competition between repressors and activators on the Mcm1 platform. *Mol. Cell 38*, 1–2.
30. Kumar, R., and Thompson, E. B. (2003) Transactivation functions of the N-terminal domains of nuclear hormone receptors: Protein folding and coactivator interactions. *Mol. Endocrinol. 17*, 1–10.
31. Meijsing, S. H., Pufall, M. A., So, A. Y., Bates, D. L., Chen, L., and Yamamoto, K. R. (2009) DNA binding site sequence directs

glucocorticoid receptor structure and activity. *Science 324*, 407–410.

32. Biggadike, K., Bledsoe, R. K., Coe, D. M., Cooper, T. W., House, D., Iannone, M. A., Macdonald, S. J., Madauss, K. P., McLay, I. M., Shipley, T. J., Taylor, S. J., Tran, T. B., Uings, I. J., Weller, V., and Williams, S. P. (2009) Design and X-ray crystal structures of high-potency nonsteroidal glucocorticoid agonists exploiting a novel binding site on the receptor. *Proc. Natl. Acad. Sci. U.S.A. 106*, 18114–18119.
33. Hill, K. K., Roemer, S. C., Jones, D. N., Churchill, M. E., and Edwards, D. P. (2009) A progesterone receptor co-activator (JDP2) mediates activity through interaction with residues in the carboxyl-terminal extension of the DNA binding domain. *J. Biol. Chem. 284*, 24415–24424.