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Quantity of partial agonist activity for antiglucocorticoids complexed with mutant glucocorticoid receptors is constant in two different transactivation assays but not predictable from steroid structure

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Abstract

An unsolved question in steroid hormone action is why the amount of agonist activity displayed by antisteroids is not constant but varies with the assay conditions. Receptor mutations have provided insight into hormone action, presumably due to changes in the tertiary structure of the receptor that alter its interaction surfaces with the transcriptional machinery or/and co-factors. We have now employed two mechanistically different induction assays to determine whether disparate transactivation processes are similarly altered by receptor mutations. The two activation assays studied were (i) the standard induction of GREtkLUC in transiently transfected CV-1 cells and (ii) a novel modulation of endogenous receptor activity by transiently transfected receptors in HeLa cells. Five different mutations in the ligand binding and DNA binding domains of the rat glucocorticoid receptor (CS1, CS1/CD, 451/9, C656G, and R732Q) and seven steroids of varied structures (five antagonists and two agonists) were selected for use. The results in both induction assays were the same. However, no generalizations regarding steroid structure and activity emerged. Neither of two potent glucocorticoids were active with GR-CS1, or GR-CS1/CD, while RU 486 was the only antisteroid with appreciable agonist activity. With the GR-451/9 mutant, three antagonists afforded partial agonist activity. We confirmed that the C656G mutant is both "super-sensitive" and "super-selective" for transactivation. In contrast, the R732Q mutation caused significant decreases in activity with both antagonists and subsaturating concentrations of agonists. This inability to generalize about the behavior of any class of steroids with mutant receptors may reflect an induced fit for each receptor–steroid complex. Nevertheless, the activity of a given steroid appeared to be constant in two different transactivation assays for a given mutant receptor. Thus, disparate transactivation processes may utilize identical receptor surfaces, even in the expression of partial agonist activity for specific antiglucocorticoids. Published by Elsevier Science Ltd.

Keywords: Glucocorticoid receptors; Point mutations; Antiglucocorticoids; Activation assays

1. Introduction

The general model of steroid hormone action involves steroid binding to the cognate receptor followed by increased receptor binding to the hormone response element (HRE) of responsive genes. This steroid–receptor–DNA complex then regulates the transcription of the selected gene, presumably via interactions with the transcriptional machinery and associated co-factors. This model predicts both that steroid binding to receptors is the rate limiting step and that the activity for a given steroid is independent of the gene be regulated. However, this model has been

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unable to explain two common observations for antisteroids. The first is that virtually every steroid hormone antagonist can display some agonist activity under selected conditions. The second is that the amount of agonist activity displayed by various antisteroids is not constant but rather can vary with the promoter [1–4], the composition [4,5] or spacing [6] of the response element, the gene [4,7–9], the cell [1,3,4], cell density [10], cis-acting elements [11], and agents such as Br-cAMP [12–14] and dopamine [15]. For example, the endogenous glucocorticoid receptors (GRs) in Fu5-5 cells bound by the irreversible antiglucocorticoid dexamethasone 21-mesylate (Dex–Mes) [16] displayed, in the same cells, significantly more agonist activity for the induction of the tyrosine aminotransferase (TAT) gene than (i) for another endogenous gene (glutamine synthetase) [17], (ii) for stably transfected MMTV [8], or (iii) for transiently transfected glucocorticoid-responsive TAT–CAT reporters lacking a 21-bp element of the TAT gene [11,18–20]. Similarly, the activity in CV-1 cells of the antiglucocorticoid RU 486 with transiently transfected chimeric human GRs containing the VP16 activation domain at their N-terminus depended on the nature of the co-transfected receptor gene [4]. Finally, the amount of agonist activity seen with several antiglucocorticoids, including RU 486, was found to increase in proportion to the total amount of receptor present in the cell [21]. Thus, parameters other than those in the general model are clearly involved in the expression of receptor–steroid complex activity.

Efforts to unravel the underlying molecular mechanism for the expression of agonist vs. antagonist activity for a given receptor–steroid complex have been greatly advanced by the use of mutant receptors. While the results from receptor mutations are sometimes inextricably entwined with effects on protein folding of the mature protein [22], they are often capable of yielding valuable information concerning the role of specific residues or regions of the receptor. An early discovery was that the DNA binding activity of receptors could readily be separated from the transactivation activities in the amino and carboxyl portions of receptors and that the steroid binding activity of receptors is located in the carboxyl terminal region of receptors, which is usually called the ligand binding domain (LBD). Subsequently, it was shown that the LBD is very complex and participates in many diverse functions (reviewed in Ref. [23]). Extensive studies using partial proteolysis have revealed that ligand binding induces unique conformations at the C-terminus of receptors [23–25]. These conformational changes presumably lead to altered receptor interactions with the transcriptional machinery and/or co-factors, although it does not appear that agonist- vs. antagonist-specific changes are generally observed [26,27]. Additional studies have succeeded in dissociating glucocorticoid regulated activation from repression [28–31], thereby showing that distinct regions of the receptor protein are recruited for disparate functions.

A relatively untested hypothesis in steroid hormone action is that all instances of gene induction by a given receptor occur by the same mechanism. This is to be contrasted with numerous studies of the mechanisms for silencing and repression, which can be quite dissimilar from activation. Silencing by thyroid receptors appears to require sequences that differ from those for activation [32]. Likewise, repression by GRs can occur by several different mechanisms, most of which can be functionally separated by receptor mutations from the process of induction [29–31,33–37]. However, ligand-dependent and -independent transactivation by thyroid receptors has been reported to occur via different mechanisms, and to be selectively influenced by receptor mutations [38]. Similarly, point mutations of GRs were reported to unequally affect gene activation in mammalian vs. yeast cells [31,39]. Thus, it is not unreasonable to suspect that all inductions by glucocorticoid receptors may not utilize identical processes. In fact, we have recently described a modulation of the kinetic properties of the GRs of HeLa cells in the presence of transfected receptors that appears to involve a different induction mechanism [21]. Under these experimental conditions, the maximal activity that was induced by saturating concentrations (1 μM) of the agonist dexamethasone (Dex) did not increase with transfected receptors [21,40]. This is contrary to the usual increased transactivation seen with higher concentrations of GR. Nevertheless, the efficacy, expressed as percent of maximal induction by 1 μM Dex, both for a subsaturating concentration of Dex and for a saturating concentration of antiglucocorticoid did increase in HeLa cells with transfected receptors. More importantly, while truncated GRs have some activity in the classical induction assay of a glucocorticoid responsive gene, such as GREtkLUC, in CV-1 cells, they have little or no activity in the HeLa cell modulation assay. Also, the ability of GRs to bind to a glucocorticoid response element (GRE) is absolutely required in the classical CV-1 assay but not in the HeLa cell modulation assay [21].

The differences in the above two transactivation assays with glucocorticoid receptors thereby enabled us to examine whether changes in receptor structure could differentially affect the induction activity of a variety of steroids. Because the underlying principles governing antagonist activity are the least well understood, most of the steroids selected were antiglucocorticoids with assorted structures. The receptor mutations were concentrated in the LBD, as this is the region is known to undergo ligand-induced conformational changes [23,27] that are proposed to reposi-

tion receptor sequences for interaction with the transcriptional machinery and various co-factors [26,41]. However, one DNA binding domain mutant was also selected to probe the different requirements for GR binding to DNA in the two assays. Our studies indicated that the consequences of altering steroid and receptor structure were the same in the two dissimilar transactivation assays. Nevertheless, the activity of various glucocorticoid steroids was not interpreted uniformly by the mutant receptors. Thus, the relative activity of two antisteroids was often quite different with the assorted mutant GRs. These results argue for the involvement of the same receptor sequences of a specific receptor-steroid complex in the two transactivation assays but against a common tertiary structure of the GR protein in the expression of antiglucocorticoid steroid activity.

2. Materials and methods

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

2.1. Chemicals

Deacylcortivasol (DAC), RU 486, and ZK 98,299 were gifts from Roussel-UCLAF (Romainville, France), Dr. Etienne Baulieu (Paris, France), and Dr. David Henderson (Schering, Berlin, Germany), respectively. Dexamethasone 21-mesylate (Dex–Mes) [42,43] was prepared as previously described. CPRG (chlorophenol red β-D-galactopyranoside) was from Boehringer–Mannheim (Indianapolis, IN). The reporter construct pCMBβ-galactose was obtained from Clonetech (Palo Alto, CA). PBS (Mg^{2+} and Ca^{2+}-free) was purchased from Quality Biological (Rockville, MD), while all restriction enzymes were obtained through New England Biolabs (Beverly, MA), Boehringer Mannheim (Indianapolis, IN), or Stratagene (La Jolla, CA). All other chemicals, including progesterone (PG), deoxycorticosterone (DOC), and dexamethasone (Dex) were obtained from Sigma (St. Louis, MO).

2.2. Preparation of reporter plasmid

GREtkLUC was constructed as follows. The HindIII/BamHI fragment from the original thymidine kinase-luciferase plasmid (designated tkLUC, a gift from Keiko Ozato, NIH, Bethesda, MD), as well as the SmaI/XhoI fragment from the original GREtkCAT plasmid (originally named PRE-PBL7 [44] and a gift from Jon Ashwell, NIH, Bethesda, MD) were treated with Klenow polymerase to produce blunt ends, and then ligated. The resulting GREtkLUC construct, which was confirmed by digestion with EcoRI, contains two inverted repeats of the 23-base pair glucocorticoid response element (GRE) II in front of the thymidine kinase promoter driving the LUC gene.

2.3. Preparation of mutant receptors

pSVLGR [45] was a gift from Keith Yamamoto (UCSF, San Francisco, CA). The single mutants of the rat GR, C656G and R732Q, were constructed in a pSVL vector backbone as previously described [21,22,46]. The double GR mutant H451N/S459G (451/9) [47], the double mutant M770A/L771A (CS1), as well as a derivative mutant containing a further deletion of two residues (P780 and K781) (CS1/CD) [48], were kindly provided by Sandro Rusconi (University of Fribourg, Fribourg, Switzerland). All enzymatic manipulations for plasmid construction were performed according to the supplier's recommendations. The constructions were transformed in DH5α competent cells (Life Technologies, Gaithersburg, MD), and plasmid DNAs were extracted and purified by a Qiagen (Chatsworth, CA) procedure, using their MaxiKit. All plasmids were verified by restriction enzyme digestion, and the point mutation in R732Q was confirmed by sequencing using the Sequenase T7 DNA polymerase kit, version 2.0 (USB, Cleveland, OH).

2.4. Cell cultures and transient transfections

Monolayer cultures of either CV-1 cells (African green monkey kidney from ATCC, Rockville, MD) or HeLa cells (epithelial adenocarcinoma from human cervix; gift of Gordon Hager, NCI, NIH) were plated at a density of 2.5×10^5 cells or 5×10^5 cells/60 mm dish, respectively, in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD) and maintained in 5% CO_2 atmosphere at 37°C. Triplicate dishes (60 mm) were transfected by the calcium phosphate method [49] with either (i) 2 μg/dish of GREtkLUC reporter ±0.1 μg/dish of receptor plasmid for CV-1 cells or (ii) 0.125 μg/dish of GREtkLUC reporter ±0.2 μg/dish of receptor plasmid for HeLa cells. In both instances, the total amount of DNA was adjusted to 3 μg/dish with an unrelated DNA, pBluescript K(+) (pBSK(+)) (Stratagene, La Jolla, CA). Thirty six hours after transfection, when the cells were approximately 75% confluent, the cells were treated for 18–22 h with either vehicle (ethanol) or steroids (final ethanol concentration = 1%).

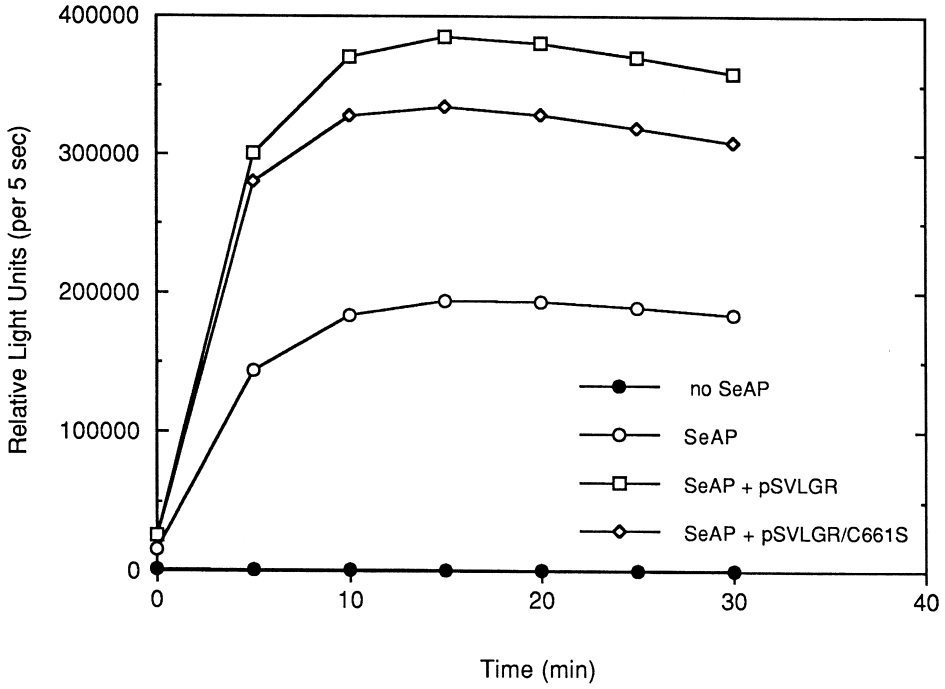

Fig. 1. Effect of composition of transfected DNA on kinetics of appearance of SeAP activity. CV-1 cells were transiently transfected with GREtkLUC±glucocorticoid receptor cDNA (pSVLGR or pSVLGR/C661S) and/or CMV-SeAP1 with the total DNA being brought to 50 μg/T150 flask with Salmon testes DNA and then split into three dishes as described in Section 2. The average value of triplicate samples for the detection of SeAP by chemiluminescence over time was plotted for the indicated samples. Similar results were obtained in five other experiments.

2.5. β-Galactosidase and secreted alkaline phosphatase (SeAP) assays for standardization of transfections

The assay for β-galactosidase was performed on a Beckman DU-8 Spectrophotometer according to Eustice et al. [50,51]. For the SeAP assays, T150 tissue culture flasks of CV-1 cells were transfected with GREtkLUC reporter (33 μg)±6.7 μg pSVL plasmid containing glucocorticoid receptor cDNA and/or 8.3 μg CMV-SeAP1 (from Tropix, Bedford, MA), with the balance of the DNA being Salmon testes DNA up to a final amount of 50 μg. The cells were split after 18 h into triplicate 60 mm dishes and incubated for 24 h, at which time SeAP activity in the cell media was detected by chemiluminescence as recommended by Tropix.

2.6. Luciferase assay

Following steroid treatment, the cells were washed twice with 1× phosphate buffered saline (PBS), detached from culture plates using sterile cell scrapers, transferred to microfuge tubes and centrifuged at 15,000 rpm for 2 min. The cell-containing pellets were resuspended in 0.25 M Tris–HCl buffer, pH 7.4 and lysed by three freeze (−80°C)–thaw cycles. After centrifugation (15,000 rpm for 15 min), the supernatant (cell lysate) was transferred to new tubes. Twenty μl of cell lysate from each tube was transferred to white opaque flat bottom 96-well plates (Corning Costar, Cambridge, MA). The LUC enzymatic activity was determined by a chemo-luminescence assay, using a MicroLumat LB96P luminometer (EG&G Berthold, Wellesley, MA) and the procedure described in the Luciferase Assay System kit (Promega, Madison, WI), which contained firefly luciferin. The luciferase reaction light signal was measured by using a 20 s delay and a 20 s measurement time. As a positive control, a solution of 5 ng (in a volume of 20 μl) of luciferase (Sigma) in 0.25 M Tris–HCl buffer, pH 7.4, with 1.0% BSA was used for the high end detection limit; 20 μl of 0.25 M Tris–HCl buffer was used as a negative control. All values were normalized for protein content in the cell lysate and expressed as "absolute LUC activity" in (RLU) (s^{-1} mg^{-1}). All reactions included cell lysates of simultaneously mock-transfected cells. Protein measurements for the cell lysates were performed using the BioRad Protein Assay (BioRad Labs, Hercules, CA) with various dilutions of 0.1% BSA as standards.

2.7. Analysis of data and statistics

The "fold induction" by various steroids was calculated as the LUC activity observed with 1 μM Dex divided by the basal activity obtained with ethanol. Thus, fold induction by ethanol was always 1. The "relative LUC activity" with various steroids was

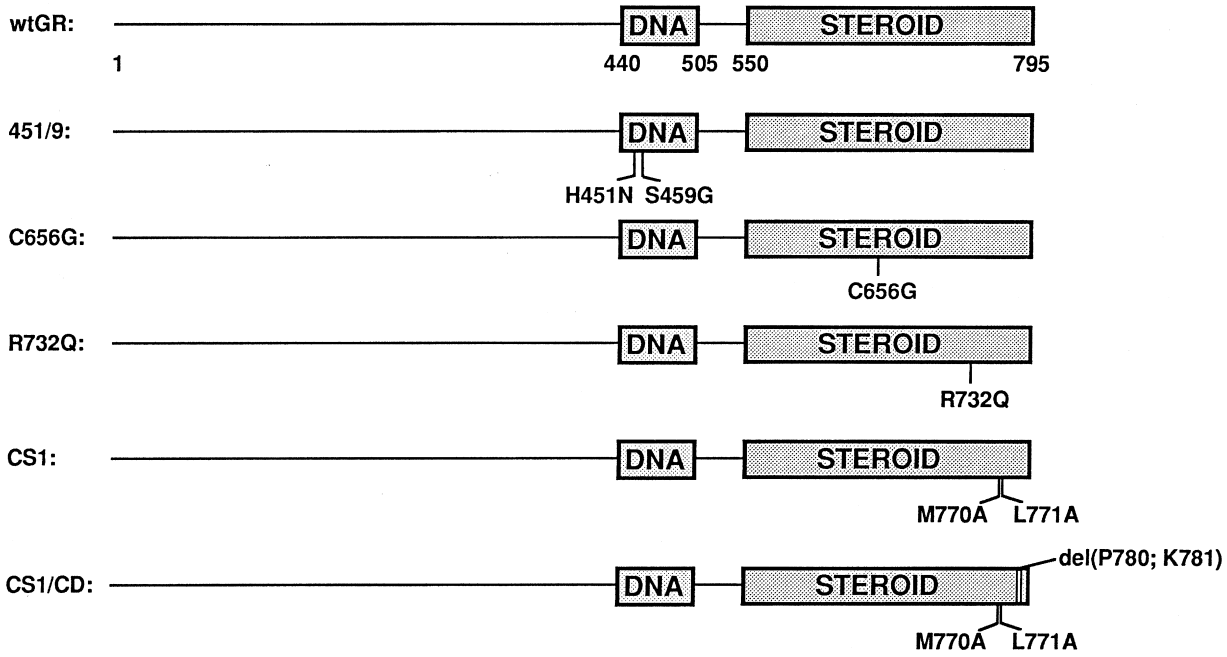

Fig. 2. Structure of wild type glucocorticoid receptor (wtGR) and mutant receptors used in this study. The numbers below the boxed regions of DNA and steroid correspond to the end points of the DNA binding [53] and steroid binding [22] domains. The position and nature of the various mutations are indicated above and/or below each mutant receptor.

expressed as a percentage of maximal activity with 1 μM Dex, except when expressing the data from cells containing receptors that do not bind Dex (and hence do not show any appreciable activity with 1 μM Dex), such as the mutants CS1 and CS1/CD. Thus, relative LUC activity for cells treated with ethanol was 0% and for cells treated with 1 μM Dex was 100%. Unless otherwise noted, all statistical analyses were performed with the unpaired two-tailed Student *t*-test using the program InStat 2.03 for Macintosh (GraphPad Software, San Diego, CA). When the standard deviations of the two populations were significantly different, the Mann–Whitney test was used. All values are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Normalization of transient transfection assays

In order to compare the fold induction of gene expression in cells that are transiently transfected with different receptors, it is helpful to have a internal control for transfection efficiency. Unfortunately, cotransfection with the often used β-galactosidase gene was not suitable as the reporter activity was found to be weakly induced by 1 μM concentrations of either Dex or Dex–Mes (induction = 1.28 ± 0.05 (SEM, $n = 28$) and 1.36 ± 0.08 (SEM, $n = 24$) for Dex and Dex–Mes respectively). The fact that the fold induction by an agonist (Dex) and an antagonist (Dex–Mes) was indistinguishable ($P = 0.53$ by two tailed Mann–Whitney test) argues that the effect was not mediated by glucocorticoid receptors. However, it did mean that we could not use the co-transfected β-galactosidase gene as an internal control. Others have also reported problems with using the β-galactosidase gene as an internal control [52].

In order to avoid possible steroid-mediated changes in an internal control, we turned to the secreted alkaline phosphatase (SeAP) assay, in which the alkaline phosphatase in the tissue culture medium is assayed after the usual 36 h of co-transfection but before the addition of steroid. This time, the total activity of the control was influenced by the precise composition of the transfected DNA, even though the total amount of DNA was constant (Fig. 1). Because we have been unable to find a control that was not influenced either by steroid or by DNA composition, we have normalized all of our transfection assays by the total protein of the assayed cells. It should be noted, however, that internal controls are less crucial in determinations of the relative activity of a given steroid. This is because the steroid's activity is expressed as percent of maximal induction by saturating concentrations of a full agonist by the same receptor in the same cells, thus eliminating the problems of inter-receptor or inter-cell comparisons.

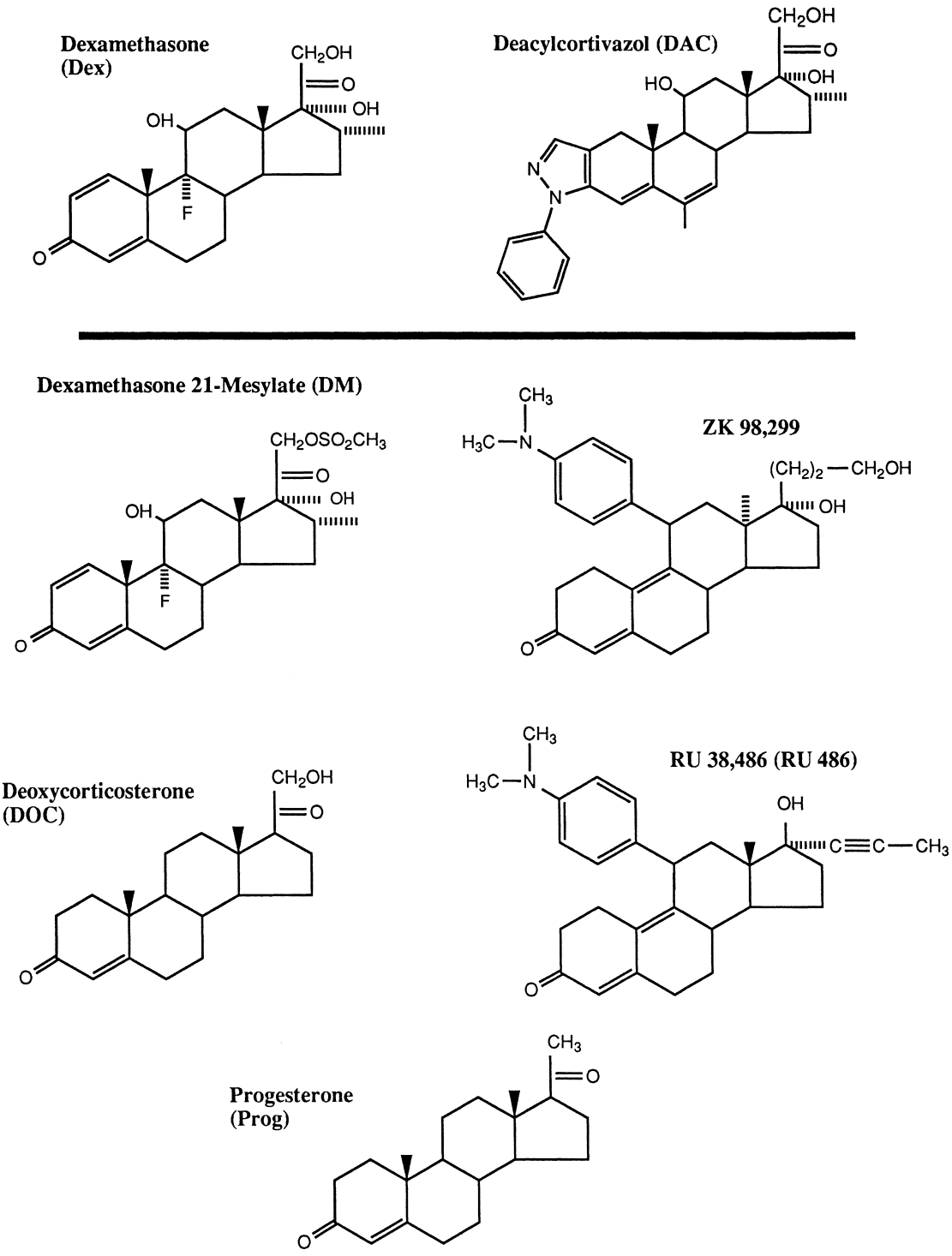

Fig. 3. Structure of different agonists (above horizontal line) and antagonists (below horizontal line) used.

3.2. Glucocorticoid induction of GREtkLUC reporter in CV-1 cells

The standard assay for GR-dependent transactivation is based on the quantification of induction by transiently transfected GR of a simple reporter construct, such as GREtkLUC. For this assay, we transfected either wild type (wt) GR or one of five GR mutants (CS1, CS1/CD, 451/9, C656G, and R732Q of Fig. 2) with assorted properties. The CS1 and CS1/CD mutants are inducible by RU 486 but inactive with Dex [48] and Dex–Mes [21]. 451/9 contains a double mutation in the GR DNA binding domain, which conferred increased transactivation (and DNA binding

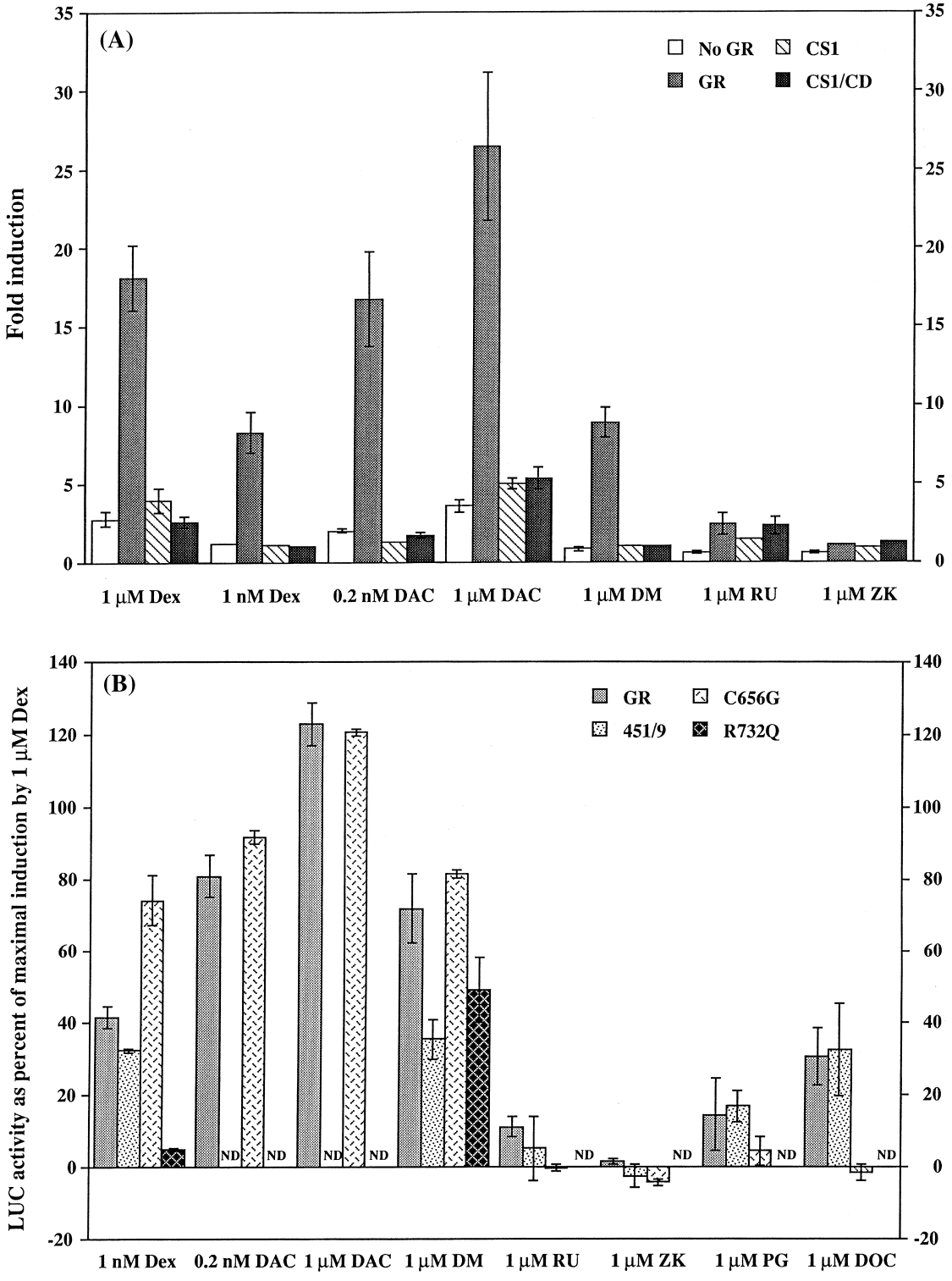

Fig. 4. Transcriptional activity of transfected receptors with various steroids in CV-1 cells. (A) Fold induction in cells ± transfected wild type, CS1, or CS1/CD GR after treatment with different steroids. Triplicate dishes were assayed as described in Section 2 and the average fold induction ± SEM for $n = 3$–9 separate experiments was plotted for the indicated steroids. (B) Relative transcriptional activity in cells ± transfected wild type, C656G, 451/9, or R732Q GR after treatment with different steroids. Triplicate dishes were assayed as in (A) and the relative activity, expressed as percent of full induction by 1 μM Dex, was determined. The average values ± SEM for $n = 3$–9 separate experiments except for PG and DOC with 451/9 and C656G, where $n = 2$, were then plotted for the indicated steroids (ND = not determined). In all cases, a lack of error bar indicates an SEM < 0.1.

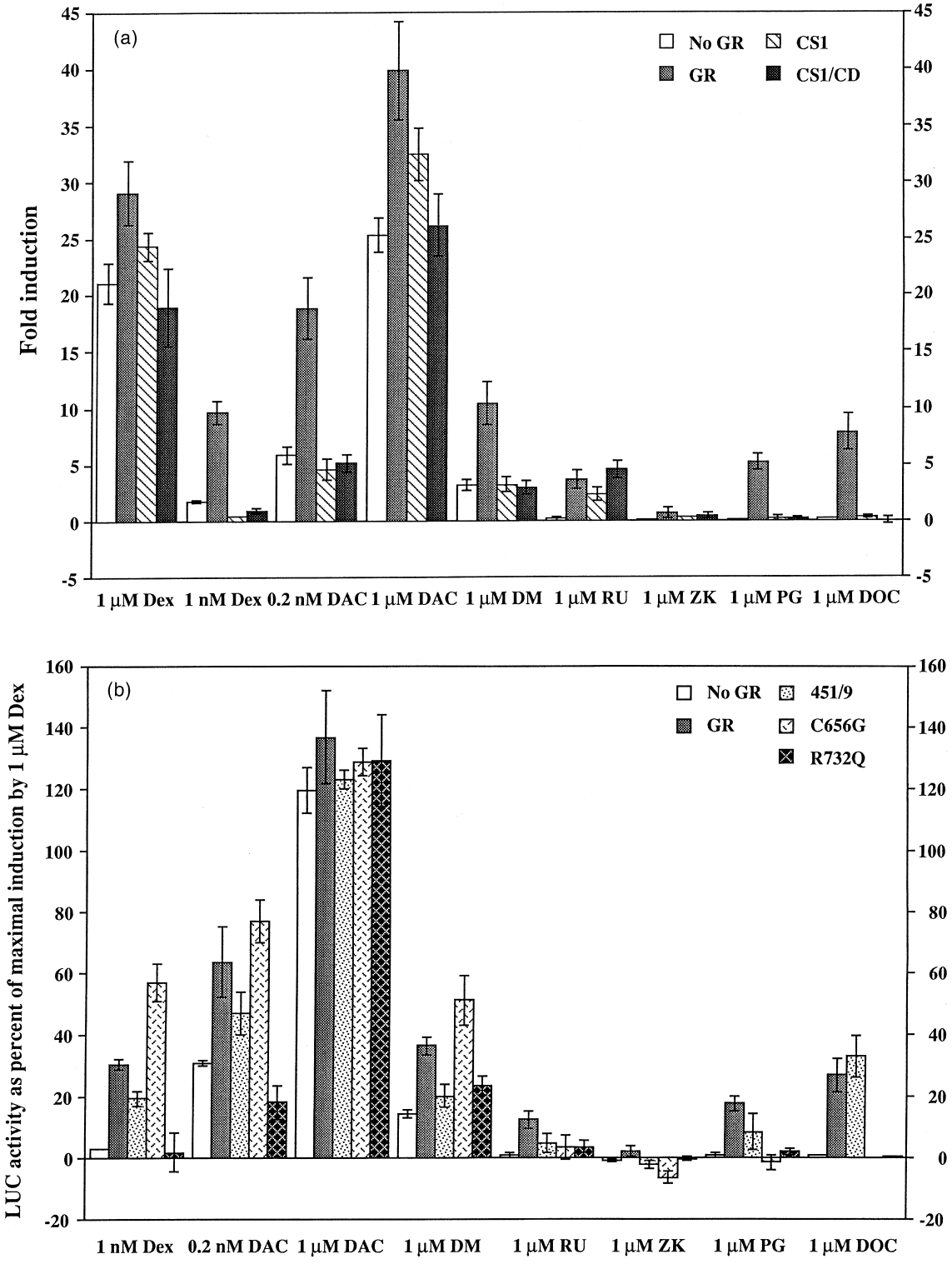

Fig. 5. Modulation of endogenous HeLa cell GR activity by transfected receptors in the presence of various steroids. (A) Fold induction of endogenous GR ± transfected wild type, CS1, or CS1/CD GR after treatment with different steroids. Triplicate dishes were assayed as described in Section 2 and the average fold induction ± SEM for $n = 3$–19 separate experiments was plotted for the indicated steroids. (B) Relative transcriptional activity of endogenous GR ± transfected wild type, C656G, 451/9, or R732Q GR after treatment with different steroids. Triplicate dishes were assayed as in (A) and the relative activity, expressed as percent of full induction by 1 μM Dex, was determined. The average values ± SEM for $n = 3$–19 separate experiments were then plotted for the indicated steroids.

affinity) in the context of the DNA binding domain fragment of 407–556 [47]. The C656G is a "super-GR" with increased sensitivity to Dex [54] while the R732Q mutant possessed decreased sensitivity to Dex [21]. The steroids used were both saturating (1 μM) and subsaturating (1 nM) concentrations of one of two agonists (Dex and DAC), as well as saturating (1 μM) concentrations of one of five antagonists (DOC, Dex–Mes, progesterone, RU 486, and ZK 98,299) with a variety of structures (Fig. 3). The first four antagonists are type I antiglucocorticoids, displaying varying amounts of partial agonist activity in classical transactivation systems. ZK 98,299 has been proposed as a "pure antiglucocorticoid", or type II antagonist, that prevents the DNA binding of receptor–steroid complexes [55], although modified gel shift conditions disclosed good DNA binding of ZK 98,299 bound progesterone receptors [56].

The endogenous GRs of CV-1 cells are insufficient for significant levels of GR-mediated induction from the transiently transfected GREtkLUC reporter gene. Co-transfection of GR led to a dramatic increase in fold induction values with saturating concentrations (1 μM) of Dex or DAC (Fig. 4A).

The activities of the two agonists, Dex and DAC, were next examined with various mutant GRs. Both steroids failed to induce transactivation in cells that were co-transfected with either the CS1 or CS1/CD mutants (Fig. 4A). This is consistent with the inability of these receptors to bind Dex, and consequently to induce transactivation [48], and suggests that DAC, like Dex, also does not bind to these receptors. This inactivity was not due to a lack of expression of the receptors as RU 486 was able to induce reporter activity (Fig. 4A and Ref. [48]). Furthermore, the CS1 and CS1/CD receptors can be covalently labeled by Dex–Mes in transiently transfected cells [48]. However, no induction of either mutant was observed for Dex–Mes (Fig. 4A). A consistently weak, but not statistically significant ($n = 3$), induction was seen for ZK 98,299 with CS1 and CS1/CD. It should be noted that these data are presented in terms of fold induction for each steroid as opposed to the more usual manner of percent of maximal induction by saturating concentrations of Dex. This was necessitated by the inactivity of Dex with the CS1 and CS1/CD mutants.

When the 451/9 mutation was tested in the context of the full length receptor, there was no augmentation in the activation by subsaturating concentrations of Dex (1 nM) compared to the wild type receptor (Fig. 4B). This is to be compared to the increased activity of the receptor fragment 407–556 containing the 451/9 mutation [48]. In contrast, the percentage of maximal activity of C656G following treatment with subsaturating concentrations of agonists was considerably greater than that for wild type receptors. These results confirmed and extended our earlier reports that C656G was a "super-sensitive" GR mutant [46,54]. As was previously observed for the R732Q mutant, low concentrations of Dex were essentially inactive (Fig. 4B), due to a "right shift" in the dose–response curve [21].

Treatment of the 451/9 mutant with Dex–Mes, progesterone, and DOC, but not RU 486 or ZK 98,299, resulted in partial agonist activity. Interestingly, Dex–Mes was the only antagonist to give a response with the 451/9 mutant that was significantly different from that of the wild type receptor (Fig. 4B). Conversely, the only antagonist that had the same activity with wild type and C656G receptors was Dex–Mes. RU 486, progesterone, and DOC displayed significant amounts of agonist activity with the wild type receptor but none with the C656G (Fig. 4B). Dex–Mes also retained significant levels of agonist activity when bound to R732Q (Fig. 4B).

These results show that, with the exception of ZK 98,299, there is no consistent behavior of all of the antagonists between individual mutant GRs. Thus, the various mutant receptor–steroid complexes present a variety of transcriptionally active complexes with which to probe the process of gene activation.

3.3. Modulation of endogenous GR transactivation activity by transiently transfected GR

Our second transactivation assay, with transiently transfected GRs in HeLa cells, is significantly different from the above described transactivation of a reporter gene in transiently transfected CV-1 cells. For example, the binding of transfected GR to the GRE was not necessary to produce a response in the HeLa cell assay but is absolutely required in the assay of Fig. 4 with CV-1 cells [21]. This, along with the other properties discussed in Section 1, differentiate our HeLa cell induction assay from the conventional induction assays, such as the above assay for GREtkLUC activation in CV-1 cells.

As we reported earlier [21], transfection of concentrations of GR expression plasmid sufficient to cause an approximately 7-fold increase in the induction of co-transfected GREtkLUC reporter in CV-1 cells (Fig. 4A) generated less than a 50% increase in fold induction of the same reporter in HeLa cells (Fig. 5A; fold induction with 1 μM Dex after transfection of GR in HeLa cells = 25.2 ± 1.9 (SEM, $n = 36$) vs. 20.6 ± 1.4 (SEM, $n = 36$) in untransfected HeLa cells, values non-significantly different ($P = 0.054$)). Nonetheless, co-transfected GR produced a significant increase in the percent of maximal activity seen with subsaturating concentrations of Dex or DAC. At the same time, transfected GR caused a dramatic increase in the percent agonist activity displayed by antiglucocorticoids. Even the response to ZK 98,299 was increased,

although the absolute increase was small. This response to transfected GR was relatively specific, in that transfected progesterone receptors were without effect [21].

Co-transfection of HeLa cells with the CS1 or CS1/CD mutants did not afford any significant increase in the fold induction by 1 μM Dex or DAC, in contrast to the increases seen with wild type GR (Fig. 5A). Transfected 451/9 and C656G mutant receptors each led to increased activity with subsaturating concentrations of both agonists (Fig. 5B). Conversely, the R732Q mutant bound by subsaturating concentrations of Dex or DAC displayed no effect.

All antagonists tested with transfected CS1 or CS1/CD, with the exception of RU 486, failed to produce an increase in the percent agonist activity in HeLa cells. Treatment of the 451/9 mutant only with progesterone, or DOC, led to any appreciable increase in the percent agonist activity. The augmentation seen with Dex–Mes in the presence of 451/9 was reproducible but not statistically significant ($P = 0.15$, $n = 3$). In contrast, Dex–Mes was the only antagonist able to afford increased agonist activity with C656G (Fig. 5B). Treatment of R732Q transfected cells with Dex–Mes and RU 486 led to a weak, but not statistically significant, increase in the percent agonist activity ($P = 0.1$ or 0.07 respectively, $n = 4$).

3.4. Comparison of activities of mutant receptors in the two transactivation assays

The activity of each steroid with the various GRs was virtually identical in the two different transactivation assays. For the CS1 and CS1/CD receptors, the only steroid to show any significant activity was RU 486. Consistently higher values than background were seen with ZK 98,299 but the level was much less than that with RU 486. To the extent that one can generalize from a limited number of steroids, it appears that CS1 and CS1/CD are inactive with agonists and display activity with only one antagonist, RU 486. What is unusual about RU 486 is presently unknown. Interestingly, the absolute level of RU 486 activity with CS1 or CS1/CD was equivalent to that seen with wild type GR in both assays (Fig. 4A vs. Fig. 5A). Thus, we conclude that the CS1 and CS1/CD mutations do not convert the wild type receptor to one that is now activated by RU 486 but rather that these mutations eliminate the ability of GR to respond to any other steroid that we have tested.

The activity of the 451/9 mutant with agonists was identical to that of the wild type receptor (Fig. 4B vs. Fig. 5B). The activity of the five antagonists with the 451/9 mutant was generally less than that with the wild type receptor except for progesterone and DOC, which displayed about the same activity with both receptors. Likewise the C656G receptor, which has many properties that diverge from those of the wild type receptor, responded similarly in the two assays. C656G was more active than wild type receptor with both agonists and with Dex–Mes but had little or no activity with the four other antiglucocorticoids (Fig. 4B vs. Fig. 5B). This inactivity with at least progesterone was not due to an inability to bind progesterone, although the selectivity was certainly higher with the C656G [54]. The R732Q receptor had reduced activity with subsaturating concentrations of agonists, apparently due to a decreased binding affinity [21], and with saturating concentrations of Dex–Mes. We have not tested the other antisteroids with R732Q in CV-1 cells but predict that they will be inactive, just as they were in the transactivation assay with HeLa cells.

In both activation assays, no generalizations could be made with regard to a relationship between steroid structure and biological activity for either the wild type or mutant GRs. However, the activity of each steroid with the various receptors, relative to full induction by 1 μM Dex, was the same in the two activation assays. Thus, while there may be multiple mechanisms for repression [29–31,33,34,36], we could not detect any differences in our two transactivation assays. Therefore, it may be that the possibly diverse mechanisms of activation all require similar conformations/structures of the GR receptor–steroid complex.

4. Discussion

This study shows that the expression of agonist activity either by two glucocorticoids or by five antiglucocorticoids, all of dissimilar structure, can vary between wild type and mutant receptors but remains the same among two dissimilar transactivation assays in mammalian cells. These results argue that those features of receptor–steroid complex tertiary structure that are required for the induction of biological activity are the same in our two assays. Whether this constancy will be maintained among other, yet to be discovered, transactivation assays is an intriguing prospect that must await the characterization of such future activation systems. To the best of our knowledge, our study is the first to examine the behavior of different steroids with glucocorticoid receptors in two different transactivation assays in mammalian cells.

The identical activity of each receptor in HeLa cells, with endogenous glucocorticoid receptors, and CV-1 cells, with almost no receptors, argues against any effects being due to heterodimerization of receptors. Furthermore, our conclusions should not be affected by possible differences in receptor concentration in HeLa vs. CV-1 cells. We have previously found that

the absolute activity of a given steroid, as a percent of maximal agonist activity, does depend upon the absolute level of receptors. However, the relative activity of any two steroids moved up, or down, together with varying amounts of receptor and thus was independent of receptor concentration [21].

Our observations of equal activity in two mammalian cell transactivation assays are not to be confused with the unequal activity of some steroid–glucocorticoid receptor complexes in yeast vs. mammalian cells. In some cases, the differences could reflect modifications of the receptor protein. Thus, the activity of Dex in CV-1 cells (Fig. 4B) but not in yeast [57] appears to be due to the $\approx$1000 fold lower affinity of Dex for cell free glucocorticoid receptors from yeast vs. rat HTC cells [57]. Furthermore, transactivation differences between yeast and mammalian cells have also been seen in the absence of steroid binding. For example, the constitutive activities of three individual mutants (R488Q, R489K, and N491S) in the context of the truncated rat GR (amino acids 1–556) were very dissimilar in yeast vs. mammalian cells [28]. Similarly, other C-terminal truncated receptors (amino acids 1–525) containing multiple mutations displayed different properties in yeast vs. mammalian cells [31]. Most likely, these inequalities stem from differences in the transcriptional machinery and/or abundance of co-activators, as suggested by the ability of GRIP1 to augment GR action in yeast but to have no effect [58] or squelch transactivation [59] in mammalian cells. Therefore, it is difficult to separate the discrepancies in transactivation in yeast vs. mammalian cells from interesting variations in the basic transcriptional machinery. In contrast, our two assays, both in mammalian cell lines, offer a way to probe for possible different interactions of a given receptor steroid complex with the same transcriptional components in disparate activation systems.

Our data also demonstrate that one is unable to predict a relationship between steroid structure and activity among wild type or mutant GRs in either of our mammalian transactivation assays. Conversely, the effect of a given receptor mutation was not interpreted uniformly, but rather varied among the various antiglucocorticoids. Several reports have appeared where limited modifications in receptor sequence were capable of converting an antagonist to an agonist [48,60,61]. However, as shown in the present study, it may not be possible to generalize from the behavior of one antisteroid to that of all antisteroids. The C656G mutant displays good activity with Dex–Mes but not RU 486, progesterone, or DOC (Fig. 4B and Fig. 5B). Even more dramatic is that only RU 486, and maybe ZK 98,299, displayed any agonist activity with the CS1 and CS1/CD mutant receptors (Fig. 4A and Fig. 5A). This suggests that subtle modifications in receptor structure are involved in the expression of agonist vs. antagonist activity, as might be expected for an induced fit following the binding of structurally diverse steroids to receptors [62,63].

It should also be noted that the amount of transcriptional activity induced by RU 486 (and ZK 98,299) with either CS1 or CS1/CD was very similar to that observed in RU 486-treated cells transfected with wtGR. Thus, it seems more reasonable to propose that, in this situation, the CS1 and CS1/CD mutations did not "convert" an antisteroid to an agonists but rather eliminated the ability of other steroids to cause any activation. The low level of activity of ZK 98,299, which had been previously noted [64], also suggests that ZK 98,299 should not be considered as a type II antagonist giving complexes incapable of binding to GRE sequences. This conclusion is consistent with the recent observation that ZK 98,299 bound progesterone receptors can bind to DNA [56].

It is interesting that the previously described increased biological activity of the 451/9 double mutant in the context of the truncated GR (amino acids 407–556) [47] was not maintained in the full length receptor in either transactivation assay (Fig. 4B and Fig. 5B) or in its ability to increase the fold induction (fold-induction with 1 μM Dex after transfection of 0.1 μg of pSVLGR into CV-1 cells = 12.27 ± 3.01-fold vs. 12.59 ± 5.69-fold with GR 451/9 ($\pm$ SD, $n = 3$); $P = 0.94$). This was particularly relevant in the CV-1 cell assay of Fig. 4, where DNA binding is required for biological activity. On the other hand, one might not have expected mutations at positions 451 and 459, which are involved in DNA backbone contacts and the specificity of DNA-binding [65], to effect biological activity in view of the generally poor correlation between the affinity of DNA binding and the magnitude of the transcriptional activation response [66]. For this reason, it was somewhat surprising that this mutation did modify the amount of activity displayed by both 1 nM Dex and 1 μM Dex–Mes in each assay (Fig. 4B and Fig. 5B). Thus, there may be some communication between the GR DNA binding domain and the transactivation domains at either ends of the receptor. A similar ability of the DNA binding domain to alter the activity of the full length receptor has been described by Yamamoto et al. [39].

The C656G "super-GR" displayed increased induction with Dex and DAC, but was inactive with most antagonists. These results support our previous findings of a decreased relative affinity of the C656G GR for progesterone and aldosterone [54]. The Cys-656 residue lies within the steroid-binding cavity of GR but appears to be closest to the C-17 side chain of the steroidal D-ring [46,67]. Therefore, a molecular explanation for the increased specificity of the C656G mutant for modifications in other regions of the ster-

oid structure is not yet clear but may involve ligand binding induced, but currently unpredictable, reorganizations of the ligand binding domain [62,63].

The R732Q mutant possessed decreased induction with subsaturating concentrations of both agonists (Dex and DAC) and was inactive with most antagonists. The Arg-732 (R732) residue is highly conserved in the nuclear receptor family and represents the first amino acid of helix 10 of the proposed structure of the GR ligand binding domain [41]. Although helix 10 is thought not to participate directly in ligand binding or transactivation, secondary effects on protein structure are always possible, as have been observed for a point mutation within the glucocorticoid receptor DNA binding domain [68,69]. Indeed, it has been previously demonstrated that the point mutant R732Q shows approximately the same amount of transcriptional activity with wtGR in the presence of Dex–Mes, but a right-shifted dose–response curve for Dex induction in CV-1 cells [21]. Hence, R732Q has now been further characterized, and indeed meets the criteria of a ''right shifted'' receptor, in direct antithesis with the ''left shifted'' C656G mutant.

In summary, the present studies show that no simple generalizations are possible about receptor–steroid complex activity. While the properties of a given complex were the same in two transactivation assays, the various transcriptional responses with different steroids suggest that a continuum of tertiary structures, perhaps due to induced fits of the receptor ligand binding domain, are obtained in response to the binding of the assorted steroid structures. Further variety of responses is possible if the ability of receptor to interact with the ever increasing number of co-activators and co-adaptors [70] is also sensitive to the continuum of steroid-induced tertiary structures. It will be interesting to see whether these predictions can be experimentally confirmed.

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