Transactivation Specificity of Glucocorticoid vs. Progesterone Receptors: Role of Functionally Different Interactions of Transcription Factors with Amino- and Carboxyl-terminal Receptor Domains

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Running title: Transactivation specificity among steroid receptors
Abstract

A major unanswered question of glucocorticoid and progesterone action is how different whole cell responses arise when both of the cognate receptors can bind to, and activate, the same hormone response elements (HREs). We have previously documented that the EC$_{50}$ of agonist complexes, and the partial agonist activity of antagonist complexes, of both glucocorticoid receptors (GRs) and progesterone receptors (PRs) are modulated by increased amounts of homologous receptor and of coregulators. We now ask whether these components can differentially alter GR and PR transcriptional properties. To remove possible cell-specific differences, we have examined both receptors in the common environment of a line of mouse mammary adenocarcinoma (1470.2) cells. In order to segregate the responses that might be due to unequal nucleosome reorganization by the two receptors from those reflecting interactions with other components, we chose a transiently transfected reporter containing a simple glucocorticoid response element, or GRE (i.e., GREtkLUC). No significant differences are found with elevated levels of either receptor. However, major, qualitative differences are seen with the corepressors SMRT and NCoR, which afford opposite responses with GR and PR. Studies with chimeric GR/PR receptors indicate that no one segment of PR or GR is responsible for these properties and that the composite response likely involves interactions with both the N- and C-termini of receptors. Collectively, the data suggest that GR and PR induction of responsive genes in a given cell can be differentially controlled, in part, by unequal interactions of multiple receptor domains with assorted nuclear cofactors.
Introduction

Among the longstanding conundrums of steroid hormone action is why different whole cell responses are observed for androgen, glucocorticoid, mineralocorticoid, and progestin steroid hormones (1) even though each receptor-steroid complex can bind to the same DNA sequences to induce gene transcription (2, 3). Steroid hormone regulated gene transactivation requires ligand binding to the cognate intracellular receptor. After binding to biologically active DNA sequences, called hormone response elements (HREs), the activated complexes are thought to recruit transcriptional coregulators and components of the transcriptional complex prior to modifying the transcription rates of target genes.

Different ligands bind to the various steroid receptors with different affinities. However, this specificity for the different steroid hormones would seem to soon disappear as the activated form of each complex of the above four steroid receptors can bind to, and activate transcription from, the same HREs.

The actions of glucocorticoid receptors (GRs) and progesterone receptors (PRs) have been extensively studied in an effort to understand how biological diversity can be maintained when the activated receptor complexes act on a common HRE. Several explanations have been proposed, including different levels of the receptor within a given cell (4). HRE mutations have also been reported to differentially affect GR vs. PR transactivation (5), although the differences seem to be less pronounced when receptor concentrations are about equal (6). Nelson et al. found that the flanking and spacer DNA of the palindromic HRE can contribute to the affinity and specificity of receptor binding (7). Similarly, DNA binding specificity has been implicated in the androgen receptor (AR) vs. GR specific induction of the probasin gene (8) and in GR vs. AR activation of the aspartate aminotransferase gene (9). Thus, subtle differences in HRE sequence may regulate the relative activities of GR and PR.

Chromatin architecture has recently emerged as another promising modifier for receptor-regulated expression of some, but not all (10), genes. Chromatin structure can repress gene expression (11, 12), thereby increasing the fold induction by receptor-steroid
complexes, especially in cell-free systems (13, 14). Chromatin environment can control gene inducibility by PR (15-17) and chromatin structure has been proposed to be a determinant for PR induction (10, 15). However, alterations in chromatin structure do not appear to be a prerequisite for all steroid receptor-induced gene transactivation. In several cases, chromatin reorganization appears to precede the actions of receptor-steroid complexes in inducing gene expression (14, 18-20) while in other cases, chromatin disruption or remodeling is not sufficient for transactivation, which requires a second step (21). In T47D cells lacking both PR and GR, or expressing only GR, the responsive B nucleosome of the MMTV enhancer is in a constitutively “open” state, indicating that GR transcriptional activation is independent of chromatin remodeling (20).

The low homology of the amino terminal halves of GR and PR (<15%) has been advanced as an additional possible cause for selective gene expression (22). One mechanism by which this could be achieved is through differential interactions with the recently discovered transcriptional coregulators such as coactivators and corepressors. While the initially determined interactions of these coregulators were with the ligand binding domain (LBD) of receptors (23-25), several recent reports describe interactions with the amino terminal domain of receptors (26-31). Unfortunately, receptor-specific interactions with coregulators appear limited. ARA70 (((32) vs. (33, 34)) and FHL2 (35) have been described to be specific coactivators for ARs while a 68K protein (p68) appears to be a coactivator for estrogen receptor (ER) α but not for ERβ, AR, mineralocorticoid receptors, or retinoic acid receptor α (36). REA selectively interacts with estrogen receptors to decrease both the activity of agonists and the concentration of antiestrogens required for half-maximal inhibition of estrogens (37). NRIF3 is a ligand-dependent specific coactivator that binds (and augments transactivation of) TR and RXR but not RAR or VDR or steroid receptors (38).

More evidence exists for differential effects of coactivators and corepressors on GR vs. PR activities. The nuclear orphan receptor ERRS is described to be a repressor of GR transcriptional activity but does not affect PR activity (39). Conversely, PIAS1 enhances
GR transactivation but represses PR transactivation in the same system (40). A 130K auxiliary protein increases the DNA binding of full length GR but not truncated PRs (41). Similar quantitative differences have been noted for other steroid receptors. SRC-1 has a greater effect on ER than AR action in the brain of developing rats (42), which may result from SRC-1 interacting with the ER LBD via the LxxLL motifs of SRC-1 but with the amino terminal region of AR in a manner that does not depend on the SRC-1 LxxLL motifs (34). SRC-1e interacts with the fragments containing the DNA binding domain and LBD of ER but not of AR (28). RIP140 represses GR (43) but increases AR (44) transactivation. Whether Zac1 augments or represses the activity of GRIP1 with AR vs. ER depends on the target gene and cell (45).

Recently we have found that varying concentrations of the homologous receptor, coactivators, and corepressors can alter the EC$_{50}$ of agonist complexes, and the partial agonist activity of antagonist complexes, for both GRs (46-48) and PRs (49). Preliminary evidence suggested that the responses of GR and PR to these factors might be different (47-49). The purpose of this study was, therefore, to examine whether the quantitative activities of those factors known to alter PR and GR transactivation properties are the same or different for PR and GR. To answer this question, we have performed multiple dose-response curves to determine the EC$_{50}$ of agonists and the partial agonist activity of antagonists. It was important to conduct these assays in the same cells so that cell-specific contributions to transcriptional activities could be eliminated. Similarly, we needed to use the same reporter so that effects of chromatin organization would be minimized. In the context of such an assay system, we found two instances in which the induction properties of PR and GR were qualitatively different, with almost opposite effects being produced by the same added component. Further studies with PR/GR chimeras indicated that no one segment of PR or GR was responsible for these differences. Collectively, the data suggest that the differences between GR and PR induction in a selected cell can be controlled, in part, by unequal responses from the combination of amino- and carboxyl-terminal domains of each receptor to assorted nuclear components.
Materials and Methods

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

*Chemicals, buffers, and plasmids:* [³H]dexamethasone (Dex, 91 Ci/mmol) was obtained from NEN (Boston, MA) and non-radioactive Dex from Sigma (St. Louis, MO). Dex-Ox (50) and Dex-Mes (51) were prepared as described. Restriction enzymes and digestions were performed according to the manufacturer’s specifications (New England Biolabs, Beverly, MA).

The Renilla null luciferase reporter was purchased from Promega (Madison, WI). GREtkLUC (52) has been previously described. The cDNA plasmids of GR (pSVLGR from Keith Yamamoto, UCSF, San Francisco, CA), MMTVLuc (pLTRLuc; Gordon Hager, NIH, Bethesda, MD), TIF2 and the B form of human progesterone receptor (hPR-B) (Hinrich Gronemeyer, IGBMC, Strasbourg, France), NCoR (Michael Rosenfeld, University of California, San Diego), and s-SMRT (53) (Ron Evans, Salk Institute, La Jolla, CA) were each received as gifts.

Construction of chimeras: The cDNA encoding GR or PR was recombined through a compatible site (NspI/SphI) that coincides with the position of cysteine 495 of the rat GR. No amino acids were added or subtracted or changed at this junction. Both expression vectors for the chimeric receptors start with the unrelated sequence ASGSWP, which is due to the tk AUG followed by a BamH1 linker. The PR/GR chimera bears a PR, which is lacking the first 24 amino acids of PR. This deletion has no observable influence on the transactivation capacity of the PR (Barbara Huse, unpublished). The GR/PR chimera starts with a rat GR that misses 3 amino acids of the amino terminus. Various experiments since this construct was first employed (54) have confirmed that there is no substantial difference between this GR and the wild type GR (S. Rusconi, unpublished).

*Cell culture and transfection:* Monolayer cultures of COS-7 and 1470.2 cells were grown at 37°C with 5% CO₂ in Dulbecco’s modified Eagles medium (DMEM, GibcoBRL
Life Technologies, Inc., and DMEM with 4.5g glucose/L, Quality Biologicals, Inc., respectively) supplemented with 5% and 10% of fetal bovine serum (FBS), respectively. We had previously used charcoal-stripped serum with 1470.2 cells to prevent any PR-mediated induction by endogenous progestins (49). However, we have confirmed the observations of others (55) that this is not necessary (data not shown). Therefore, charcoal-stripped serum was no longer used with 1470.2 cells. CV-1 cells were grown as described (46). Coregulator plasmids were transiently co-transfected into 1470.2 cells using Lipofectamine (Life Technologies, Inc.) with hPR-B receptor-containing plasmid, 1µg of GREtkLUC, and 50ng Renilla null luciferase, with the total transfected DNA brought up to 3 µg/60 mm dish with pBSK+ DNA (56). In experiments with varying amounts of receptor or coregulator cDNA plasmids, equimolar amounts of the same plasmid vector were cotransfected to control for artifacts of the vector DNA. The cells were treated for 24 hr with 1% ethanol ± steroids in media containing 10% FBS and harvested in 1x Passive Lysis Buffer (0.5 ml / dish, Promega). Fifty µl of the cell lysates were used to assay for luciferase activity using the Dual-Luciferase Assay System from Promega (Madison, WI) according to the supplier. The data were then normalized for the cotransfected Renilla activity.

Steroid Binding Assays. Transient transfection of COS-7 cells with 1 µg/10 cm plate of GR or PR/GR plasmid DNA and 19 µg of single stranded DNA was performed as described (57). Cytosols of transfected cells containing the steroid-free receptors were obtained by the lysis of cells on dry ice and centrifugation at 15,000 xg (58). Thirty percent cytosol with 20mM sodium molybdate was added to varying concentrations of [3H]Dex ± 100 fold excess of non-radioactive Dex and incubated at 0°C for 18 h. Unbound [3H]Dex was removed by dextran-coated charcoal.

Whole cell steroid binding was performed by incubating suspensions of cells (1.5-2 x 10^6) with increasing concentrations of [3H]Dex (1.5 to 50 nM) in 200 µl of serum-free medium in the presence or absence of a 100-fold molar excess of unlabeled Dex (each with 1.2% ethanol) for 30 to 45 min at 37°C. The binding was terminated by the addition of 2 ml of PBS, followed by centrifugation for 15 seconds, all at room temperature. Cells were
washed for additional three times with PBS at room temperature. In both cases, the total binding was determined by liquid scintillation counting. The specific binding was calculated by subtracting the background dpms (100-fold Dex) from the total [3H]Dex binding. The binding capacity and affinity were determined by Scatchard plot analysis by plotting the ratio of bound steroid/free steroid vs. bound steroid.

**Analysis of data:** The activity for subsaturating concentrations of agonist, or saturating concentrations of antagonist, was expressed as percent of maximal activity with saturating concentrations of agonist (30 nM R5020 or 1 µM Dex unless otherwise noted). The fold induction was calculated as the luciferase activity (relative firefly light units/relative Renilla light units) with 30 nM R5020, or 1 µM Dex, divided by the basal activity obtained with ethanol. Individual values were generally within ±20% of the average, which was plotted.

The dose-response curves were constructed from the theoretical sigmoidal curve for the binding isotherm, which is described by the equation of \( y = \frac{x}{x+k} \), where \( y \) = the fractional response, \( x \) = the concentration of free steroid, and \( k \) = an arbitrary value for the binding affinity of steroid to receptor. This theoretical curve was then aligned with the experimental data so as to give the best visual fit.

Unless otherwise noted, all statistical analyses were by two-tailed Student’s t test using the program “InStat 2.03” for Macintosh (GraphPad Software, San Diego, CA).

**Results**

**Selection of assay system:** A bioassay with transiently transfected receptors and reporters to analyze possible differences in the biological properties of PR and GR was chosen for several reasons. First, bioassays measure the cumulative effect of the proceeding steps in the induction of protein synthesis. Bioassays are also often more sensitive than other assays, like DNA binding (8). Furthermore, not all *in vitro* interactions are sufficiently strong to elicit an effect in whole cell bioassays (59). A transiently transfected template, in which nucleosome reorganization does not occur (60), was used in
order to minimize the possible complications of differential chromosomal reorganization by receptors.

The ideal cells for this study would lack both GR and PR but would display dynamic, induction responses over a range of transiently transfected receptors. CV-1 cells lack both GR and PR and respond well to transfected GR (46). However, in our hands, CV-1 cells give a low fold induction with transfected PR over a very narrow range of transfected receptors (49) (Szapary and Simons, unpublished results). The 1470.2 mouse mammary adenocarcinoma cells do contain some GR but possess excellent properties regarding gene induction by transfected human PR-B (49). As both transiently and stably transfected PR induce transactivation with transiently transfected MMTVLUC reporters (49, 55, 61), the use of transiently transfected PR should not pose a problem. PR is limiting for transactivation, and capable of displaying increasing levels of gene induction, over a range of transiently transfected receptors. PR also responds to a variety of coactivators and corepressors in these cells (49).

*Induction properties of PR and GR in 1470.2 cells:* Increasing concentrations of transiently transfected PR produce a progressive left-shift in the dose-response curve to lower EC$_{50}$s for R5020 induction of transiently transfected MMTVLuc reporters in 1470.2 cells (49). Steroid hormone induction of MMTV is complicated, due to the binding of NF1 and Oct1 to the MMTV promoter (62). In order to avoid these additional complications, we elected to use the simpler GREtkLUC reporter, which does not contain cis-acting binding sequences for other transcription factors. We first determined that higher concentrations of hPR-B plasmid afford increased total reporter activity, indicating that PR is limiting in this range ([Fig. 1A](#)). Under these conditions, the dose-response curve (or EC$_{50}$) for R5020 induction of the transiently transfected GREtkLUC reporter is shifted to lower steroid concentrations and the partial agonist activity of the antiprogestin dexamethasone mesylate (Dex-Mes) (49) is increased ([Fig. 1B](#)). Thus, we see the same responses to changing PR concentrations with the GREtkLUC reporter as for the MMTVLUC reporter (49). We
conclude that the ability of added PR to reposition the dose-response curve is independent of the GRE and promoter sequences.

It should be noted that the endogenous GR of 1470.2 cells does not interfere with the quantitation of PR induction. Not only does Dex display negligible activity with PR but also Dex-Mes and R5020 have little or no activity with the endogenous GR (49).

We previously reported that increasing amounts of transiently transfected GR produce a left-shift for the induction of a GREtkLUC reporter in HeLa and CV-1 cells (46). However, no further left-shift (or increased partial agonist activity of antiglucocorticoids) is seen with high concentrations of GR (>1 µg of plasmid), indicating that there is a limit to the effects of added GR (48). 1470.2 cells contain some GR (49). Under conditions where added GR increases the total transactivation of cotransfected GREtkLUC in 1470.2 cells, the higher levels of GR also afford about a two-fold left-shift in the dose-response curve with dexamethasone (Dex) (Fig. 1C). At the same time, the very low amount of partial agonist activity of Dex-Mes and dexamethasone oxetanone (Dex-Ox) with GR in 1470.2 cells (49) increases (Fig. 1C and data not shown). Thus, both the dose-response curve for glucocorticoids and the partial agonist activity of antiglucocorticoids are modulated by added GR in 1470.2 cells just as has been observed in HeLa and CV-1 cells (46-48). These data indicate that the transactivation properties of GR and PR are similarly altered by elevated levels of receptor.

Modulation of PR and GR activities by NCoR: We recently observed that added corepressor NCoR (63) had no effect on GR transactivation of GREtkLUC in CV-1 cells (47) but a significant effect on PR transactivation properties in 1407.2 cells with the MMTVLuc reporter (49). We therefore asked whether this behavior of the two receptors was maintained with a common reporter in the same cells. With the GREtkLUC reporter, NCoR decreases the maximal transactivation of GREtkLUC by PR by 41 ± 8% (S.E.M., n = 4) while slightly increasing the fold induction (18 ± 13%, S.E.M., n = 4). As indicated by the representative experiment of Fig 2A, NCoR simultaneously produces a 3.9 ± 0.8 (S.E.M., n = 4, P = 0.033) fold left-shift in the dose-response curve and a 70 ± 17% (S.E.M., n = 3)
increase in the partial agonist activity of Dex-Mes. This is comparable to the effects of NCoR on the transactivation properties of PR with MMTVLUC (49) and shows that NCoR modulation of PR properties in 1470.2 cells is independent of the enhancer and promoter sequences.

With the endogenous GR of 1470.2 cells, added NCoR also reduced the total transactivation by 27 ± 11% (S.E.M., n = 4) and increased the fold induction (54 ± 18%, S.E.M., n = 4), just as was observed above for PR. However, the effect on the other GR transactivation properties was almost exactly the reverse as seen with PR (Fig. 2B). NCoR afforded an average of a 2.1 ± 0.2 fold (S.E.M., n = 4, P = 0.011) right-shift in the dose-response curve. The slight decrease in the low partial agonist activity of Dex-Ox was not statistically significant (to 84 ± 31%, n=3, P = 0.47).

Modulation of PR and GR activities by SMRT: The transfection of SMRT cDNA causes a right-shift in the dose-response curve, and decreases the partial agonist activity of antisteroids, both for GR induction of GREtkLUC in CV-1 cells (47) and for PR induction of MMTVLuc in 1407.2 cells (49). When PR induction properties were determined with a different reporter, GREtkLUC, SMRT still affords a right-shift (3.2 ± 0.6 fold, n = 2) in the EC$_{50}$ and a 54 ± 1% (n = 2) decrease in the partial agonist activity of Dex-Mes (Fig. 3A). Interestingly, when GR induction was examined, the addition of SMRT no longer yields a right-shift. Instead, a weak left-shift (1.4 ± 0.2 fold, S.E.M., n = 5) is obtained (Fig. 3B) while causing a 33 ± 10% (S.E.M., n = 5, P = 0.032) decrease in the total transactivation. The magnitude of the left-shift of the GR dose-response curve with SMRT is not statistically significant (P = 0.11). However, the observation that SMRT causes a right-shift with PR and little or no left-shift with GR is significant (P = 0.025). At the same time, the low partial agonist activity of Dex-Ox (8.0 ± 0.7%, S.E.M., n = 5) is increased by SMRT to 9.9 ± 0.6% (S.E.M., n = 5, P = 0.048 in paired Student t Test). This result further supports a left shift in the GR dose-response curve by SMRT because a shift in the dose-response curve to the left has always been accompanied by increased partial agonist activity of an antisteroid (46-48, 64-66) The ability of added SMRT to produce a decrease in Dex-Mes partial
agonist activity with PR and an increase in the partial agonist activity of Dex-Ox with GR is also significant ($P = 0.0014$ by Alternate Welch t Test). These combined data indicate that the effects of SMRT on the dose-response curve and partial agonist activity of antagonists for PR complexes are the inverse of that seen with GR complexes.

Responses of chimeric GR/PR receptors: In an effort to understand the origins of the divergent responses of GR and PR transactivation properties to NCoR and SMRT, we asked whether the individual biological effects require specific domains of each receptor. For this, we selected chimeras in which the amino terminal and DBD domains of one receptor were fused to the LBD of the other to give the hybrid receptors that we call PR/GR and GR/PR (Fig. 4A). In both cases, the junction is seamless so that no mutations have been introduced in the body of the receptors. The construction of the chimeras did result in changes at the amino termini (see Fig 6A). However, these changes have not been observed to affect any properties of the receptors (Rusconi, data not shown). The experiments with PR/GR are not compromised by the endogenous GR of 1470.2 cells as the total activity, and fold induction, with PR/GR is $3.3 \pm 0.6$, and $2.8 \pm 0.6$ (S.E.M., $n = 7$), times greater, respectively, than the values with the endogenous GR (data not shown).

With both chimeras, 3 to 30 ng of transfected receptor yielded increasing total amounts of induced Luciferase activity (data not shown). This confirms that each receptor is limiting under our transfection conditions, as is the case for all of the above experiments. The dose-response curve for GR/PR is left-shifted by a factor of $2.36 \pm 0.65$ (range, $n=2$) in going from 3 to 30 ng of plasmid (Fig. 4B). There is no increase in the partial agonist activity of Dex-Mes. Instead, there is a slight decrease of $21 \pm 3\%$ (range, $n=2$).

A much larger response to changing concentrations is observed with the PR/GR chimera. In a preliminary experiment in 1470.2 cells, the dose-response curve of 30 ng of PR/GR plasmid is shifted to 6.8 fold lower Dex concentrations than that with 3 ng of plasmid while the partial agonist activity of Dex-Mes and Dex-Ox each increased (data not shown). At the same time, we noticed that the $EC_{50}$ for GREtkLUC induction by 30 ng of PR/GR plasmid was unexpectedly 5-10 fold lower than that for the endogenous GR (data
not shown). To be sure that these properties are not influenced by the endogenous GR of 1470.2 cells, the PR/GR chimera was further examined in CV-1 cells, which contain much lower levels of functional GR (46-48). Here, the same two concentrations of PR/GR cause a 7.13 ± 0.07 (S.E.M., n = 3, P = 0.0001) fold left-shift in the dose-response curve and a 31 ± 7% (S.E.M., n = 3, P < 0.050) increase in the partial agonist activity of Dex-Mes (Fig. 4C). Interestingly, under conditions where the total transactivation of GREtkLUC by 30 ng of PR/GR and 100 ng of GR is the same (consistent with equivalent amounts of transcriptionally active GR and PR/GR complexes), the dose-response curves and partial agonist activities with the two receptors are dramatically different (see also below).

Added SMRT plasmid causes a slight left-shift with GR and a right-shift with PR (Fig. 3). With the chimeric receptors, SMRT produces a weak right-shift with PR/GR and a stronger right-shift with GR/PR (Fig. 5A and B). The receptors can thus be ordered by decreasing ability of SMRT to afford a left-shift as follows, with the value for the fold left-shift in parentheses: GR (1.41 ± 0.2) > PR/GR (0.79 ± 0.11) > GR/PR (0.46 ± 0.09) > PR (0.33 ± 0.06) (± S.E.M. with n = 5 for all samples except PR, where ± range, n = 2). These differences are significant with P = 0.027 for GR vs. PR/GR, P = 0.049 for PR/GR vs. GR/PR, and P = 0.0027 for GR vs. GR/PR. This ordering is maintained when the effect of SMRT on the partial agonist activity of the various antisteroids is expressed in terms of increased agonist activity, with 100% being the control value: GR (127 ± 10%) > PR/GR (75 ± 3%) > GR/PR (58 ± 8%) > PR (46 ± 1%) (P = 0.009 for GR vs. PR/GR and 0.08 for PR/GR vs. GR/PR, both using Welch’s t Test) (see Figs. 3 and 5A&B). We therefore conclude that not only can the action of SMRT differ among receptors but also the eventual response results from a combination of the amino and carboxyl terminal regions of PR and GR as opposed to the actions of any one domain.

Similar conclusions were obtained from experiments on NCoR action with the chimeric receptors. NCoR shifted the dose-response curve of PR/GR to the left by a factor of 1.52 ± 0.14 (S.E.M., n=3) (Fig. 5C) but had no significant effect on GR/PR (Fig. 5D). The rank order of altered dose-response curve by NCoR, with the fold left-shift in parentheses, was
thus PR (3.9 ± 0.8, n=4) > PR/GR (1.52 ± 0.14, n=3) > GR/PR (1.19 ± 0.19, n=4) > GR (0.48 ± 0.04, n=4) (all values are ± S.E.M.) (see Figs. 2 and 5C&D). These values all differ from each other at the level of \( P \leq 0.05 \) except for PR/GR vs. GR/PR (\( P = 0.24 \)). The order for increased partial agonist activity with added NCoR of PR \( \approx \) GR/PR > PR/GR > GR (see Figs. 2 and 5C&D) is slightly different from that for the left-shift in the dose-response curve. Nevertheless, neither the LBD nor the amino terminal half of the receptor (including the DBD) appears to be dominant in the responses to NCoR. Instead, the final behavior of the chimeric receptor is an amalgam of the properties of each wild type protein with both halves of the receptor contributing to the final activity.

**Comparison of the PR/GR chimera with wild type GR:** We noted above (Fig. 4C) that the dose-response curve for PR/GR is considerably left-shifted from that for GR. This was unexpected because steroid binding to the receptor is thought to be the determining factor for the dose-response curve and both GR and PR/GR contain the same LBD (67). This question was therefore examined in greater detail. As shown for 1470.2 cells in **Fig. 6A**, the presence of 30 ng of transfected PR/GR affords a dose-response curve for Dex that is left-shifted more than 10 fold from that for the endogenous GR and more than 3 fold left-shifted from that for cells containing an additional 200 ng of transfected GR. This difference in dose-response curves could be due to unequal levels of expressed, biologically active receptors because the total transactivation by 30 ng of PR/GR is about twice that by 200 ng of GR (data not shown). To examine this possibility, the experiments were repeated in CV-1 cells, in which the ability of increasing amounts of transfected GR to cause a progressive left-shift in the dose-response curve, and higher amounts of partial agonist activity with antisteroids, reach nearly plateau values with 1000 ng of transfected GR plasmid (48). We therefore compared the effects of near saturating amounts of GR to those obtained with 3 and 30 ng of PR/GR plasmid. As seen in **Fig. 6B**, the dose-response curve for 3 ng of PR/GR is the same as that with 300 times more GR plasmid, which yields close to the maximal left-shift seen with added GR (48). In contrast, 30 ng of PR/GR is able to further shift the Dex dose-response curve another 7 fold to the left. Similarly,
marginal increases in the partial agonist activity of Dex-Mes are seen with 100 vs. 1000 ng of GR plasmid while substantial additional increases occur with 3 and 30 ng of PR/GR. This suggests that the differences in transactivation properties of GR vs. PR/GR are not limited by the concentration of expressed receptors but rather reflect intrinsic transactivation properties of the wild type GR and the PR/GR chimeric receptors.

One trivial explanation for the different properties of PR/GR vs. GR is that the presence of the PR amino terminal domain and/or DBD somehow increases the affinity of Dex for the GR LBD. This explanation can be eliminated, though, because the cell-free affinity of Dex is the same for both receptors in a cell-free Scatchard assay (Fig. 6C). Similarly, whole cell binding assays at 37°C reveal that there is no appreciable difference in Dex binding to PR/GR and GR under the conditions of the whole cell bioassay (Fig. 6D). Therefore, the lower EC$_{50}$ of PR/GR, compared to GR, is not the result of any difference in affinity between the receptors for ligand at either 0 or 37°C.

Discussion

This study presents evidence that changes in the concentration of the corepressors NCoR and SMRT can modify selected transcriptional properties of GR differently than those of PR. The most commonly examined transcriptional properties of steroid receptors are the total transactivation and the fold induction. None of the species in this study influence these properties of GR appreciably more or less than those for PR. However, notable differences can be seen between GR and PR with regard to changes in the dose-response-curve, or EC$_{50}$, and the partial agonist activity of antisteroids. These two properties of receptor-steroid complexes have important physiological and pathological consequences. Physiological levels of steroids are rarely sufficient for maximal induction and commonly correspond to the concentrations required for half-maximal induction, or the EC$_{50}$. Therefore, changes in EC$_{50}$ of a gene will result in significant differences in the expressed activity under physiological conditions. We do not know how similar the magnitude of responses in intact animals will be to our results in transfected cells.
However, the effects could even be greater. In transgenic mice with about a 50% higher level of GR protein, due to a 100% increase in the GR gene dosage, the dose-response curve for glucocorticoid induced, DNA-binding dependent apoptosis of primary thymocytes (68) is left-shifted to lower steroid concentrations by a factor of greater than 10 (69). Conversely, in cells from transgenic flies carrying the human estrogen receptor and an estrogen receptor responsive reporter and no known coactivators, the EC\textsubscript{50} for reporter induction by estradiol was right shifted by a factor of about 100 (70). The other transactivation parameter that we investigated, i.e., changes in the partial agonist activity of antisteroids, is highly relevant for endocrine therapies. An antisteroid that displays high levels of partial agonist activity for the very gene that one wishes to suppress would clearly be contraindicated. Alternatively, increased levels of partial agonist activity, which is characteristic of selective receptor modulators (SRMs), is often desirable. To the extent that side effects of antisteroid therapies result from the suppression of all responsive genes, an antisteroid with partial agonist activity for as many genes as possible, other than the one to be repressed, would be extremely useful.

In this study, we find that the corepressors NCoR and SMRT affect both the EC\textsubscript{50} of agonists and the partial agonist activity of antisteroids complexed with GR and PR for induction of GRE\textsubscript{tkLUC}-based reporters in 1470.2 cells. More significantly, these factors differentially affect these responses for the two receptors. The corepressor SMRT was previously found to shift the dose-response curve to higher EC\textsubscript{50}s, and decrease the partial agonist activity of antisteroids, both for GR induction of GRE\textsubscript{tkLUC} in CV-1 cells (47) and for PR induction of MMTVLuc in 1470.2 cells (49). As anticipated, the responses of PR to SMRT in 1470.2 cells are independent of the sequence of the glucocorticoid response element and are the same for a GRE\textsubscript{tkLUC} (Fig. 3A) and MMTV (49) reporter. Unexpectedly, the response of GR to added SMRT for induction of GRE\textsubscript{tkLUC} in 1470.2 cells is the opposite of PR. The GR dose-response curve is repositioned to lower EC\textsubscript{50}s and the partial agonist activity of antisteroids increases (Fig. 3B). This argues not only that the responses of a given receptor can vary among cell types (i.e., GR in CV-1 vs. 1470.2
cells) but also that the same cofactor can produce diametrically opposite effects for the transactivation properties of two different steroid receptors (GR and PR) acting on the same gene in the same cell. Similarly, the effects of NCoR with GR and PR are cell- and receptor-selective. The response of PR to added NCoR is the same for GREtkLUC (Fig. 2A) and MMTVLuc (49) and thus is independent of the reporter. In contrast, NCoR modulates GR the transactivation properties for GREtkLUC induction in 1470.2 cells (Fig. 2B) but not in CV-1 cells (47). Furthermore, NCoR shifts the dose-response curves for GR and PR induction of GREtkLUC in 1470.2 cells in opposite directions and has opposite effects on the partial agonist activity of antisteroids (Fig. 2). Earlier studies have also documented specificity in receptor binding of coactivators and corepressors (71, 72).

The net result is that two factors have been identified that cause unequal modifications of GR vs. PR induction properties of the same gene in the same cell. Furthermore, these changes are biologically relevant. The normal variations of glucocorticoid and progestin hormones are in the range of the EC$_{50}$ for induction of most genes, thus assuring significant alterations in gene expression. Within this context, the amount of corepressor in each cell will inversely affect the response of GR and PR to physiological concentrations of steroid, making the position of the agonist dose-response curves and the partial agonist activity of antagonists less similar. Thus, the presence of corepressors within a cell appears to constitute a novel mechanism for imparting different transcription properties to receptors that bind to the same HRE, such as GR and PR.

Many of the activities of steroid receptors can be localized to unique domains of the proteins. Using chimeras containing the amino terminal plus DNA binding domain of one receptor and the LBD of the other, it is apparent that no one domain of either receptor is dominant for the differential responses of PR and GR to NCoR and SMRT. Rather, the chimeras have properties that are intermediate between the two wild type receptors. This result supports the notion that the transcription properties of GR and PR are modified by the interactions, either indirect or direct, of corepressors with the N- and C-terminal portions of both wild type and chimeric receptors. Such inter-domain interactions and signaling
appear to be common among the steroid/nuclear receptors and have been observed for GR (73), PR (74), androgen receptors (44, 75) estrogen receptors (76), and PPAR receptors (77). Similarly, we propose that the combination of N- and C-terminal sequences forms a surface that differentially interacts with cell-specific factors, thereby giving rise to the unequal transcriptional responses regarding the EC_{50} and partial agonist activity (Fig. 7). These cell-specific factors would be expected to include the corepressors, given the above consequences of added NCoR and SMRT. We do not yet have any evidence for the corepressors directly contacting GR. Initially, it was thought that NCoR and SMRT bound only to the nuclear receptors. More recently, however, NCoR and SMRT have been shown to bind to antagonist complexes of PR and ER (26, 78-80) and the amino terminal domain of receptors may be involved (31) in addition to the LBD (81-83). Significantly, others (78, 79), in addition to ourselves (47, 49), have reported effects of corepressors on the total transactivation of agonist-bound ER, PR, and GR complexes. These data support an interaction of corepressors with agonist-, as well as antagonist-, bound receptors. Further support for the importance of N- and C-terminal sequence interactions in determining the transactivation properties of GR and PR comes from the PR/GR chimera. While the steroid binding affinity of PR/GR at 0 and 37°C is, as expected, the same as that of wild type GR (Fig. 6), the EC_{50} for Dex induction of GREtkLUC, and the partial agonist activities of antisteroids, are quite different (Fig. 4C). The simplest explanation is that the combination of domains alters steps in transactivation that follow steroid binding to the receptor in a way that increases the sensitivity to circulating steroids, as outlined in Fig. 7.

A differential effect of cofactors on steroid receptor regulated-gene transcription is an attractive mechanism for conveying unequal transactivation properties to receptors that bind to common DNA targets. As is described in the present study with GR and PR, this mechanism can help to account for the different responses between two classes of steroid receptors within the same and different cells. Another apparent example of differential responses to a common cofactor has recently been reported for PIAS1. In CV-1 cells, PIAS1 increased the fold induction by both androgen receptors and GR but decreased
progesterone receptor fold induction (40). In light of the variation of cofactor levels among cell types (84-89), the model of Fig. 7 helps to explain the cell-to-cell differences for a given receptor, as has been seen here for GR in CV-1 vs. 1470.2 cells. Finally, the present modulation of EC$_{50}$, and partial agonist activity of antisteroids, in the same and different tissues offers yet another method for achieving the differential control of gene expression that is required during the differentiation, development, and homeostasis of complex organisms. These results suggest that additional studies on the differential consequences of cofactor binding to steroid receptors represent a fertile area for future research.

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Figure Legends

Fig. 1: Effect of steroid receptor concentration on GREtkLUC induction properties. Triplicate cultures of 1470.2 cells were transfected with GREtkLUC and Renilla null luciferase reporters plus the indicated amounts of hPR-B (A, B) or rat GR (C) cDNA plasmids, induced with varying concentrations of agonist (R5020 (A, B) or Dex (C)), or antagonist (Dex-Mes (A, B) or Dex-Mes (C)), and assayed for luciferase and Renilla activities as described in Materials and Methods. The average values, normalized for cotransfected Renilla, were then plotted as either total luciferase activity (A) or the percent of maximal induction above EtOH controls (B, C). The error bars represent the S.D. within a given experiment. Similar results were obtained in one (A, B) and two (C) additional experiments.

Fig. 2: Modulation by exogenous NCoR of steroid receptor induction properties for GREtkLUC. Triplicate cultures of 1470.2 cells were transfected with GREtkLUC and Renilla null luciferase reporters plus 30 ng of hPR-B cDNA plasmid (A). The endogenous GR, with no transfected receptor plasmids, was used in (B) to examine glucocorticoid induced responses. Both sets were cotransfected with either 1.2 µg of NCoR plasmid or the molar equivalent of empty vector, induced with varying concentrations of agonist (R5020 (A) or Dex (B)), or antagonist (Dex-Mes in (A) and (B)), and assayed for luciferase and Renilla activities as described in Materials and Methods. The average values, normalized for cotransfected Renilla, were then plotted as percent of maximal induction above EtOH controls. The error bars represent the S.D. within a given experiment. Similar results were obtained in three additional experiments with each receptor.

Fig. 3: Ability of SMRT to modify receptor-regulated induction properties of GREtkLUC. The behavior of transfected hPR-B (30 ng of plasmid DNA; A) or endogenous GR (B) was assessed in triplicate cultures of 1470.2 cells that were cotransfected with GREtkLUC and Renilla null luciferase reporters plus 0.4 µg of SMRT plasmid or the molar equivalent of
empty vector, induced with varying concentrations of agonist (R5020 (A) or Dex (B)), or antagonist (Dex-Mes in (A) and Dex-Ox in (B)), and assayed for luciferase and Renilla activities as described in Materials and Methods. The average values, normalized for cotransfected Renilla, were then plotted as percent of maximal induction above EtOH controls. The error bars represent the S.D. within a given experiment. Similar results were obtained in one (A) and four (B) additional experiments.

Fig. 4: Induction properties of chimeric receptors. (A) Cartoon of composition of chimeric receptors. The linear structures of the wild type rat GR and human PR-B are displayed on top. The individual domains of the receptors are identified by the letters A-E on top of the GR structure. The C and D domains, corresponding to the DNA binding domain and the hinge region respectively, are differentially shaded. The chimeric receptors are joined at the interface of the C and D domains, indicated by the vertical line. The precise amino acids from each receptor are shown above and below the two segments comprising each chimera. (B) Effect of concentration of transfected GR/PR on GREtkLUC induction properties in 1470.2 cells. (C) Effect of steroid receptor concentration of GR vs. PR/GR on GREtkLUC induction properties in CV-1 cells. In (B) and (C), triplicate cultures were co-transfected with the indicated amounts of receptor plasmids and GREtkLUC plus Renilla null luciferase reporters, induced with varying concentrations of agonist (R5020 (B) or Dex (C)), or antagonist (Dex-Mes in (A) and (B)), and assayed for luciferase and Renilla activities as described in Materials and Methods. The average values, normalized for cotransfected Renilla, were then plotted as percent of maximal induction above EtOH controls. The error bars represent the S.D. within a given experiment. Similar results were obtained in one (A) and two (B) additional experiments.

Fig. 5: Effect of corepressors (SMRT and NCoR) on the induction properties of GREtkLUC by chimeric receptors in 1470.2 cells. The consequences of cotransfected SMRT (0.4 µg; A, B) and NCoR (1.2 µg; C, D) on the properties of PR/GR (A, C) and
GR/PR (B, D) were examined. Triplicate cultures were co-transfected with 30 ng of each chimeric receptor plasmid, an equimolar amount of corepressor plasmid or vector, and GREtkLUC plus Renilla null luciferase reporters, induced with varying concentrations of agonist (Dex (A, C) or R5020 (B, D)), or antagonist (Dex-Ox (A, C) or Dex-Mes (B, D)), and assayed for luciferase and Renilla activities as described in Materials and Methods. The average values, normalized for cotransfected Renilla, were then plotted as percent of maximal induction above EtOH controls. The error bars represent the S.D. within a given experiment. Similar results were obtained in four (with SMRT) and two or three (with NCoR) additional experiments.

Fig. 6: Comparison of properties of PR/GR with those of wild type GR. Induction properties of PR/GR vs. GR in 1470.2 (A) and CV-1 (B) cells. Triplicate cultures were co-transfected with the indicated amounts of each receptor plasmid and GREtkLUC plus Renilla null luciferase reporters, induced with varying concentrations of agonist (Dex in (A) and (B)), or antagonist (Dex-Ox (A) or Dex-Mes (B)), and assayed for luciferase and Renilla activities as described in Materials and Methods. The average values, normalized for cotransfected Renilla, were then plotted as percent of maximal induction above EtOH controls. The error bars represent the S.D. within a given experiment. Similar results were obtained in two (A) and three (B) additional experiments. Affinity of Dex binding to GR and PR/GR in cell-free (C) and whole cell (D) systems. Scatchard plots of Dex binding to GR or PR/GR in cytosols from transfected COS-7 cells, or intact transfected COS-7 cells, were performed as described in Materials and Methods. The best fitting lines were determined by least squares analysis to give the indicated $K_d$ values. Similar results were obtained in a second experiment.

Fig. 7: Proposed model for role of receptor domains in differential modulation of transactivation properties by various cofactors. For simplicity, only one of the two receptor molecules that bind to a single GRE of GREtkLUC are shown. Those factors that interact
with the structurally discrete N-and C-terminal domains of GR, PR, and the chimeric receptors (only PR/GR is shown) may undergo a binding-induced conformational modification. These factors may interact alone or as part of a multi-protein complex. The now different tertiary structures of these interacting molecules or complexes (indicated by the altered shape and shading of the species contacting the N- and C-terminal domains of the receptors) will possess unequal spatial relationships with, and/or will have altered affinities for, different components of the transcriptional machinery (lightly shaded species). These varied protein-protein contacts, which may be mediated by additional nuclear proteins, would result in the non-equal effects on the EC$_{50}$ and partial agonist activity that are seen for each of the illustrated receptors. See text for further description.
Fig. 1A

Fig. 1B

Fig. 1C
Fig. 2A

Luciferase Activity as Percent of Maximal Response with 30 nM R520

R520 Concentration (pM)

- NCoR
- NCoR

Fig. 2B

Luciferase Activity as Percent of Maximal Response with 1 μM Dex

Dex Concentration (nM)

- NCoR
- NCoR

Dex-Ox (nM)
Transactivation Specificity of Glucocorticoid vs. Progesterone Receptors: Role of Functionally Different Interactions of Transcription Factors with Amino- and Carboxyl-terminal Receptor Domains

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