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Modulation of transcriptional sensitivity of mineralocorticoid and estrogen receptors

Qi Wang[a], Sarah Anzick[b], William F. Richter[a,1], Paul Meltzer[b], S. Stoney Simons Jr.[a,*]

[a] *The Steroid Hormones Section, NIDDK/LMCB, National Institutes of Health, Bethesda, MD, USA*
[b] *Cancer Genetics Branch, NHGRI, National Institutes of Health, Bethesda, MD, USA*

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Abstract

Recent reports describe the ability of factors to modulate the position of the dose–response curve of receptor–agonist complexes, and the amount of partial agonist activity of receptor–antagonist complexes, of androgen, glucocorticoid (GRs), and progesterone receptors (PRs). We now ask whether this modulation extends to the two remaining steroid receptors: mineralocorticoid (MRs) and estrogen receptors (ERs). These studies of MR were facilitated by our discovery that the antiglucocorticoid dexamethasone 21-mesylate (Dex-Mes) is a new antimineralocorticoid with significant amounts of partial agonist activity. Elevated levels of MR, the co-activators TIF2 and SRC-1, and the co-repressor SMRT do modulate the dose–response curve and partial agonist activity of MR complexes. Interestingly, the precise responses are indistinguishable from those seen with GRs in the same cells. Thus, the unequal transactivation of common genes by MRs versus GRs probably cannot be explained by differential responses to changing cellular concentrations of homologous receptor, co-activators, or co-repressors. We also find that the dose–response curve of ER–estradiol complexes is left-shifted to lower steroid concentrations by higher amounts of exogenous ER. Therefore, the modulation of either the dose–response curve of agonists or the partial agonist activity of antisteroid, and in many cases the modulation of both properties, is a common phenomenon for all of the classical steroid receptors.
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Keywords: Mineralocorticoid receptors; Estrogen receptors; Dose–response curve; Partial agonist activity; Co-activators; Co-repressors

1. Introduction

The basic steps for steroid-regulated gene induction have been known for many years and are similar for all of the steroid receptors [1]. After the steroid enters the cell by passive diffusion and binds to an intracellular receptor, the receptor–steroid complex is activated to a form that binds with high affinity to biologically active DNA sequences, called hormone response elements (HREs), to alter the rates of transcription of nearby promoters. More recently, this model has been embellished by numerous co-factors and co-modulators that associate with the DNA-bound receptor–steroid complexes [2–5]. While the presence of these additional transcriptional co-factors further complicates the elucidation of steroid–hormone action, they also offer the possibility of additional control of various receptor properties.

One critical parameter of steroid receptor regulated gene induction is the absolute amount of induced gene product. Two other properties that are of utmost importance for mammalian physiology and human endocrine therapy are the dose–response curve of agonist steroids and the partial agonist activity of antisteroids [6,7]. The dose–response curve gives the amount of gene induction by any concentration of steroid, with half of the maximal induction occurring at a value called the EC_{50}. The lower the EC_{50} of a given gene,

* Corresponding author. Room B2A-07, Bldg. 8, NIDDK/ LMCB, NIH, Bethesda, MD 20892, USA. Tel.: +1 301 496 6796; fax: +1 301 402 3572.
[1] University of Maryland, College Park, Bethesda, MD, USA.

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the greater is the level of induction that is achieved with the sub-saturating concentration of circulating steroid in an animal or cell. Antisteroids, or antagonists, block the action of agonist steroids. However, virtually all antisteroids retain some partial agonist activity with selected genes. Recently, it has been appreciated that this mixed activity of antisteroids is therapeutically useful. If one can selectively eliminate the transactivation of a target gene, while retaining the expression of most of the other genes that are regulated by a given receptor, then the number of undesirable side-effects that usually result from the indiscriminate repression of all responsive genes will be greatly reduced. While the mechanism for changes in the amount of partial agonist activity is not yet known, it is evident that studies of this phenomenon are of great theoretical and clinical importance.

Among the various receptor-associated co-factors, two of the most extensively studied classes are the p160 co-activators (SRC-1, TIF2/GRIP1 and AIB1/pCIP/ACTR/ RAC3/ TRAM1 [8–11]) and the co-repressors NCoR and SMRT [12,13]. Co-activators are defined as co-factors that increase the levels of transactivation. Conversely, co-repressors decrease the absolute amount of gene product. Recently, co-activators and co-repressors have also been reported to modulate the EC_{50} and/or the partial agonist activity of androgen receptors (ARs) [14], glucocorticoid receptors (GRs) [15–20] and progesterone receptors (PRs) [17,21–23]. Co-activators and co-repressors each physically interact with both agonist- and antagonist-bound forms of receptors, thus offering interesting molecular mechanisms for regulating the EC_{50} and partial agonist activity of at least some steroid receptors (reviewed in [7]). Because the modulatory responses are independent of the effects of the co-factors on the absolute levels of induced gene product, they probably proceed via different mechanisms [19,20].

Cofactor-induced changes in EC_{50} and partial agonist activity may help to resolve the persistent question of how the selectivity of steroid binding to the cognate receptor is maintained, when all of the classical steroid–receptor complexes, except those of estrogen receptors (ERs), can bind to the same HREs [24]. This is particularly important for GRs and mineralocorticoid receptors (MRs) because of the relatively high homology between the two receptors and the fact that the endogenous glucocorticoid of humans (cortisol) and rats or mice (corticosterone) binds even more tightly to MRs than to GRs [25,26]. Furthermore, cortisol appears to be a mineralocorticoid in the brain [27–29]. One attractive explanation for the different activities of the assorted receptor–steroid complexes (RS) is that they have unequal affinities for the various co-factors, which modify the activity of HRE-bound receptors [30–33]. An interesting variant of this explanation is that, while co-activators and co-repressors may each bind to all of the receptor–steroid complexes, the activities of these quaternary complexes (steroid-receptor-cofactor-HRE) may be unequal. For example, the co-repressors NCoR and SMRT alter the EC_{50} and partial agonist activity for GR and PR induction of the same transfected Luciferase reporter in the same cells (1470.2 mouse mammary adenocarcinoma cells) [17]. However, not only does each co-repressor evoke opposite responses for the same receptor–steroid complex, but also each co-repressor produces diametrically opposed effects with the two receptors. Thus, NCoR left-shifts the PR dose–response curve to a lower EC_{50}, and increases the partial agonist activity of antiprogestins, while the GR dose–response curve is right-shifted to a higher EC_{50} and the activity of antiglucocorticoids is decreased. Furthermore, studies with PR/GR chimeras suggest that both the amino- and carboxyl-halves of the receptor contribute to the final activity with added co-repressors [17].

Another method for modifying the EC_{50} and partial agonist activity of receptor–steroid complexes is simply to change the amount of homologous receptor. Elevated levels of GR [15,16,34] and PR [22] lower the EC_{50} of agonist complexes and increase the partial agonist activity of antagonist complexes. This result was unexpected in view of the current equilibrium models of steroid–hormone action. While more receptor usually results in greater amounts of induced gene product, the concentration of steroid required for half–maximal induction of the gene product (the EC_{50}) would not be expected to change. This can be appreciated from a consideration of the equilibrium binding of steroid (S) to receptor (R) to give RS, where the equilibrium dissociation constant (K_d) is defined as [R][S]/[RS]. When half of the receptors are bound by steroid, RS = 0.5 × (total R) and K_d = [0.5 × (total R)][S]/[0.5 × (total R)] = [S], which shows that the K_d is independent of receptor concentration. Similar calculations predict that the EC_{50} for gene induction by agonist complexes is independent of receptor concentration. Thus, some other explanation is required for the changes EC_{50} and partial agonist activity. However, whatever mechanism(s) is involved, it is clear that a variety of factors are available to cells for modulating these properties of GRs and PRs.

The purpose of this study was, therefore, two-fold. First, we wanted to determine whether the modulation of the EC_{50} of agonists, and/or the partial agonist activity of antisteroids, that has been observed with varying concentrations of ARs, GRs, and PRs is general for all of the steroid receptors and can be seen with MRs and ERs. Second, we desired to know whether a selection of those factors that modulate GR activities (i.e., co-activators and co-repressors) can differentially affect the activities of MRs, as has recently been proposed [33].

2. Materials and methods

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

2.1. Chemicals and plasmids

[^{3}H]Aldosterone (Aldo, 76.4 Ci/mmol) was obtained from NEN (Boston, MA). Non-radioactive aldosterone was

from Sigma (St. Louis, MO). Dexamethasone-oxetanone (Dex-Ox) [35] and Dex-mesylate (Dex-Mes) [36] were prepared as described. The renilla null Luciferase reporter (Renilla-TK) was purchased from Promega (Madison, WI). GREtkLUC contains two tandem repeats of the GRE and has been previously described [37]. MMTVLuc (pLTRLuc) was from Gordon Hager (NIH, Bethesda, MD). The MR expression plasmid (pCMV4-MR) was a gift from David Pearce (UCSF, San Francisco, CA). The empty vector, pCMV4ΔMR was prepared by removing the MR cDNA with *Bam*HI and *Xba* I. The resulting 6.0 kb fragment was blunt-ended with DNA Polymerase I, large (Klenow) fragment, and then ligated to give the 6.0 kb circularized vector. The s-SMRT expression plasmid (pCMX-SMRT) [38] was from Ron Evans (Salk Institute, La Jolla, CA). The GR expression plasmid (pSVLGR) and vector plasmid (pSVL) were from Keith Yamamoto (UCSF, San Francisco, CA). The TIF2 and TIF2.0 expression plasmids (pSG5-TIF2 and pSG5-TIF2.0) were from Hinrich Gronemeyer (IGBMC, Strasbourg, France). The SRC-1a expression plasmid (pCR3.1-SRC1a) was from Bert O'Malley (Baylor College of Medicine, Houston, TX). The NCoR expression plasmid (pCMX-NCoR-Flag) was from Michael Rosenfeld (University of California, San Diego). The pGL3.luc.ERE, which contains three tandem copies of the estrogen response element (ERE) upstream the simian virus 40 promoter driving the luciferase gene, was the kind gift of Fern Murdoch (University of Wisconsin, Madison, WI). hSA/pSG5 and hSA/pCMX have been previously described [20]. The hSA/pCR3.1 was prepared by excising the hSA cDNA from hSA/pBSK$^-$ (Human Serum Albumin, Stratagene Liver 937224, IMAGE. Consortium Clone 83491, ATCC cat #323324) with *Eco*R1 and *Xho* 1 and directionally subcloning it into the corresponding sites of pCR3.1 (Invitrogen cat # K300001).

2.2. Cell culture and transfection

Monolayer cultures of CV1 cells (monkey kidney cells from ATCC, Manassus, VA) were grown as described [22,34]. Cells are transfected for 18 h using lipofectamine (Life Technologies, Inc.) or FuGene (Roche) as recommended by the supplier. For each 60 mm dish, we used 1000 ng of reporter (GREtkLuc or MMTV-Luc) and 200 ng of Renilla-TK (as an internal control for transfection efficiency) plus various combinations of other expression vectors. Equal molar amounts of expression vectors lacking MR, GR or co-factors (i.e., pCMV4ΔMR, pSVL, hSA/pSG5, hSA/pCR3.1, hSA/pCMX) are included to keep the molar amount of each vector constant, with the total transfected DNA brought to 3000 ng/dish with pBSK$^+$ unless otherwise indicated. With ER, CV-1 cells were seeded one day before transfection at a density of 1.5×10^5 cells/well into 6-well culture dishes (20 mm). Using LipofectAMINE transfection reagent (Invitrogen, Carlsbad, CA) and the manufacturer's instructions, cells were transfected with 20 ng pRL-CMV (Renilla luciferase internal control; Promega, Madison, WI), 1.5 μg pGL3.luc.ERE, and varying concentrations of an expression vector encoding the estrogen receptor-α pHEGO-hyg (ATCC, Manassas, VA). The day following transfection, all cells were incubated in the absence or presence of varying concentrations of aldosterone or 1 μM Dex-Mes (for MR), or 17β-estradiol or 100 nM 4-hydroxytamoxifen for ER, for 24 to 36 h in media containing 10% FBS and harvested in 1X Passive Lysis Buffer (600 μl/60 mm dish, Promega). One hundred microliters of the cell lysates per 60 mm dish are used to assay for Luciferase activity using the Dual-Luciferase Assay System (Promega) and an EG and G Berthold's luminometer (Microlumat LB96P) according to the supplier. The Luciferase activity is divided by the Renilla value from the same well to normalize the data to a constant transfection efficiency.

2.3. Calculation of dose–response curves and partial agonist activity

To obtain dose–response curves, the normalized Luciferase activity values with different concentrations of agonist steroid are each reduced by the basal activity seen in the absence of steroid. These values are expressed as a percent of the maximal induction by saturating concentrations of agonist steroid in each experiment and then plotted against the steroid concentration. To obtain the partial agonist activity, the normalized Luciferase activity values for 1 μM antisteroid and saturating concentrations of agonist in the same experiment are reduced by the basal activity and then plotted as a percent of the maximal induction by saturating concentrations of agonist steroid in each experiment. These methods of plotting greatly facilitate comparisons both of the positions of the dose–response curves and of the partial agonist activity of antisteroids [7]. In those cases, where the level of gene induction for ER with 100 nM 4-hydroxytamoxifen is less than that seen with the EtOH control, the value with 4-hydroxytamoxifen was used as the basal level of gene expression.

2.4. Steroid binding assay

Transient transfection of COS-7 cells with 1.5 μg/15 cm dish of MR expression plasmid DNA is performed with FuGene. To obtain cytosols containing the steroid-free receptors, the transfected cell pellets are lysed with a freeze–thaw cycle in HEPES buffer (20 mM HEPES, 1 mM EDTA, 10% glycerol, pH 7.5 at 0 °C), followed by centrifugation at 15,000 $\times$ *g* for 20 min at 0 °C. Thirty percent cytosol with 20 mM sodium molybdate is adjusted to 13 nM of [^{3}H]aldosterone ± 1, 2, and 100-fold excess of non-radioactive aldosterone or 100- and 800-fold excess of non-radioactive Dex-Mes, which are incubated at 0 °C for 18 h. Unbound [^{3}H]aldosterone is removed by dextran-coated charcoal at 0 °C and the supernatant is counted by scintillation counting.

2.5. Western blotting

Transfected COS-7 cells were lysed with 1 × SDS loading buffer (Quality Biological, Inc.). The lysates were sonicated briefly (20 s at 400 W). Equal amounts of total protein were separated on 6% SDS–PAGE gel (150 V for 1 h) and then transferred to a nitrocellulose membrane (Schleicher and Schuell BioScience). The MR protein was detected by rabbit anti-MR antibody (MCR[H-300] from Santa Cruz, 1:1000 dilution) and visualized by ECL detection reagents as described by the manufacturer (Amersham Biosciences).

2.6. Statistical analysis

Unless otherwise noted, all experiments were performed in triplicate several times. KaleidaGraph 3.5 (Synergy Software, Reading, PA) was used to determine a least-squares best fit (R^2 was almost always $\geq$0.95) of the experimental data to the theoretical dose–response curve, which is given by the equation derived from Michaelis–Menton kinetics of y = [free steroid]/([free steroid] + K_d) (where the concentration of total steroid is approximately equal to the concentration of free steroid because only a small portion is bound), to yield a single EC_{50} value. The values of n independent experiments were then analyzed for statistical significance by the two-tailed Student's t-test using the program "InStat 2.03" for Macintosh (GraphPad Software, San Diego, CA). When the difference between the S.D.s of two populations is significantly different, then the Mann–Whitney test or the Alternate Welch t-test is used.

3. Results

3.1. Effects of MR concentration on the dose–response curve of MR-agonist complexes

CV-1 cells that had been transiently transfected with the simple GREtkLUC reporter and three different concentrations of MR-containing plasmid were treated with a range of concentrations of the mineralocorticoid aldosterone. No induction of Luciferase over that seen with vehicle (EtOH) is observed with 1 μM aldosterone in the presence of the empty vector, thus demonstrating that there are no functional MRs in CV-1 cells (Fig. 1A). With increasing amounts of MR-containing plasmid, a progressively higher amount of induced gene product is obtained (Fig. 1A). This demonstrates that MR is limiting in this concentration range of receptor. At the same time, there is an increased left-shift of the dose–response curve to lower EC_{50}s with higher amounts of transfected MR (Fig. 1A). The dose–response curve with 33 ng of MR plasmid is 3.30 ± 0.16 (± range, n = 2) fold left-shifted from that for 3.3 ng of MR.

A different reporter, MMTVLuc, was then used to determine, whether the nature of the reporter influences the results. The fold induction by low amounts of MRs with MMTVLuc is 4–10-fold higher than with GREtkLUC (data not shown). More total gene expression is again achieved with more MR plasmid, thus demonstrating that MR is limiting under these conditions too. Also, a left-shift in the dose–response curve is obtained with elevated levels of MR (Fig. 1B). Increasing the amount of MR from 0.5 ng to 10 ng causes a 2.95 ± 0.24 (±S.E.M., n = 5, P = 0.0013) fold left-shift in the dose–response curve. Thus higher concentrations of MR cause a shift in the dose–response curve for gene induction to a lower concentration of agonist in a manner that is independent of promoter organization. Because of the greater amount of induction with the MMTVLuc reporter, all of the subsequent experiments with MRs were conducted with this reporter.

3.2. Effects of MR concentration on the partial agonist activity of MR-antagonist complexes

A common consequence of increased receptor levels for GRs [15,16,34] and PRs [22] is that the partial agonist activity of antisteroids is also increased. Such changes are of great clinical interest (see Discussion). Spironolactone is the classical antimineralocorticoid [39]. However, it displays no partial agonist activity in either of the above two assay systems (data not shown). Therefore, spironolactone is not useful for assessing the ability of co-factors to increase the partial agonist activity of antimineralocorticoids. Given the high sequence homology between the ligand binding domains (LBDs) of GR, PR, and MR, and the fact that Dex-Mes and dexamethasone-oxetanone (Dex-Ox) yield significant amounts of partial agonist activity with both GR and PR, we asked if these steroids might also be antimineralocorticoids with appreciable amounts of partial agonist activity. In fact, Dex-Mes does afford significant amounts of partial agonist activity. Furthermore, elevating the amount of MR plasmid from 0.5 ng to 10 ng increases the partial agonist activity of Dex-Mes, relative to 100 nM aldosterone under the same conditions, by 1.85 ± 0.27-fold (±S.E.M., n =5, P = 0.037) (Fig. 2A). The partial agonist activity of Dex-Ox was usually much less than that of Dex-Mes (data not shown) and was not pursued.

To determine whether Dex-Mes is acting as an antimineralocorticoid, we looked at its ability to block the biological activity of aldosterone. Dex-mesylate inhibits the ability of aldosterone, both to induce the MMTVluc reporter (Fig. 2B) and to bind to cell-free MRs (Fig. 2C). Therefore, Dex-Mes qualifies as a new antimineralocorticoid because it competitively inhibits both aldosterone binding to MRs and aldosterone-induced transactivation by MRs.

3.3. Modulation of MR induction properties by co-activators

The effects of 50 ng and 200 ng of exogenous TIF2 on MR transactivation properties with a MMTVluc reporter in transiently transfected CV-1 cells are about equal. The total

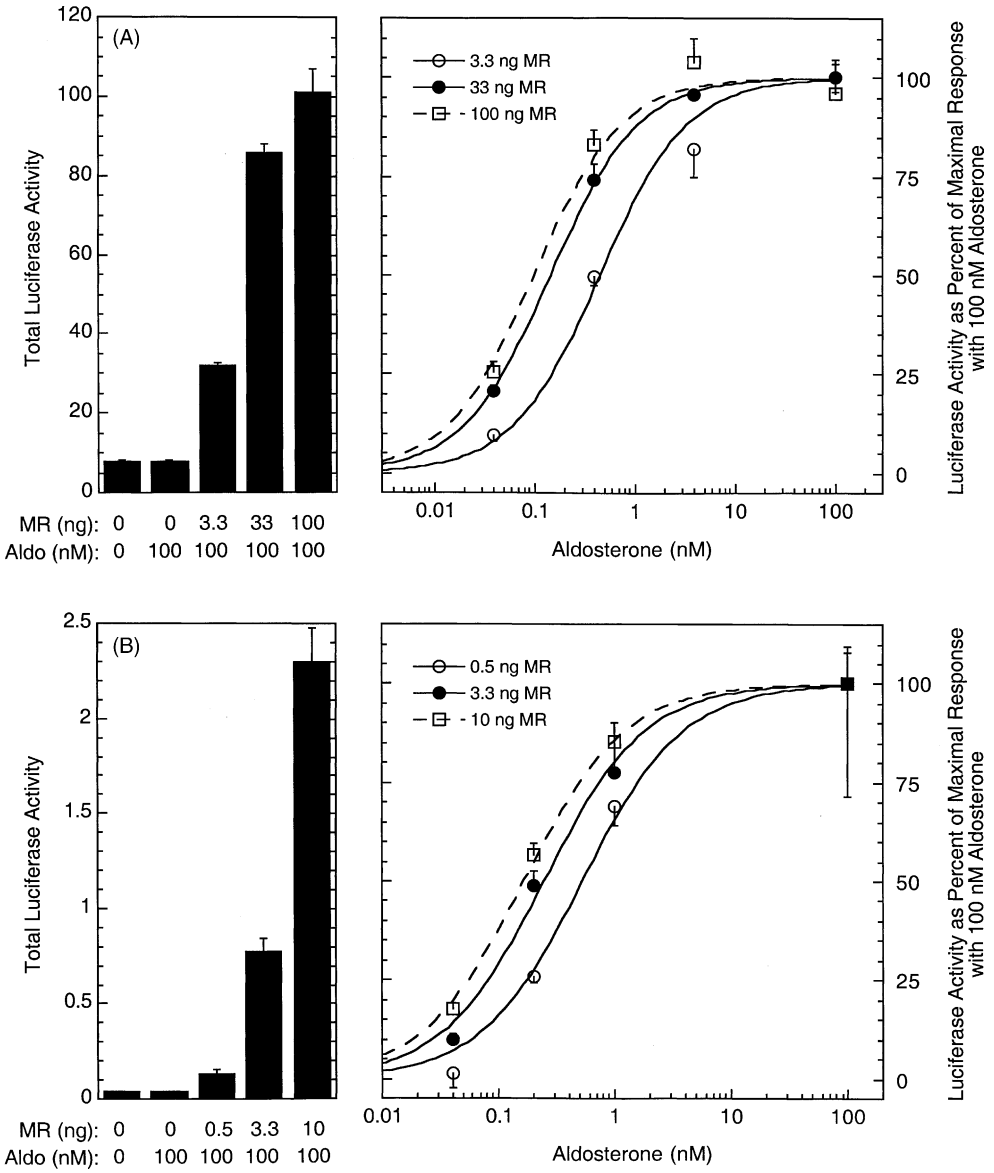


Fig. 1. Influence of increasing MR concentrations on the transactivation of transiently transfected reporters. Triplicate 60 mm dishes of CV-1 cells were transiently transfected with the listed amounts of MR plasmid plus enough empty vector to maintain a constant molar amount of vector DNA, 1 μg of GREtkLUC (A), or MMTVluc (B), and 200 ng of Renilla TK. After 18 h, the indicated concentrations of aldosterone in EtOH (final concentration = 0.1%) were added for 24 h before the assays were harvested and Renilla and Luciferase activities were measured as described in the Materials and Methods. The Luciferase values at each steroid concentration were normalized for Renilla expression and presented either as total Luciferase activity (left figure) or as percent of the maximal response seen with 100 nM aldosterone (right figure). In the right figure, the average values (±S.D.) were plotted against the concentration of aldosterone to give the dose–response curve. Similar results were obtained in a second independent experiment with GREtkLUC and in four additional experiments with MMTVluc.

amount of transactivation with 100 nM aldosterone is increased by 3.6 ± 1.2 (n = 3) and 5.0 ± 0.6 (n = 4)-fold (errors = S.E.M.) in the presence of 50 ng and 200 ng TIF2, respectively. In contrast, 140 ng of TIF2.0 plasmid (Fig. 3A), which lacks the receptor interaction domains (RIDs), and the LxxLL sequences that are required for TIF2 binding to steroid receptors [40], causes a 25 ± 7% (±S.E.M., n = 2) decrease in total activity. More importantly, the addition of 50 ng of TIF2 shifts the EC_{50} to lower steroid concentrations by a factor of 2.34 ± 0.31-fold (±S.E.M., n = 3, P = 0.048) and increases the partial agonist activity of Dex-Mes by 7.1 ± 2.2-fold (±S.E.M., n = 3, P = 0.048) (Fig. 3B). This modulation is not seen with TIF2.0 (Fig. 3B), which lacks the RIDs of TIF2, even though Western blots show that it is expressed at high levels (data not shown).

Cotransfection of 50 ng of SRC-1a plasmid also increases the total amount of MR transactivation but only weakly (1.42 ± 0.18-fold; ±S.E.M., n = 6). However, SRC-1a is about as effective as TIF2 in causing a left-shift in the dose–response curve (2.45 ± 0.46-fold; ±S.E.M., n = 6, P = 0.025) and

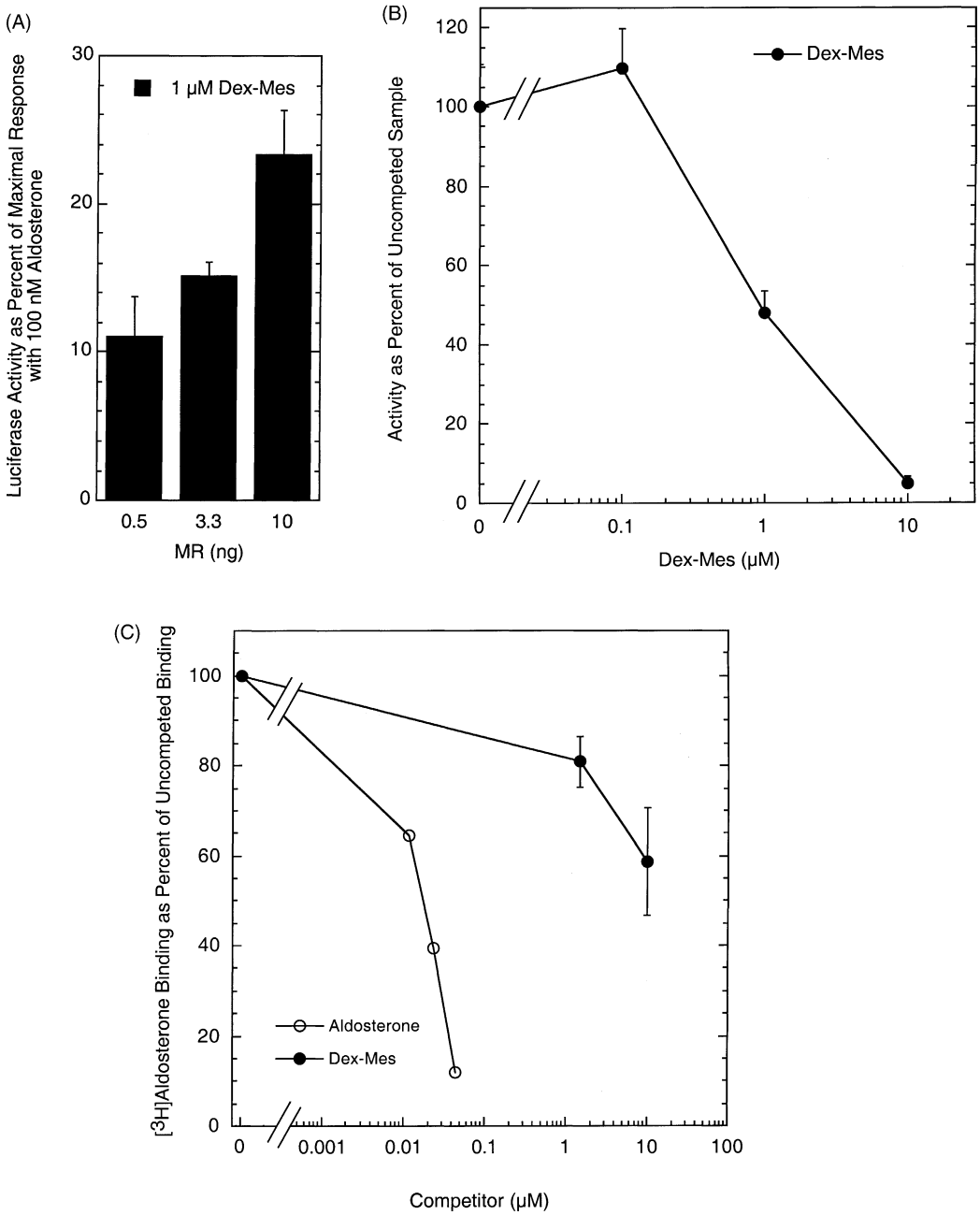


Fig. 2. Characterization of Dex-Mes as a new antimineralocorticoid. (A) Changes in partial agonist activity for Dex-Mes induction of MMTVluc in the presence of increasing amounts of transiently transfected MR. Triplicate dishes of CV-1 cells were transiently transfected with the given amounts of MR plasmid plus enough empty vector to maintain a constant molar amount of vector DNA, 1 μg of MMTVluc, and 200 ng of Renilla TK. The cells were induced and assayed as in Fig. 1. The Luciferase values for 100 nM aldosterone and 1 μM Dex-Mes with each concentration of MR plasmid were normalized for Renilla expression and presented as percent of the maximal response seen with 100 nM aldosterone (±S.D.). Similar results were obtained in four additional experiments. In the absence of transfected MR plasmid, there is no induction by either 100 nM aldosterone or 1 μM Dex-Mes (data not shown). (B) Whole cell competition of MR–aldosterone complex induction of a MMTVluc reporter by Dex-Mes. Triplicate dishes of CV-1 cells were transiently transfected with 3.3 ng of MR plasmid, 1 μg of MMTVluc, and 200 ng of Renilla TK. The cells were induced with 1 nM aldosterone plus the indicated concentrations of Dex-Mes and assayed as in Fig. 1. The Luciferase values at each concentration of Dex-Mes were normalized for Renilla expression and presented as percent of the uncompeted response seen with 1 nM aldosterone (±S.D.). Similar results were obtained in a second independent experiment. (C) Cell-free competition of [^{3}H]aldosterone binding to MR by Dex-Mes. Duplicate samples of COS-7 cell cytosol containing over-expressed MR were incubated at 0 °C with 13 nM [^{3}H]aldosterone ± the given concentrations of non-radioactive steroid. The samples were processed as described in Materials and Methods. The specific binding was determined by subtracting the non-specific binding of 13 nM [^{3}H]aldosterone + 1.5 μM non-radioactive aldosterone from each sample and then expressing the difference as percent of uncompeted binding. The average of two independent experiments (±range) was then plotted against the concentration of each competitor.

an increase in the partial agonist activity of Dex-Mes (5.2 ± 1.6-fold; ±S.E.M., n = 3) (Fig. 3C). Thus, co-activators in general appear both to shift the EC_{50} for transactivation by agonists to lower steroid concentrations and to increase the partial agonist activity of antisteroids, just as has been reported for GRs and PRs [7,15,16,19,22].

More MR causes both increased amounts of total transactivation and changes in the EC_{50} of agonists and the partial agonist activity of antagonists (see Fig. 1). While, co-activators have been reported not to influence the levels of ERs and GRs [11,41,42], it is formally possible that the present coactivator-induced responses reflect an elevation in the number of functional MR receptors by added co-activators. That this is not the case is indicated by Western blots showing no change in MR protein levels ± cotransfected TIF2 (Fig. 3D). However, protein levels determined by Western blotting are not indicative of the amount of functionally active receptors. For example, ≤10% of the GR protein over-expressed in SF9 cells is functionally active ([43] and data not shown). We therefore examined the amount of biologically active MR ± cotransfected co-activators. If added co-activators are increasing the amount of functional MRs, then there should be a direct correlation between amount of transfected co-activator plasmid and the total amount of induced gene product, similar to that seen in Fig. 1. In this case, the magnitude of changes in EC_{50} and partial agonist activity will be proportional to the increases in total gene activity by MR. This approach is analogous to that where the ability of estrogens to induce PRs [44] is used routinely to determine the functional ER levels in breast cancer tissues [45]. Therefore, we compared the total levels of transactivation at saturating concentrations of aldosterone, which would indicate the quantity of functional receptors, to the changes in EC_{50} and partial agonist activity in cells containing different amounts of factor added to a constant level (3.3 ng) of MR plasmid. As shown in Table 1, either 50 ng of TIF2 or 6.7 ng of extra MR each augment, the total amount of transactivation of 3.3 ng MR to a similar level (P = 0.9), consistent with the presence of similar quantities of functional MR. Nevertheless, the change in EC_{50} to lower steroid concentrations is significantly greater for TIF2 than for added MR (P = 0.011). Conversely, 50 ng of SRC-1 is much less effective in increasing the total levels of transactivation than is 6.7 ng of MR (P = 0.0002), which indicates fewer functional MRs with added SRC-1. However, SRC-1 causes a greater shift in the dose–response curve to lower EC_{50}s, than does additional MR (P = 0.026). Finally, 50 ng of TIF2 and SRC-1 each produce dramatically larger changes in the amount of Dex-Mes partial agonist activity than does 6.7 ng of MR even though their ability to augment gene expression (and, by these assumptions, the total functional MR) is the same or less than with added MR (Table 1). This lack of correlation between the ability of MR, TIF2, and SRC-1 to alter the dose–response curve (and partial agonist activity) of MR complexes and their ability to increase the level of transactivation, which is used as a measure of the amount of functional MR, indicates that the responses seen with added co-activators are not a consequence of co-activators altering the levels of functional MRs. Therefore, both by Western blotting (Fig. 3D) and by the more rigorous bio-activity assay, we conclude that the modulatory activity of TIF2 and SRC-1 is not due to their ability to increase the amount of transcriptionally active MR protein.

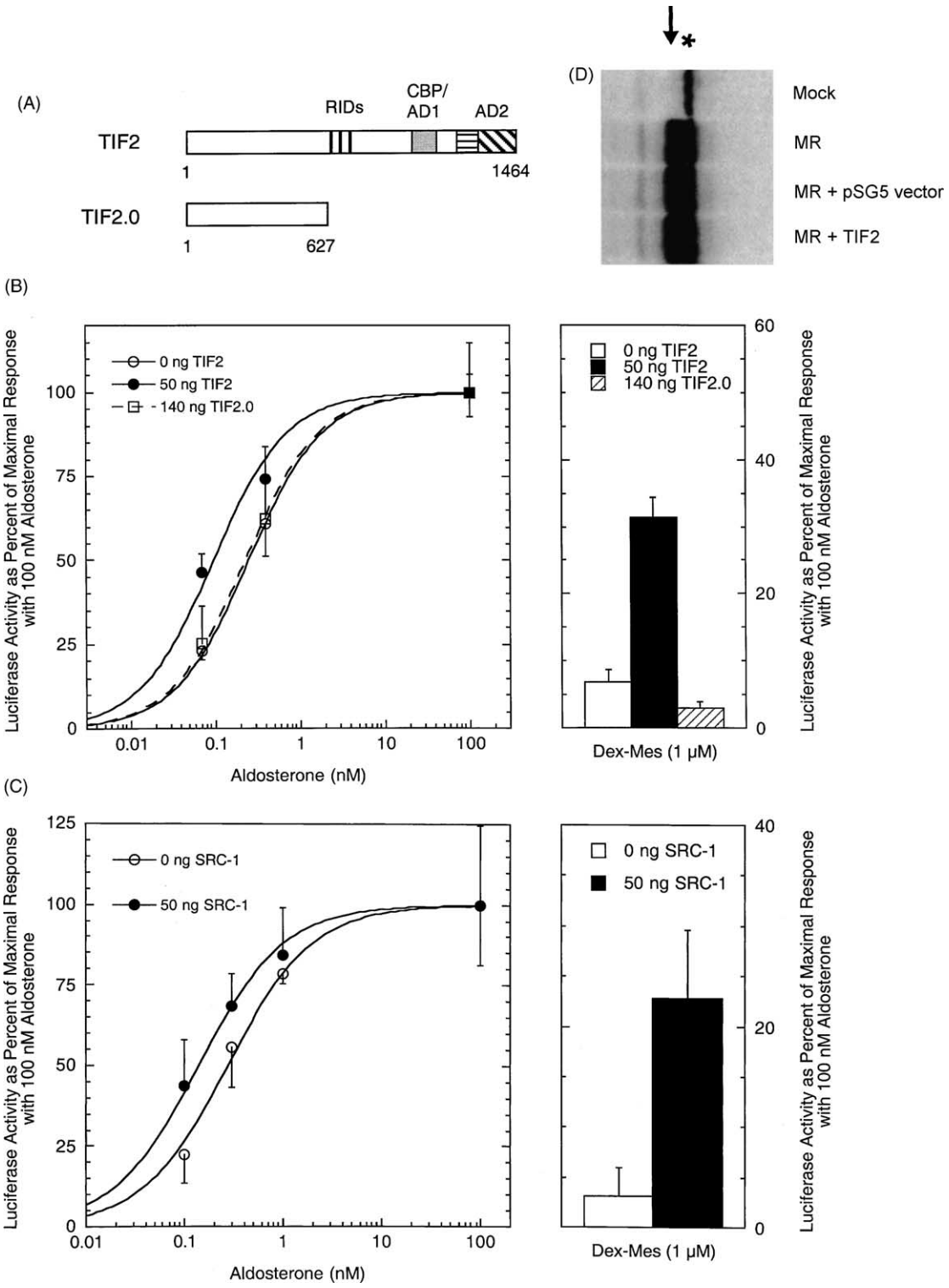
3.4. Modulation of MR induction properties by co-repressors

Co-repressors have not been reported to affect MR transactivation properties. However, we find that cotransfected co-repressor SMRT (40 ng) reduces the maximal amount of MR–aldosterone complex transactivation to 17 ± 3% (±S.E.M., n = 7, P < 0.0001) of the levels seen with an equimolar amount of plasmid containing human serum albumin (hSA) instead of SMRT. Thus, SMRT displays the expected behavior of a co-repressor by reducing the total

Table 1
Test of ability of added factors to increase levels of functionally active MR

Fold increase in total		Fold reduction	DM agnoist activity (%)	
3.3 ng MR plus	Transactivation	in EC_{50}	No factor	Plus factor
None	1	1	–	–
6.7 ng MR (n = 6)	2.96 ± 0.19	1.28 ± 0.16	24.1 ± 4.0	26.0 ± 4.3
50 ng TIF2 (n = 3–4)	3.63 ± 1.16 (P = 0.90*)	2.34 ± 0.31 (P = 0.011)	8.30 ± 2.18	39.8 ± 8.4
50 ng SRC-1 (n = 5–6)	1.42 ± 0.18 (P = 0.0002)	2.45 ± 0.46 (P = 0.026*)	2.39 ± 1.40	17.2 ± 2.0

The data from all of the experiments portrayed in Fig. 1B, 3B, and 3C are averaged (S.E.M.). The fold increase in total transactivation equals the total activity with 100 nM aldosterone in the presence of added factor divided by that seen with no factor. The fold reduction in EC_{50}, which is equivalent to the fold left-shift of the dose–response curve, equals (EC_{50} without factor)/(EC_{50} with factor). The changes in the partial agonist activity of Dex-Mes are listed as the absolute values for each treatment. The variations in partial agonist activity with no added factor (anywhere from 2.39 to 24.1%) arise because unequal amounts of various vectors were added to each control (3.3 ng MR plus no added factor) to compensate for the addition of the different vectors of the MR, TIF2, and SRC-1 plasmids. The values in parentheses indicate the number (n) of experiments and the P values for the comparison of each value (with TIF2 or SRC-1) to that with added MR (asterisk [*]indicates Mann–Whitney test, all others are Student t-test).



amount of induced gene transcripts [2–5]. Under these conditions, SMRT also causes a 2.77 ± 0.45-fold (P = 0.0077) right-shift in the dose–response curve and a decrease in the partial agonist activity of Dex-Mes to 44 ± 19% (P = 0.025) of the control value (Fig. 4A). This behavior is virtually identical to what we see with SMRT and GRs [15,20].

Under comparable conditions, the related co-repressor NCoR [12,13], has much less of an effect on MR transcriptional properties. The maximal amount of transactivation by MR is reduced by 25 ± 4% and 44 ± 4% (S.E.M., n = 5, $P \leq 0.0041$) with 17 and 100 ng of NCoR plasmid, respectively. This reduction of gene product is the classical behavior of a co-repressor [2–5] and indicates that functional NCoR

is being over-expressed. Additionally, there is a very weak, but statistically significant, right-shift of the dose–response curve to higher EC_{50}s with 17 ng of NCoR (1.43 ± 0.11-fold, ±S.E.M., n = 5, P = 0.017), but not with higher amounts (100 ng) of NCoR (1.27 ± 0.20-fold, P = 0.26) (Fig. 4B). Thus, conditions that further decrease the levels of total activation by saturating concentrations of aldosterone, and could arise from reduced amounts of functional MR, have no effect on the position of the dose–response curve. This result further supports the above conclusion from Table 1, that the changes in MR EC_{50} and partial agonist activity produced by exogenous co-factors are not simply the result of varying the amount of functionally active MRs.

Neither concentration of NCoR causes any significant change in the partial agonist activity of the antimineralocorticoid Dex-Mes (Fig. 4B). A similar unresponsiveness of GR induction of a GREtkLUC reporter in the presence of added NCoR has been communicated as data not shown [17]. In order to directly compare the behavior of GRs and MRs, we now examined the effect of added NCoR on GR using the same MMTVLuc reporter as for MRs above. As with MR, there is a very weak right-shift in the GR dose–response curve with 17 ng of added NCoR (1.11 ± 0.03-fold, S.E.M., n = 4, P = 0.037), but no significant effect with 100 ng of NCoR (0.92 ± 0.05-fold, ±S.E.M., n = 4, P = 0.21). With both amounts of NCoR, there is no consistent effect on the partial agonist activity (0.89–0.99 ± 0.04-fold reduction, n = 4) (Fig. 4C). The relative inability of NCoR to modulate GR induction properties of GREtkLUC and MMTVLuc argue that this behavior of GR with NCoR is independent of the reporter construct. More importantly, the sensitivity of MR transactivation properties to the co-repressors SMRT and NCoR are significantly different but, in each case, essentially identical to what is seen with GRs [15,17].

3.5. Effects of ER concentration on the dose–response curve of ER-agonist complexes

Recently, some of us have reported that increased concentrations of transiently transfected ERα in a line of human breast cancer (MDA-MB-436) cells expressing low levels of the co-activator AIB1 [11] causes a left-shift in the dose–response curve for estradiol induction of an estrogen-responsive reporter [46]. Unfortunately, the antiestrogen 4-hydroxy-tamoxifen did not show any partial agonist activity under any conditions in the breast cancer cells. We therefore looked at the effects of increased levels of transiently transfected ERα in the same CV-1 cells that were used above with MRs to see if we could extend these observations. Using concentrations of ERα plasmid that are not limiting and gave progressively higher amounts of induced gene transcripts, a five-fold higher amount of transfected ER causes a 4.29 ± 0.58-fold (±S.E.M., n = 11, P = 0.0002) left-shift in the dose–response curve in CV-1 cells (data not shown). Thus, the ability of higher concentrations of ER to modulate the ER dose–response curve appears to be independent of the cell line. Again, however, the antagonist 4-hydroxy-tamoxifen did not show any partial agonist activity. Therefore, we cannot yet say whether higher ER concentrations also affect the partial agonist activity of antiestrogens.

Fig. 3. Effect of added co-activators on MR transactivation properties of transiently transfected reporters. (A) Cartoon of TIF2 co-activator constructs used. The relative size of full length TIF2, and the truncated TIF2.0, is shown along with various domains of TIF2 (solid bars = RIDs, stippled box = activation domain 1 [AD1] and CBP binding domain, horizontally striped box = polyglutamine region, diagonally striped box = activation domain 2 [AD2]). (B) Modulatory effects of wild-type and truncated TIF2 on MR transcription properties. Triplicate dishes of CV-1 cells were transiently transfected with 3.3 ng of MR plasmid, the indicated amount of TIF2 plasmid or enough hSA in the same vector to have a constant molar amount of vector, 1 μg of MMTVluc, and 200 ng of Renilla TK. The cells were induced with different concentrations of aldosterone or Dex-Mes and assayed as in Fig. 1. The Luciferase values were normalized for Renilla expression and expressed as percent of the maximal response seen with 100 nM aldosterone (±S.D.) and plotted against the concentration of steroid. Similar results were seen in two (one for TIF2.0) additional experiments. (C) Modulatory effects of SRC-1 on MR transcription properties. Triplicate dishes of CV-1 cells were treated, analyzed and plotted as in Fig. 3B using SRC-1 instead of TIF2 plasmid, or enough hSA in the same vector to have a constant molar amount of vector. Similar results were seen in five additional experiments for the dose–response curve and two further experiments for the partial agonist activity of Dex-Mes. (D) MR protein levels ± cotransfected TIF2. COS-7 cells in 60 mm plates were transiently transfected with no DNA (Mock) or 1.5 μg of MR plasmid ± 1.5 μg of TIF2 plasmid or 0.75 μg of vector (pSG5) plasmid. Cytosols prepared and the MR proteins were separated on SDS–PAGE gels and detected by Western blotting as described in the Materials and Methods. The position of MR protein is indicated by the arrow at the side of the blot. The asterisk (*) marks a non-specifically detected band.

4. Discussion

Recent reports indicate that the EC_{50} of agonists, and/or the partial agonist activity of antagonists, bound to several steroid receptors (AR, ER, GR, and PR) are modified by varying the concentration of the homologous receptor, of co-activators, and of co-repressors (reviewed in [7]). We now report that a similar behavior is displayed by MR and by ER in a second cell line. The breadth of this study with MR was made possible by our discovery of Dex-Mes as a new antimineralocorticoid with much more partial agonist activity than the antimineralocorticoid spironolactone. Thus, the ability to modulate the partial agonist activity of antagonist complexes and/or the EC_{50} of agonist complexes is general for all of the classical steroid receptors.

The antimineralocorticoid spironolactone displays no partial agonist activity under any condition in our systems. For this reason, our characterization of Dex-Mes as a new antimineralocorticoid with partial agonist activity was of major importance because it allowed us to ask whether higher concentrations of MR or co-factors also increase the partial agonist activity of Dex-Mes. As seen in Figs. 2–4, they do. The affinity of Dex-Mes for MRs is low (about 750-fold less than that of aldosterone [Fig. 2C]), but the higher amount of

partial agonist activity than other common antimineralocorticoids at the same concentration [39] makes Dex-Mes a very useful research tool.

The capacity of several transcriptional properties of steroid receptors to be modulated by receptor and co-factor concentration confers numerous regulatory benefits to cells. It is well-known that elevated levels of receptor and co-activators can augment the amount of total gene activation seen with saturating, or pharmacological, concentrations of agonist [2–5,15,16,22]. Our data indicate that the EC_{50} for the induction of a given responsive gene can be significantly altered when tissues possess dissimilar quantities of receptor or co-factors. The resulting unequal positioning of the dose–response curve changes the level of induction of the same gene in various tissues in response to a single sub-saturating, physiological concentration of steroid hormone. Consequently, the common circulating concentration of steroid can differentially regulate the expression of the same gene amongst a variety of cells and tissues. Within a given cell, the differential expression of multiple genes can be accomplished through the action of gene-specific DNA elements and their associated proteins [7,47–50] along with

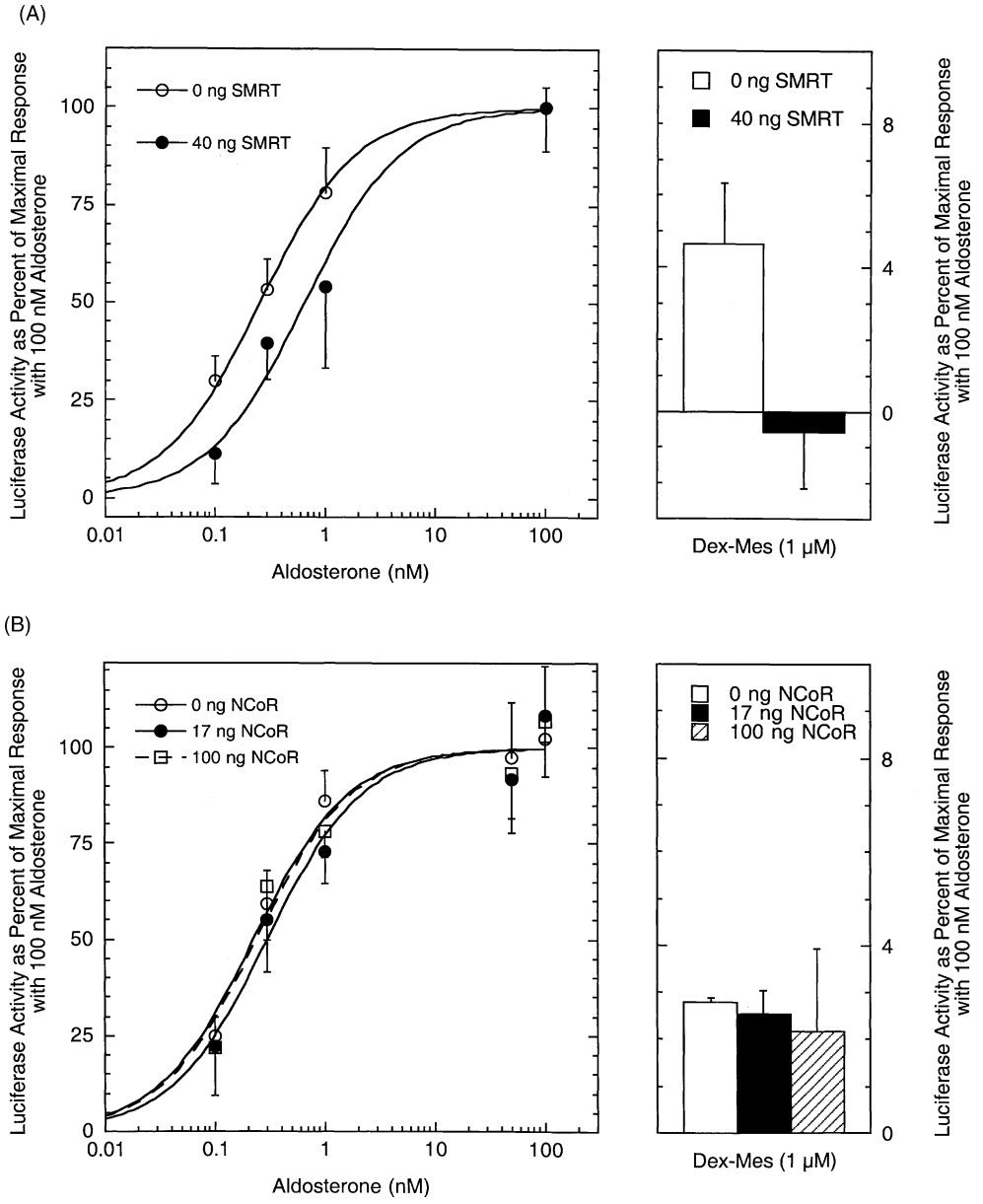


Fig. 4. Effect of added co-repressors on MR and GR transactivation properties of transiently transfected reporters. Modulatory effects of SMRT (A), and NCoR (B), on MR transcription properties. Triplicate dishes of CV-1 cells were treated, analyzed and plotted as in Fig. 3B using SMRT or NCoR instead of TIF2 plasmid, or enough hSA in the same vector to have a constant molar amount of vector. Similar results were seen in six additional experiments with SMRT and four additional experiments with NCoR. (C) Modulatory effects of NCoR on GR transcription properties. Triplicate dishes of CV-1 cells were treated, analyzed and plotted as in Fig. 3B using 40 ng of GR plasmid and the indicated amounts of NCoR instead of TIF2 plasmid, or enough hSA in the same vector to have a constant molar amount of vector. Similar results were seen in three additional experiments.

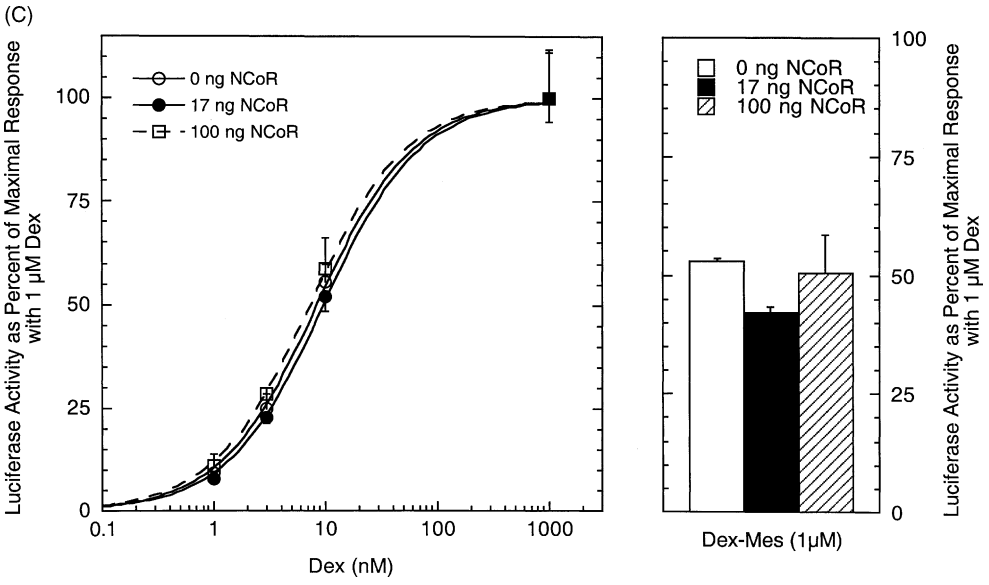


Fig. 4. (*Continued*).

DNA-induced conformational changes that modify co-factor affinity for the DNA-bound receptors [51–53]. Similarly, variations in the partial agonist activity of antisteroids are of immense importance for endocrine therapies of a variety of human conditions such as conception, breast cancer, inflammation, salt and water retention. An antisteroid that possesses partial agonist activity for many genes and blocks the induction of only a small number of genes will have many fewer adverse side-effects than an antagonist that inhibits all of the genes that are induced by a given steroid–hormone. The fact that the modulation of the EC_{50} of agonists, and the partial agonist activity of antagonists, is now documented for MR indicates that expanded regulatory mechanisms for the control of gene expression is a general feature in the action of all classical steroid receptors. However, the phenomena may not be limited to the steroid receptors. It has been reported that the p160 co-activators cause a left-shift in the dose–response curve of agonist complexes of the vitamin D receptor [54]. Therefore, it will be very interesting to see, if the modulation of steroid receptor transactivation properties is also possible for other nuclear receptors, such as the thyroid, retinoic acid, and PPAR receptors.

The co-activator GRIP1/TIF2 was reported to cause a left-shift in the dose–response curve of MR in yeast [41]. However, the properties of receptors in yeast and mammalian cells can be subtly different. For example, the potent glucocorticoid dexamethasone has little affinity for GRs over-expressed in yeast [55,56], even when transport of the steroid out of the cell is reduced [57]. Also, the antiglucocorticoids deoxycorticosterone and progesterone [58], often show more agonist activity in yeast than the conventional glucocorticoid agonists such as cortisol, dexamethasone, and triamcinolone acetonide [56]. Thus, it was not clear what the response of MRs would be in mammalian cells to increased levels of co-activator. Here, we have demonstrated that the co-activator TIF2/GRIP1 causes a left-shift in the MR dose–response curve in mammalian cells, just as described in yeast cells. Likewise, the co-activator SRC-1 also shifts the MR dose–response curve to the left to lower steroid concentrations. Therefore, we predict that the final p160 co-activator, AIB1, will also reposition the MR dose–response curve to lower steroid concentrations.

The co-repressor SMRT can move the dose–response curve of PR- and GR-agonist complexes to the right to higher steroid concentrations and decrease the partial agonist activity of antisteroid complexes [15,22]. A very similar response is seen with MRs in the current study. As far as we are aware, this is the first report of co-repressors affecting MR transactivation properties. Interestingly, the co-repressor NCoR has little activity with either MR or GR (Fig. 4B and C). This appears to be due to the inability of transfected NCoR to cause a significant increase in the already high level of endogenous NCoR in CV-1 cells [20].

The modulatory activity of exogenous MR, and co-activators and co-repressors, on the MR dose–response curve and partial agonist activity is independent of the ability of each factor to augment the total amount of transactivation (Table 1). In fact, the common practice of plotting dose–response curves as a percent of maximal induction by saturating concentrations of agonist eliminates all references to the absolute amount of gene activation. This separation of responses has also been seen in the modulation of the transcriptional properties of GRs [15,16,19,34,48,59,60] and PRs [17,22], and argues that the modulation of receptor properties occurs via a different mechanism than the elevation of the total level of receptor-mediated gene transactivation.

So far, we have not been able to find any differences in the responses of MRs versus GRs to changing concentrations of homologous receptor, the co-activators TIF2 or SRC-1, or the co-repressors SMRT or NCoR. This contrasts with the

unequal effects of the co-repressors SMRT and NCoR on GR and PR induction of a common reporter in the same cells [17]. Our inability to find any factor that affects MRs differently from GRs leads us to conclude that the unique transcriptional activities of GRs and MRs [26,29] derive from the interactions of either other co-factors or other mechanisms. For example, GR and MR are known to heterodimerize and several reports suggest that the responses of the heterodimers are different from those of the homodimers [61–64]. Also, protein kinase C-related kinase 1 augments agonist-induced gene transactivation by progesterone and mineralocorticoid receptors but not glucocorticoid receptors [65].

Estrogen receptors (ERs) are classical steroid receptors that bind steroidal ligands but, in many ways, are more similar to the nuclear receptors than to the other steroid receptors, AR, GR, MR, and PR. Like the nuclear receptors, ligand-free ERs are predominantly nuclear. The three amino acids in the distal knuckle of the first zinc finger of the DNA-binding domain, which determine the binding specificity of receptors to DNA sequences, are more closely related for ER and nuclear receptors (glutamic acid/glycine/alanine [EGA] versus EGS [S = serine], respectively) than is the glycine/serine/valine (GSV) sequence that is employed by the other steroid receptors. Finally, the consensus DNA sequence to which ER binds is the same as that for nuclear receptors (TGACCT) and significantly different from that for the other steroid receptors (TGTTCT) [24]. Therefore, it was very interesting to find that the dose–response curve of ER-agonists is also shifted to lower EC_{50} values by increasing concentrations of ERα. This behavior is not unique to ER in CV-1 cells, but is also seen for ER in a line of human breast cancer (MDA-MB-436) cells expressing low levels of the co-activator AIB1 [46]. In both studies, the lack of partial agonist activity with Tamoxifen-bound ERs unfortunately prevented our determining if there are effects of receptor concentration on the partial agonist activity of ER-antagonist complexes. We have not examined the effects of added TIF2 or SRC-1 co-activators. However, Hanstein et al. have described an ERβ variant (β2) that contains an 18 amino acid insert in helix 6, has an 8-fold lower affinity than the wild-type ERβ for estradiol, and no longer binds the co-activator SRC-1. Interestingly, the dose–response curve for estradiol-bound ERβ2 is right-shifted from that of ERβ by a factor of 100–1000 [66]. This additional right-shift, which is 10- to 100-fold greater than that expected from the decreased steroid binding affinity, is precisely the predicted behavior if varied levels of SRC-1 do influence the position of the dose–response curve, as has been observed for GR, PR, and MR. Further studies are required to determine directly whether co-activators and co-repressors can alter the dose–response curve of ER complexes. Even more useful would be to employ systems in which antiestrogens display partial agonist activity and determine whether the partial agonist activity of ER-antagonist complexes can be modulated by varying the concentration of ER, co-activators, and co-repressors. Such variation would greatly expand the use of antiestrogens in the treatment of breast cancer by potentially reducing the number of non-target genes that would be suppressed.

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