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Modulation of transcription parameters in glucocorticoid receptor-mediated repression

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Abstract

Glucocorticoid receptors (GRs) affect both gene induction and gene repression. The disparities of receptor binding to DNA and increased vs. decreased gene expression have suggested significant mechanistic differences between GR-mediated induction and repression. Numerous transcription factors are known to modulate three parameters of gene induction: the total activity (V_{max}) and position of the dose-response curve with glucocorticoids (EC_{50}) and the percent partial agonist activity with antiglucocorticoids. We have examined the effects on GR-mediated repression of five modulators (coactivators TIF2 [GRIP1, SRC-2] and SRC-1, corepressor SMRT, and comodulators STAMP and Ubc9), a glucocorticoid steroid (deacylcortivazol [DAC]) of very different structure, and an inhibitor of histone deacetylation (trichostatin A [TSA]). These factors interact with different domains of GR and thus are sensitive topological probes of GR action. These agents altered the V_{max}, EC_{50}, and percent partial agonist activity of endogenous and exogenous repressed genes similarly to that previously observed for GR-regulated gene induction. Collectively, these results suggest that GR-mediated induction and repression share many of the same molecular interactions and that the causes for different levels of gene transcription arise from more distal downstream steps.

Keywords

glucocorticoid receptor; gene repression; coactivator and corepressor; STAMP and Ubc9; deacylcortivazol; dose-response curve

1. Introduction

Glucocorticoid receptor (GR)-regulated gene expression can proceed via either induction or repression. The inhibition of gene expression by GR is encountered during immunosupression and is the most commonly prescribed clinical use of glucocorticoid steroids. Thus, glucocorticoids are given to treat organ transplant rejection, autoimmune diseases (e.g., rheumatoid arthritis, myasthenia gravis, systemic lupus erythematosus, Crohn's disease, and ulcerative colitis), and non-autoimmune inflammations such as asthma (Schacke et al., 2007;

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Stahn et al., 2007). Conversely, glucocorticoid induction of surfactant protein in fetal lungs is a standard treatment of premature babies to avoid collapsed lungs (Haas et al., 2005). Despite the more numerous applications of glucocorticoids in scenarios involving gene repression, most basic research on the mechanism of GR action has utilized gene induction models.

The initial steps for GR-regulated gene induction and repression appear to be the same. Steroid binds to the predominantly cytoplasmic receptor protein. The receptor-steroid complex is then activated, binds to chromatin to recruit various cofactors to modify chromatin organization, and interacts with the transcriptional machinery to alter the basal level of gene transcription (Beato, 1989; Chen et al., 2006; Rosenfeld et al., 2006). Nevertheless, there are several salient differences. The most obvious is that repression decreases, while induction increases, gene expression. The repression of some genes, such as proliferin (Diamond et al., 1990) and POMC (Drouin et al., 1993), has been reported to occur via DNA-bound GR, as for induction. However, the majority of repressed genes are thought to involve GR tethered to other DNA-bound proteins such as AP1 and NF-κB (Webster and Cidlowski, 1999; Jakacka et al., 2001). The greater variety of DNA sequences that bind AP1 and NF-κB vs. GR has contributed to the preference of studying GR-induced genes. The potency is also often different for induction vs. repression. There is an inverse relationship between potency (i.e., the concentration of steroid required for half maximal response, also referred to as EC_{50}) and EC_{50}. Thus, the more potent the steroid, the lower its EC_{50}. Gene induction usually requires a 10-fold higher steroid concentration than gene repression, even though the same GR-steroid complex is involved (Wei et al., 1998; Nye et al., 2002; Koubovec et al., 2005; Smit et al., 2005; Tao et al., submitted; Ronacher et al., submitted). For example, the EC_{50} for gene induction in cultured cells by the potent synthetic glucocorticoid dexamethasone (Dex) is typically about 1 nM while the EC_{50} for gene repression is often around 0.1 nM. Finally, several point mutations selectively inhibit GR-mediated induction vs. repression (Liu et al., 1995; deLange et al., 1997; Reichardt et al., 1998; Ray et al., 1999; Rogatsky et al., 1999; Tao et al, submitted). These differences suggest that at least a few of the details for GR induction and repression proceed along dissimilar pathways that should be exploitable for beneficial clinical outcomes (Schacke et al., 2004; Wang et al., 2006; Miner et al., 2007). These differences also underscore the importance of examining GR actions at physiological concentrations of steroids, which could easily lead to new applications.

Our studies of GR-regulated gene induction at physiological levels of steroid have documented that the EC_{50} for gene induction by agonists can be significantly altered simply by varying the concentration of several transcription factors/comodulators, such as the receptor itself, p160 coactivators, corepressors, Ubc9, and STAMP (reviewed in refs. Simons; Jr., 2003; Simons; Jr., 2006; Simons Jr., submitted). Ubc9 is a human homolog of the yeast E2 ubiquitin-conjugating enzymes but has many effects that are independent of this enzymic activity (Kaul et al., 2002a; Yokota et al., 2007). STAMP is a co-modulator of the actions of the coactivator TIF2 (GRIP1, SRC-2) with GRs and binds GR and TIF2 (He and Simons; Jr., 2007). Many of these factors also modulate the EC_{50} of gene induction by androgen, estrogen, mineralocorticoid, and progesterone receptors (Simons; Jr., 2003; Simons; Jr., 2006; Szapary et al., 2008; Simons Jr., submitted). This suggests that this ability of transcription factors to alter the EC_{50}, and the position of the dose-response curve, may be general for all of the classical steroid receptors.

An antisteroid is defined as any steroid that prevents the action of an agonist steroid. Many antisteroids display some agonist activity and are therefore also called partial agonists or selective receptor modulators. Here, we quantitate the partial agonist activity as the percent of maximal induction by a full agonist steroid under the same conditions, i.e., the percent partial agonist activity of the antisteroid. Interestingly, the above factor-induced modifications in EC_{50} are almost invariably accompanied by reciprocal changes in the percent partial agonist

activity of the antisteroid. Thus, when the EC_{50} for gene induction by an agonist decreases, the percent partial agonist activity of an antisteroid increases, and *vice versa*. This property also seems to be general for the classical steroid receptors (reviewed in refs. Simons; Jr., 2003; Simons; Jr., 2006; Simons Jr., submitted).

The commonly prescribed doses of glucocorticoids to suppress autoimmune diseases and inflammation are hyper-physiological and greater than or equal to twice the daily output of cortisol in humans). This is in contrast to the circulating concentrations of hormone that do not saturate GR and are required for proper control of gene expression during development and homeostasis. It has been estimated that up to 10% of the genome in human T-lymphoblastoid cells is regulated by glucocorticoids (Galon et al., 2002) and that approximately 1000–2000 genes in rat cortical neuronal cells (Kino et al., 2007) and human U2OS osteosarcoma cells (Lu and Cidlowski, 2005) are under glucocorticoid control, with about an equal number being repressed and induced. Thus, extensive physiologically relevant gene repression is expected at steroid concentrations much lower than frequently prescribed dosages. This indicates a compelling need to determine whether various factors that modulate the EC_{50} and percent partial agonist activity in GR-controlled induction are similarly effective in GR-regulated repression. Surprisingly, the research to date in the field is very limited. The only two studies of which we are aware for GR-mediated repression report that increasing concentrations of GR (Zhao et al., 2003) or SRC-1 (van der Laan et al., 2008) cause a left-shift of the EC_{50} to lower steroid concentrations. Earlier studies with the GAL DNA binding domain (DBD) fused to the GR ligand binding domain (LBD), plus 25 upstream amino acids (i.e. amino acids 525 to C-terminus), to give GAL/GR525C revealed that the induction properties (EC_{50} and percent partial agonist activity) of this chimera could be modulated by increasing concentrations of the chimeric GR, the p160 coactivator TIF2, and Ubc9 (Cho et al., 2005). The GR segment of GAL/GR525C does not directly bind to DNA and thus mimics the tethered status of GRs associated with DNA-bound AP1 or NF-κB in GR-mediated repression. Why the covalently tethered GAL/GR525C induces gene transcription, while the non-covalently tethered GR-AP1 or -NF-κB complexes represses transcription, is not clear. Nevertheless, the behavior of GAL/GR525C suggests that the EC_{50} and percent partial agonist activity of GR-repressed genes may also be capable of modulation.

The objective of the current study, therefore, is to determine if several modulatory factors (TIF2, SRC-1, SMRT, STAMP, and Ubc9) are as active in gene repression by GRs as they are in gene induction. A positive result would suggest that many of the molecular details of induction are preserved in repression. Thus, a positive result would greatly expand our knowledge of the mechanism of action of GRs during the repression of gene transcription even though precise descriptions of the factors' actions in gene induction remain unavailable. We report that a variety of factors modulate GR repression properties in much the same manner as seen earlier for gene induction. We conclude that several of the molecular mechanisms responsible for altering the V_{max}, EC_{50}, and percent partial agonist activity during gene induction by GRs are shared by GRs causing gene repression.

2. Materials and Methods

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

2.1. Chemicals

Dexamethasone (Dex) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Promegestone (R5020) was from PerkinElmer Life Sciences (Boston, MA). Cortivazol (gift from Roussel UCLAF) was converted to deacylcortivazol (DAC) by Dr. Craig Thomas (NIDDK/NIH, Bethesda, MD. The dual-luciferase reporter assay system is from

Promega (Madison, WI). Restriction enzymes and digestions were performed according to the manufacturer's specifications (New England Biolabs, Beverly, MA).

2.2. Plasmids

The Renilla null luciferase reporter was purchased from Promega. Rat GR (pSG5-GR) and GREtkLUC (He et al., 2002), pSG5/rUbc9 (Kaul et al., 2002b), and hemagglutinin (HA) tagged STAMP (pSG5/HA-STAMP) (He and Simons; Jr., 2007) have been previously described. TIF2/GRIP1 (Hinrich Gronemeyer, IGBMC, Strasbourg, France, and Michael Stallcup, UCS School of Medicine, Los Angeles, CA), NCoR (Michael Rosenfeld, University of California, San Diego, CA), s-SMRT (Ron Evans, Salk Institute, La Jolla, CA) were generously donated. pSG5/SRC1 was constructed by digesting pCDNA3-SRC1 (Sergio Onate, Univ. of Pittsburgh, Pittsburgh, PA) with Bam H1 and Xba I and cloning into BamH1/Bgl II digested pSG5 vector (Michael Stallcup).

2.3. Cell culture, transient transfection, and reporter analysis

U2OS human osteosarcoma cells containing stably transfected rat GR gene (U2OS.rGR cells) (Rogatsky et al., 1997) were transiently transfected at 37 °C with a slight modification of the original procedure (Rogatsky et al., 2002). Thus, triplicate samples were seeded into 24-well plates in Dulbecco's modified Eagle's medium (DMEM) with10% fetal bovine serum (FBS) at 30,000 cells per well and transfected with reporter (100 ng of GREtkLUC or 20 ng of AP1Luc), 10 ng of phRG-TK Renilla (Promega) as an internal control, and the indicated amounts of plasmids for various factors on the following day in FBS-free DMEM by using 0.8μl of Lipofectamine and 1.6μl of PLUS reagent (Invitrogen) per well according to the manufacturer's instructions. The molar amount of plasmids expressing different protein constructs was kept constant with added empty plasmid or plasmid expressing human serum albumin (hSA). The total transfected DNA was adjusted to 150 ng/well of a 24-well plate with pBluescriptII SK (Stratagene). After transfection (4 h), cells were refed with DMEM/10% FBS, allowed to recover for 2 h, and fresh DMEM/10% FBS was added containing the appropriate dilutions of steroid hormones. Sixteen hours later, the cells were lysed (for induction), or treated with 25 ng/ml PMA or vehicle for 2 hr (for repression), and assayed for reporter gene activity using dual luciferase assay reagents according to the manufacturer's instructions (Promega, Madison, WI). Luciferase activity was measured by an EG&G Berthold's luminometer (Microlumat LB 96P). In all cases, the data were normalized for Renilla TS activity, expressed as a percentage of the maximal response above basal activity, and then plotted to determine the EC_{50} and percent partial agonist activity. In gene repression, the maximal response is set as the activity without agonist steroid and the basal activity is the lower plateau activity seen with saturating concentrations of agonist steroid. For gene induction, the basal activity is that without steroid and maximal activity is that produced by saturating agonist steroid concentrations. The fold induction is (induced value)/(basal activity). Fold repression is similarly calculated as (basal activity)/(repressed value). The percent partial agonist activity of each antisteroid was calculated by expressing the activity of a saturating concentration of antagonist (1 μM) as percent of maximal repression (or induction) under the same conditions. For dose-response curves, each point is the average of triplicate samples ± S.D. For bar graphs giving average values of V_{max}, EC_{50}, and percent partial agonist activity, the average of n replicates (each in triplicate but considered, statistically, as one observation) was plotted ± S.E.M. (n observations) unless otherwise noted.

2.4. mRNA, total RNA extraction, and reverse transcription-PCR (RT-PCR)

RNA in U2OS.rGR cells was prepared by growing cells at 37 °C to confluence in 6-well plates for 2 days, lysing the cells with TRIzol (Invitrogen) reagent, and extracting the total RNA according to the manufacturer's instructions. First-strand cDNA was synthesized by

SuperScript III RNase H reverse transcriptase (Invitrogen). For quantitative real-time PCR (qRT-PCR), the relative levels of target mRNAs were quantitated using Taqman and the ABI 7900HT real-time PCR system. The primers for collagenase 3 and glyceraldehyde-3-phosphate dehydrogenase from ABI are Hs00233992-m1 and 4310884E respectively.

2.5. Statistical Analysis

Unless otherwise noted, each steroid concentration was analyzed in triplicate and this average treated as a single result. Best-fit dose-response curves (R^2 almost always ≥ 0.95) following Michaelis-Menten kinetics were obtained for each experiment with KaleidaGraph (Synergy Software, Reading, PA). In all types of experiments, the average value of n independent experiments under one condition is compared, one at a time, to a control and is analyzed for statistical significance by the two-tailed Student's t test using InStat 2.03 (GraphPad Software, San Diego, CA). When the difference between the SDs of the two populations was significantly different, the Mann-Whitney or Alternate Welch t test was used. A nonparametric test was used if the distribution of values was non-Gaussian.

Determining precisely whether the combined effect of two agents (i.e., SRC-1 and STAMP) that cause opposite responses is greater than predicted is not yet possible in the absence of a defined molecular mechanism. Mathematical approximations were made as follow. For EC_{50} values, which are not thought to be bounded by any constraints, we used geometric averaging (nth root of the product of n terms) to obtain the predicted EC_{50}. This cannot be used for the percent partial agonist, however, because there is an upper limit of 100% agonist activity. As GR-mediated gene induction (Kim et al., 2006) and repression appear to follow Michaelis-Menten kinetics, we chose to use an "odds of happening" calculation, where the odds of the percent partial agonist activity being determined by Control conditions, by SRC-1, and by STAMP are as follows:

odds for control = x = percent activity with control/(100-percent activity with control)

odds for SRC-1 = y = percent activity with SRC-1/(100-percent activity with SRC-1)

odds for STAMP = x = percent activity with STAMP/(100-percent activity with STAMP)

then, odds for SRC-1 plus STAMP = $(y - x) + (z - x) + x = y + z - x$

the fraction of activity with SRC-1 plus STAMP is then = $(y + z - x)/(1+ y + z - x)$

and the percent of partial agonist activity with SRC plus STAMP = $100(y + z - x)/(1+ y + z - x)$

3. Results

3.1. Modulation of endogenous coll3 gene expression by TIF2 and STAMP

Collagenase 3 is an endogenous gene of U2OS.rGR cells that is repressed by GRs (Rogatsky et al., 2001; Rogatsky et al., 2002; He and Simons; Jr., 2007). Using qRT-PCR to quantitate the levels of coll3 mRNA, we found that the EC_{50} (Fig. 1A), but not the fold repression (Fig. 1B), with the glucocorticoid Dex is altered by TIF2, STAMP, and the combination of the two. We define "fold repression" as (activity without saturating agonist steroid)/(activity with saturating agonist steroid). As this is also a measure of total agonist activity when the basal levels are the same, we use "fold repression" and V_{max} interchangeably in this report. Furthermore, either factor alone decreases the value of EC_{50} for Dex repression (Fig. 1C). TIF2 and STAMP together gave an approximately additive response that was significantly greater ($P \leq 0.031$) than for either factor alone. In all cases, the transfection efficiency was about 50%, as determined with exogenous green fluorescent protein (data not shown), so that only half of the cells contain transfected factor. This heterogeneity of coll3 repression does not alter the

sigmoidal nature of the dose-response curve but does mean that that the magnitude of the change in EC_{50} is, in each case, underestimated by a factor of two. We conclude that the EC_{50} of GR-mediated repression of the endogenous coll3 gene is similarly affected by TIF2 and STAMP as was reported for GR induction of a synthetic reporter (Fig. 1 and He and Simons; Jr., 2007).

3.2. Dose-dependent modulatory activity of TIF2 during GR-controlled repression of AP1/Luc

Most of the data on the ability of modulatory factors to alter the V_{max}, EC_{50}, and percent partial agonist activity of GR-controlled gene induction is with transiently transfected reporter genes (reviewed in refs. Simons; Jr., 2003; Simons; Jr., 2006; Simons Jr., submitted). In order to compare in detail the properties of GR-regulated repression and induction, and to eliminate the reduced sensitivity associated with endogenous genes when only 50% of the cells receive transfected factors, we sought a transfected reporter that was efficiently repressed by GRs. An excellent system for this is GR inhibition of AP1 mediated induction of the transiently transfected AP1Luc reporter by phorbol esters in U2OS human osteosarcoma cells (Rogatsky et al., 1998) containing stably transfected GRs (U2OS.rGR) (Rogatsky et al., 2001; Rogatsky et al., 2002; He and Simons; Jr., 2007; Tao et al., submitted). In this model system, the coactivator TIF2 shifts the dose-response curve to lower steroid concentrations (Fig. 2A) while causing the previously reported (He and Simons; Jr., 2007) increase in fold repression (Fig. 2B). In these same experiments, the percent partial agonist activity of the antiglucocorticoid progesterone (Prog) (Szapary et al., 1996; Sarlis et al., 1999; Szapary et al., 1999) increases with added TIF2 while the EC_{50} for Dex repression decreases (Fig. 2C left and right respectively). Progesterone displays a large response in these experiments and thus is preferable to the antiglucocorticoid Dex-Mes, which usually gives ≥70% partial agonist activity even without added cofactor. The alterations by 5 and 20 ng TIF2 of both the EC_{50} and the percent partial agonist activity of Prog are about the same (Fig. 2C). Thus, the effects of 5 ng TIF2 plasmid are nearly maximal. These modulatory properties of TIF2 in GR-regulated gene repression, and the reciprocal responses of EC_{50} and percent partial agonist activity, are identical to those reported for GR in gene induction (Szapary et al., 1999; Chen et al., 2000; He et al., 2002; Vottero et al., 2002; Wang et al., 2004; Cho et al., 2005).

3.3. Ability of STAMP to modulate GR repression parameters

The protein STAMP was discovered on the basis of its capacity to augment the modulatory activity of TIF2 in gene induction by GRs (He and Simons; Jr., 2007). This appears to proceed via the formation of a ternary GR/TIF2/STAMP complex. In our GR-mediated repression system, STAMP again increases the changes in GR properties beyond those seen with the maximal amount of TIF2, which was determined in Fig. 2 to be 20 ng. TIF2 by itself decreases the EC_{50} for repression by Dex (Figs. 3A&C right, $P = 0.014$). The minimal effect of TIF2 on the fold repression and percent partial agonist activity (Figs. 3B&C left) may result from the additional 100 ng of vector. Exogenous STAMP both increases the fold repression and percent partial agonist activity and decreases the EC_{50} (for all, $P \leq 0.0043$). Importantly, the combination of added STAMP and TIF2 has a statistically significant greater effect on all of the parameters than either factor by itself (Figs. 3B&C). Thus, the ability of STAMP to augment the modulatory activity of TIF2 in GR-mediated gene induction is recapitulated in gene repression. It should be noted that the increased activity of the fold repression and percent partial agonist activity when TIF2 is added to STAMP, but not when added alone, suggests that STAMP concentrations in U2OS.rGR cells are limiting here. Conversely, endogenous TIF2 concentrations also appear to be near maximal for modulatory activity unless more STAMP is added, in which case TIF2 now becomes limiting. This would explain why the combination of TIF2 plus STAMP produces a more than additive response.

3.4. Modulation of repression accommodates different steroid structures

The steroid deacylcortivazol (DAC) lacks the C-3 ketone of Dex and possesses a bulky A-ring substituent (Fig. 4A). As these two features are thought to be required for glucocorticoid activity (Rousseau and Schmit, 1977;Bledsoe et al., 2002;Kauppi et al., 2003), it would be predicted that DAC is an inactive glucocorticoid. In fact, DAC is perhaps the most potent glucocorticoid known for gene induction (Simons; Jr. et al., 1979). A recent x-ray structure reveals that this is possible due to a rearrangement of the GR LBD to accommodate the larger bulk of DAC vs. Dex (Suino-Powell et al., 2008). This reorganization of the LBD may contribute to inability of the C-21 mesylate derivative of DAC to covalently label GR (Lamontagne et al., 1984;Simons; Jr. and Miller, 1986) while the same derivative of Dex (Dex-21-mesylate, or Dex-Mes) is a very efficient affinity label (Simons; Jr. et al., 1987;Simons; Jr., 1987).

The induction parameters of the endogenous TAT gene in Fu5-5 vs. HTC cells are identically modulated by GR-DAC and -Dex complexes (Mercier et al., 1983), despite the rearranged LBD with DAC. However, not all genes are identically regulated by Dex and DAC (Miller et al., 2007). A different, but stringent, test of gene induction properties makes use of the known ability of elevated levels of GR receptor to increase the V_{max} and decrease the EC_{50} (Szapary et al., 1996; Chen et al., 2000; Kaul et al., 2002a). We therefore inquired whether varying the concentration of GR would similarly modulate the DAC induction properties of transiently transfected GREtkLUC reporter in CV-1 cells. When the ratio of each parameter with high GR/low GR concentrations is compared (Table 1A), higher GR concentrations are seen to produce the same increase in total amount of induction (V_{max}) and fold induction, and decrease in EC_{50}, as reported with Dex (Szapary et al., 1996; Szapary et al., 1999; Chen et al., 2000; Reichardt et al., 2000; Kaul et al., 2002b).

Another test of steroid structure in GR induction stems from the unusual properties of Ubc9. With low GR concentrations and added Ubc9, the V_{max} increases with no significant modification of EC_{50}. However with high GR concentrations, there is reduced effect on the V_{max} and a large decrease in EC_{50} for Dex-mediated induction (Kaul et al., 2002a; Cho et al., 2005). As shown in Table 1B, a ratio >1 for the V_{max} of low GR (0.1 ng) + DAC, but not the fold induction or EC_{50}, indicates an increase with added Ubc9. Conversely, with high GR (10 ng) bound by DAC or Dex, the ratio (+Ubc9/−Ubc9) for V_{max} hardly increases while values <1 indicate Ubc9-generated decreases in the fold induction and EC_{50}. These combined results lead us to conclude that Dex- and DAC-bound GR complexes are comparably modulated for the induction of our endogenous and exogenous test genes in more than one cell line.

We next asked if GR-DAC complexes, with an altered protein conformation, are capable of repressing the transiently transfected AP1Luc reporter in U2OS.rGR cells. If so, might the properties of gene repression by the rearranged GR-DAC complex be similarly prone to modulation by TIF2 and STAMP as were the Dex-bound receptors in Fig. 3? The results of Fig. 4B show that exogenous TIF2 and STAMP each shift the dose-response curve for repression by GR-DAC complexes to lower levels of DAC. The magnitude of this left-shift to lower EC_{50} values is significantly greater than that seen with either TIF2 or STAMP alone (Fig. 4D, right). At the same time, the increase in both fold repression by Dex (Fig. 4C) and percent partial agonist activity by 1 μM Prog (Fig. 4D, left) is significantly greater with TIF2 plus STAMP than that of either TIF2 or STAMP alone. These results indicate that repression by GR-DAC complexes is modulated as efficiently as for GR-Dex complexes. Thus, the structural changes caused by DAC vs. Dex binding to GR do not alter ability of TIF2 and STAMP to affect GR repression and this behavior may be general for all glucocorticoid agonist steroids.

3.5. Modulation by SRC-1 of GR complexes during repression by GRs

We next asked if another p160 coactivator, the 1441 amino acid SRC-1a, was as effective with STAMP as TIF2 (Fig. 3) in modifying the parameters of gene repression by GR-Dex complexes. This coactivator was selected because it had previously been shown to modify GR induction properties similarly to TIF2, with the C-terminal 303 residues of SRC-1 retaining most of the modulatory activity of the full-length protein (He et al., 2002). This C-terminal region is also known to possess histone acetyltransferase (HAT) activity (Spencer et al., 1997) and to bind to STAMP (He and Simons; Jr., 2007). Using the system of Fig. 3, we found that SRC-1 by itself, and unlike TIF2, effects a right-shift to higher EC_{50} values (Figs. 5A&C; $P = 0.018$ in Fig. 5C right). Exogenous SRC-1 also weakly decreases the percent partial agonist activity of Prog ($P = 0.30$; Fig. 5C, left) with little change in the fold repression (Fig. 5B) while STAMP increases both ($P \leq 0.030$). Nonetheless, STAMP plus SRC-1 causes a significantly larger change in both the percent partial agonist activity and the EC_{50} than expected from the sum of the effects of both individual factors (Fig. 5C). This suggests that SRC-1 cooperates with STAMP to augment STAMP actions, just as is seen with TIF2 and STAMP in Fig. 3.

3.6. Competition between added corepressors and coactivators for modulation of parameters of GR-mediated repression

Coactivators and corepressors competitively inhibit the actions of each other both in GR-mediated gene induction (Szapary et al., 1999; Wang and Simons; Jr., 2005) and in the association with GRs (Wang et al., 2004). It was therefore of interest to see if the corepressor SMRT could reverse the modulatory activity of the coactivator TIF2 in gene repression. When exogenous TIF2 and SMRT are each added, they produce, as expected, opposite responses relative to control for the fold repression by Dex (V_{max}; Fig. 6A) and for the percent partial agonist activity of Prog and EC_{50} of Dex-bound GR-mediated inhibition (Fig. 6B, left and right respectively). Importantly, the concomitant addition of SMRT and TIF2 gives an intermediate value in each case. We conclude that, as for GR-regulated gene induction, coactivators and corepressors can competitively inhibit the actions of each other during GR-mediated gene repression.

3.7. Effect of histone deacetylation on the modulation of GR-regulated gene repression

The inhibitory effects of corepressors on steroid receptor action are thought to involve the recruitment of HDACs, among other factors, and the subsequent deacetylation of promoter-bound histones (Burke and Baniahmad, 2000; McKenna and O'Malley, 2002; Rosenfeld et al., 2006). Histone deacetylation is well known to be associated with decreased gene expression and is considered to be mediated by histone deacetylases (HDACs) that are often inhibited by trichostatin A (TSA) (Alland et al., 1997; Burke and Baniahmad, 2000). In GR-controlled gene induction, TSA often augments gene expression but does not increase the EC_{50} or lower the percent partial agonist activity of antiglucocorticoids with or without added corepressor SMRT (Wang et al., 2004; Kim et al., 2006). We therefore asked whether TSA would modify these parameters in GR-repression of AP1Luc induction by PMA. This was examined both without and with added STAMP, which increases the repressive activity of GR in this system (Fig. 3–Fig. 5 and He and Simons; Jr., 2007). There is little ability of up to 300 nM TSA to either increase PMA induction or decrease Dex repression with or without added STAMP (Fig. 7A). The same TSA concentrations ± STAMP also produce negligible change in either the percent partial agonist activity of Prog or the EC_{50} of Dex repression (Fig. 7B). Finally, TSA does not prevent STAMP from increasing the fold repression by Dex (V_{max}), augmenting the percent partial agonist activity of Prog, or decreasing the EC_{50} of gene inhibition by Dex. We conclude that, similar to GR-controlled induction, these parameters of GR-regulated repression, and the ability of STAMP to modulate them, are independent of TSA-inhibited histone deacetylation.

3.8. Activity of Ubc9 in modulating gene repression by GR

In the presence of sufficiently high GR concentrations, Ubc9 increases the percent partial agonist activity of antagonists and decreases the EC_{50} while slightly augmenting the V_{max} of gene induction by glucocorticoids (Kaul et al., 2002a; Cho et al., 2005; Szapary et al., 2008). U2OS.rGR cells appear to contain high levels of endogenous GR because additional transfected GR plasmid causes squelching (data not shown). Therefore, the effects of added Ubc9 on the repression of AP1Luc reporter by endogenous GR in U2OS.rGR cells was determined. As displayed in Fig. 8A), exogenous Ubc9 has little effect on the fold repression by glucocorticoid steroid but significantly decreases the EC_{50} for Dex repression (Fig. 8B, right) and increases the percent partial agonist activity of 1 μM Prog (Fig. 8B, left). These responses are nearly identical to those for GR-regulated gene induction (Kaul et al., 2002a; Cho et al., 2005; Kim et al., 2006). These results suggest that similar processes are modulated by Ubc9 in both gene repression and gene induction.

4. Discussion

The ability of several transcriptional cofactors (TIF2, SRC-1, SMRT, Ubc9, and STAMP) known to modulate the V_{max} and EC_{50} of agonists, and the percent partial agonist activity of antagonists, in GR-mediated gene induction have been examined for their comparable activity in gene repression by GRs. In addition, a glucocorticoid agonist of very different structure and the consequences of inhibiting histone deacetylation were investigated. As many steps in induction and repression are thought to involve different protein-protein and protein-DNA interactions, the comparison of the activities of these factors and steroids represents a stringent test for mechanistic similarities and differences. Surprisingly, most of the responses seen with each factor and steroid in gene induction are retained in the repression of exogenous and endogenous genes. We conclude that many of the same molecular interactions responsible for altering the V_{max}, EC_{50}, and percent partial agonist activity in GR-regulated gene induction are utilized in GR-mediated gene repression. Thus, there appears to be greater similarity at a molecular level between the two modes of GR action than has been appreciated to date. These data further suggest that the major differences of decreased vs. increased gene expression derive from more downstream steps in both mechanisms of action.

The factors used in this study probe numerous topological features of GR (Fig. 9). The comparison of Dex vs. DAC examines the importance of the reorganization of the interior of the GR LBD to accommodate the larger DAC molecule (Suino-Powell et al., 2008). In some situations, these molecular reorganizations appear to be transmitted to the surface of the LBD (Tao et al., submitted). Similar changes in the interior of other proteins have been found to alter the interactions and activities of associated proteins (van Tilborg et al., 2000;Shiau et al., 2002;Shulman et al., 2004;Hilser and Thompson, 2007;Zwart et al., 2007). The coactivators TIF2 and SRC-1 bind via their LxxLL motifs to the AF2 pocket in the LBD in a manner that only partially overlaps with the association site of the corepressors NCoR and SMRT (Hu and Lazar, 1999;Nagy et al., 1999;Perissi et al., 1999). In addition, the amino terminal domain of TIF2 competitively inhibits corepressor binding to an amino terminal region of GR (Wang et al., 2007). Thus, two discrete regions of GR are tested during the binding of coactivators and corepressors. Ubc9 interacts with a region of the GR LBD and hinge region that may or may not overlap with the AF2 pocket (Cho et al., 2005). STAMP requires the entire GR for the high affinity binding seen in two-hybrid assays. However, STAMP and TIF2 do not compete for each other's binding to GR and appear to use separable regions of GR to form a ternary complex (He and Simons; Jr., 2007). We also see functional competition of TIF2 and SMRT in gene repression (Fig. 6), just as in gene induction (Szapary et al., 1999). Thus our model (Szapary et al., 1999) that the ratio of coactivators to corepressors is a major determinant of the position of the dose-response curve of agonists, and the percent partial agonist activity of antagonists,

appears to apply equally well to GR-mediated repression and induction. The combined data of the present study indicate that the interactions of numerous GR-associated factors with surfaces of both GR (Fig. 9) and the transcriptional machinery are sufficiently similar that the final effects on V_{max}, EC_{50}, and percent partial agonist activity are largely the same in both gene induction and gene repression.

Not all of the effects of increased factor concentration are the same, though. The increase in fold repression of the exogenous AP1Luc reporter with added TIF2 (1–2 fold in Fig. 2–Fig. 4) is considerably less than the 2–4 fold increase seen for induction of the exogenous GREtkLUC reporter (Szapary et al., 1999;Chen et al., 2000;He et al., 2002;Cho et al., 2005). This may result from higher levels of endogenous TIF2 in the U2OS.rGR cells used for gene repression that would mask the effect of added TIF2. However, it cannot explain the ability of TIF2 plus STAMP to increase the fold repression of exogenous AP1Luc but not endogenous coll3 in the same cells (Fig. 3 vs. Fig. 1). Also, SRC-1 causes an increase in the EC_{50} (and a decrease in percent partial agonist activity) in repression (Fig. 5) vs. a decrease in EC_{50} (and an increase in percent partial agonist activity) in induction (Szapary et al., 1999). These discrepancies are not unanticipated, however, in view of the increasingly frequent differences when the properties of other factors with specific genes, both endogenous and exogenous, are compared (Chen et al., 2006;Grenier et al., 2006;Wang et al., 2006;He and Simons; Jr., 2007;Kino et al., 2007;Trousson et al., 2007;Zou et al., 2007;Zou et al., 2007; Ronacher et al., submitted).

The behavior of the coactivator SRC-1 in the present gene repression system is particularly interesting. Increased concentrations of both TIF2 and SRC-1 are known to afford an increase in percent partial agonist activity and a decrease in EC_{50} for GR-regulated gene induction (Szapary et al., 1999; Chen et al., 2000; He et al., 2002; Cho et al., 2005). SRC-1 and TIF2 both also act as coactivators to enhance GR-mediated repression of TGFβ-regulated induction of type-1 plasminogen activator inhibitor (PAI-1) in Hep3B human hepatoma cells (Li et al., 2006). In the present U2OS.rGR cell system, TIF2 and SRC-1 (weakly) both display coactivator activity to enhance the ability of GR-agonist complexes to repress PMA-induced AP1Luc reporter expression (Fig. 2–Fig. 5). Unexpectedly, the effects of added SRC-1 or TIF2 on the Dex EC_{50} and the percent partial agonist activity of Prog are diametrically opposed during gene repression. SRC-1 decreases the percent partial agonist activity and increases the EC_{50} (Fig. 5C) and thus acts more like a corepressor (Szapary et al., 1999; Song et al., 2001; Wang et al., 2004). Nevertheless, the combination of SRC-1 and STAMP does not give simply the average of the two opposing responses. Instead, the presence of SRC-1 with STAMP augments the modulatory activity of STAMP with the EC_{50} and percent partial agonist activity instead of yielding an intermediate value. Thus, additional STAMP makes SRC-1 a more effective coactivator, just as STAMP does with TIF2 (Fig. 3&Fig. 4 and He and Simons; Jr., 2007). These responses with STAMP are not unexplainable because both SRC-1 and TIF2 have similar modulator activity in gene induction (Szapary et al., 1999) and both interact directly with STAMP (He and Simons; Jr., 2007). We suspect that the differences between TIF2 and SRC-1 during gene repression derive from the unique C-terminal RID of SRC-1 that also contains repressive activity (Jenster et al., 1997). This is the same region of SRC-1 that has the highest binding affinity for GR LBD (Needham et al., 2000; Suino-Powell et al., 2008), displays modulatory activity for gene induction (He et al., 2002), and binds to STAMP (He and Simons; Jr., 2007). Thus, the presence of additional STAMP may mask the intrinsic inhibitory activity of SRC-1 in the present gene repression system and strengthen SRC-1's coactivator activity to give a final response more like that of TIF2, which lacks the C-terminal inhibitory domain. The fact that SRC-1 is reported to have opposite effects (a decrease in EC_{50} and no effect with added SMRT) on GR-mediated repression of a forskolin-induced, transiently transfected Luciferase reporter (van der Laan et al., 2008) is probably due to cell- and/or promoter-specific effects.

The ability of TSA to influence gene repression appears to be varied. Histone deacetylation is often associated with decreased activity of receptor-agonist complexes in gene induction (Burke and Baniahmad, 2000; McKenna and O'Malley, 2002; Rosenfeld et al., 2006 but not Astrand et al., 2004). Conversely, the inhibition of deacetylation increases the activity of agonist steroids in gene induction. Thus, one could argue that TSA, which prevents histone deacetylation, might also increase the activity of agonist steroids in gene repression and further reduce gene expression, thereby increasing the magnitude of gene repression. However, TSA is reported to prevent PPAR repression of the iNOS gene (Pascual et al., 2005) and decrease GR repression of lipopolysaccharide- and tumor necrosis factor-alpha-induced interleukin (IL)-8 release (Tsaprouni et al., 2007). In contrast, GR repression of AP-1 activity, and of NF-κB activity in A549 cells, was resistant to TSA (Nissen and Yamamoto, 2000). Similarly, we see no marked ability of added TSA in U2OS.rGR cells to decrease the fold repression (V_{max}) of AP1Luc by Dex, alter the EC_{50} of Dex repression, or modify the percent partial agonist activity of GR-Prog complexes. Furthermore, TSA did not reverse the ability of STAMP to modulate the V_{max}, EC_{50}, or percent partial agonist activity in this system. Thus, the effects of TSA, like those of various cofactors, appear to be gene- and cell-selective.

A recurring observation in studies of V_{max}, EC_{50}, and percent partial agonist activity is the possibility of selectively modulating each parameter (Szapary et al., 2008; reviewed in Simons, submitted). This separation of effects is further supported by the current results. TIF2 displays comparatively reduced ability to alter the V_{max} of repression of exogenous and endogenous genes with Dex compared to the greater ability to decrease the EC_{50} to lower steroid concentrations (Fig. 1–Fig. 4). Conversely, SRC-1 is unable to modify the V_{max} but is very effective in increasing the EC_{50} (Fig. 5). Whether these opposite influences on EC_{50} require the recruitment of different mechanistic pathways is not yet clear. TIF2 and/or STAMP evince marginal change in the V_{max} of the endogenous coll3 gene but cause much larger decreases in the EC_{50} (Fig. 1). This same behavior is seen for Ubc9 with the exogenous AP1Luc reporter (Fig. 8). Further studies of these responses should yield valuable insight into the mechanism of glucocorticoid hormone action in general.

In conclusion, the many of the mechanistic details of GR-mediated gene induction, not to mention GR-regulated gene repression, remain largely unknown. The current study suggests that the effects of several agents (steroid structure and various cofactors) affecting a variety of surfaces of GR are preserved in the mechanisms of both GR-controlled induction and repression. Thus, future elucidation of the details for one process should be directly applicable to the other. We predict that the major difference of raising vs. lowering gene transcription will be found to be determined by further downstream steps.

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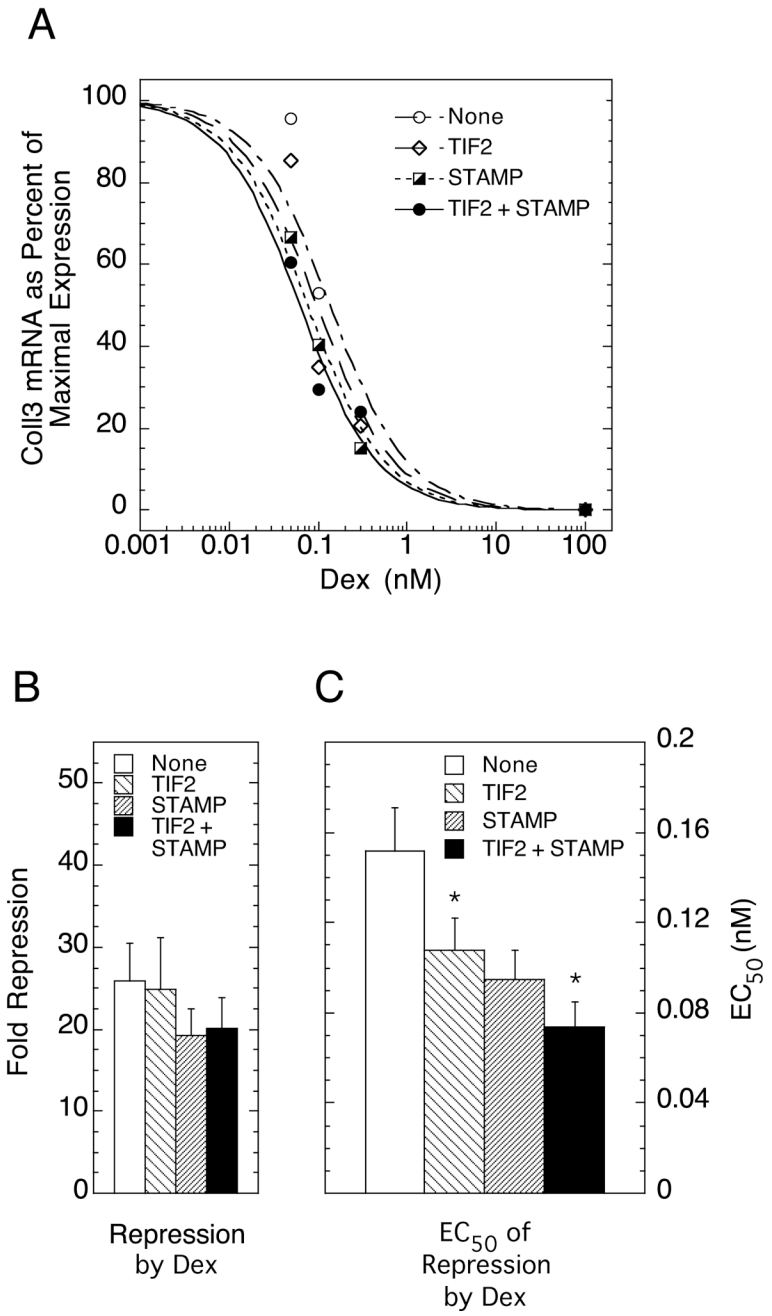


Fig. 1. Cooperation of STAMP with TIF2 in modulating properties of GR-mediated repression of the endogenous coll3 gene

Single wells of U2OS.rGR were transiently transfected with or without 80 ng TIF2 plasmid and/or 400 ng of plasmid for HA-STAMP, incubated with PMA and the indicated Dex concentrations, and processed for determination of coll3 mRNA by qRT-PCR as described in Materials and Methods. (A) Dose-response curve for Dex induction of coll3. One representative experiment is shown. The values for (B) fold repression (V_{max}) and (C) EC_{50} from 4 independent experiments as in A are averaged (± S.E.M.) and plotted. * $P \leq 0.014$ (paired t-test vs. None).

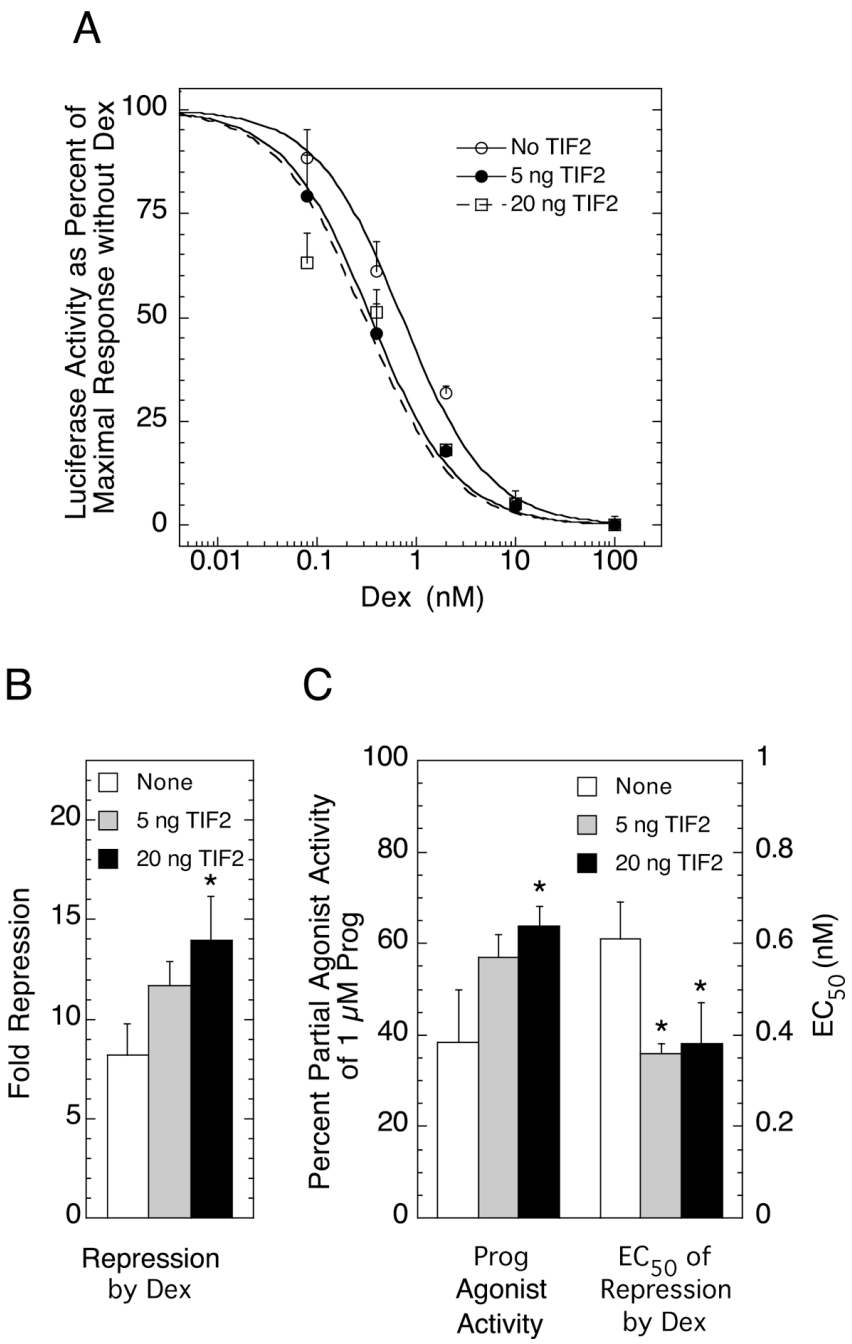


Fig. 2. Dose-dependent augmentation by coactivator TIF2 of properties of GR-mediated gene repression

As described in Materials and Methods, U2OS.rGR cells were transiently transfected with AP1Luc along with the indicated amounts of TIF2 plasmid, and induced with varying concentrations of Dex or 1 μM Prog and PMA. (A) Dose-response curve for GR-mediated repression with Dex and different amounts of TIF2. One representative experiment is shown. The values of (B) fold repression (V_{max}) and (C) percent partial agonist activity of 1 μM of the antiglucocorticoid Prog (left half of figure) and EC_{50} (right half of figure) from 4 independent experiments as in A are averaged (± S.E.M.) and plotted. * $P < 0.050$ (paired t-test vs. None).

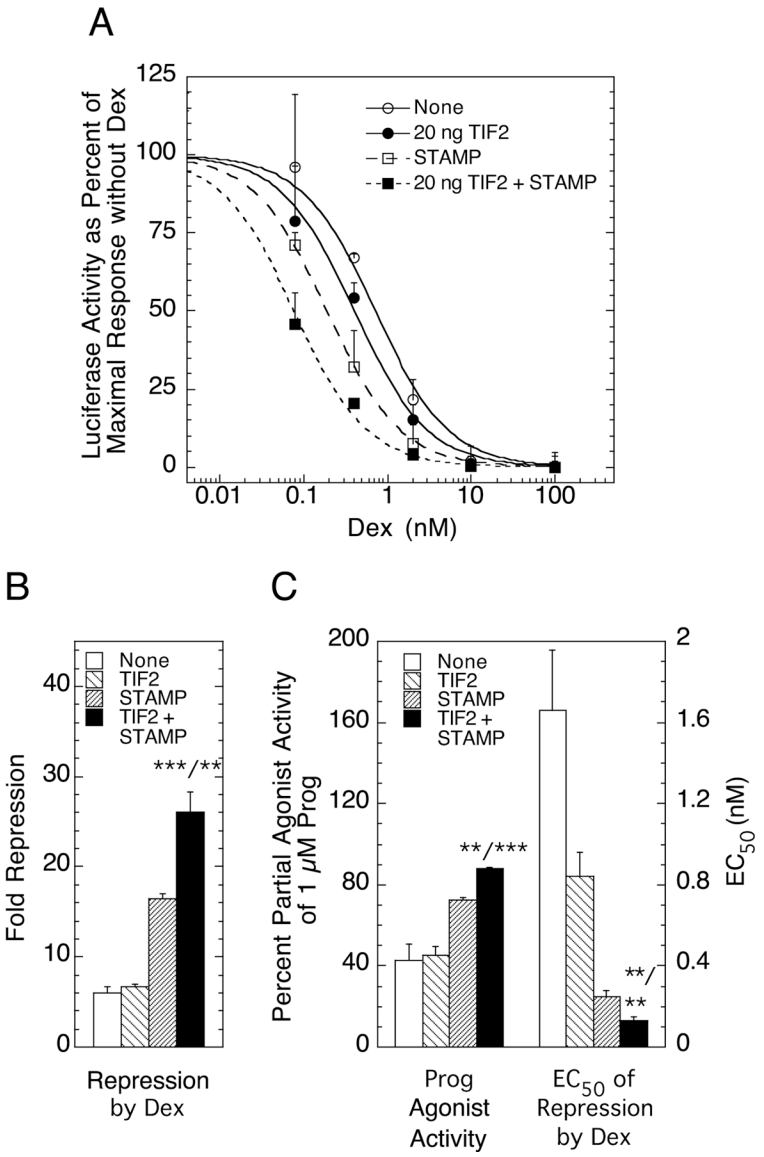
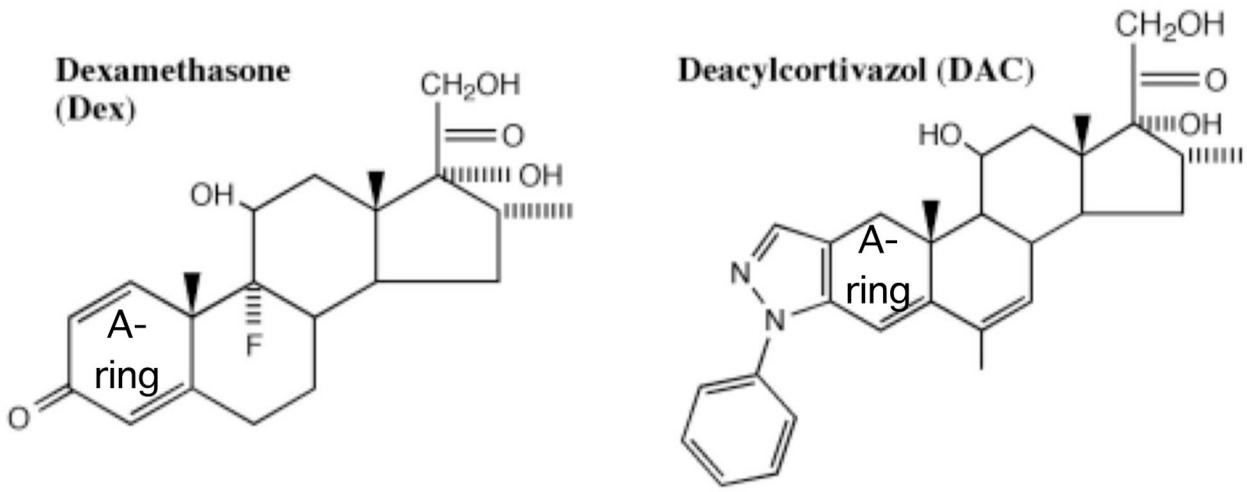


Fig. 3. Cooperation of STAMP with TIF2 in modulating properties of GR-mediated gene repression

Cells were transiently transfected with AP1Luc ± 20 ng TIF2 plasmid and/or 100 ng of plasmid for HA-STAMP, incubated with steroid and PMA, and processed as in Fig. 2. (A) Dose-response curve for repression by GR-Dex complexes with and without TIF2 and/or STAMP. One representative experiment is shown. The values of (B) fold repression (V_{max}) and (C) percent partial agonist activity of 1 μM of the antiglucocorticoid Prog (left half of figure) and EC_{50} (right half of figure) from 4–6 independent experiments as in A, or with 1 μM Prog, are averaged (± S.E.M.) and plotted. Asterisks on either side of slash above the data for GR + TIF2 + STAMP refer to the significance compared to GR + TIF2 (left of slash) and to GR + STAMP (right of slash). ** $P < 0.01$, *** $P < 0.0003$ (t-test, paired t-test, and Mann-Whitney).

A



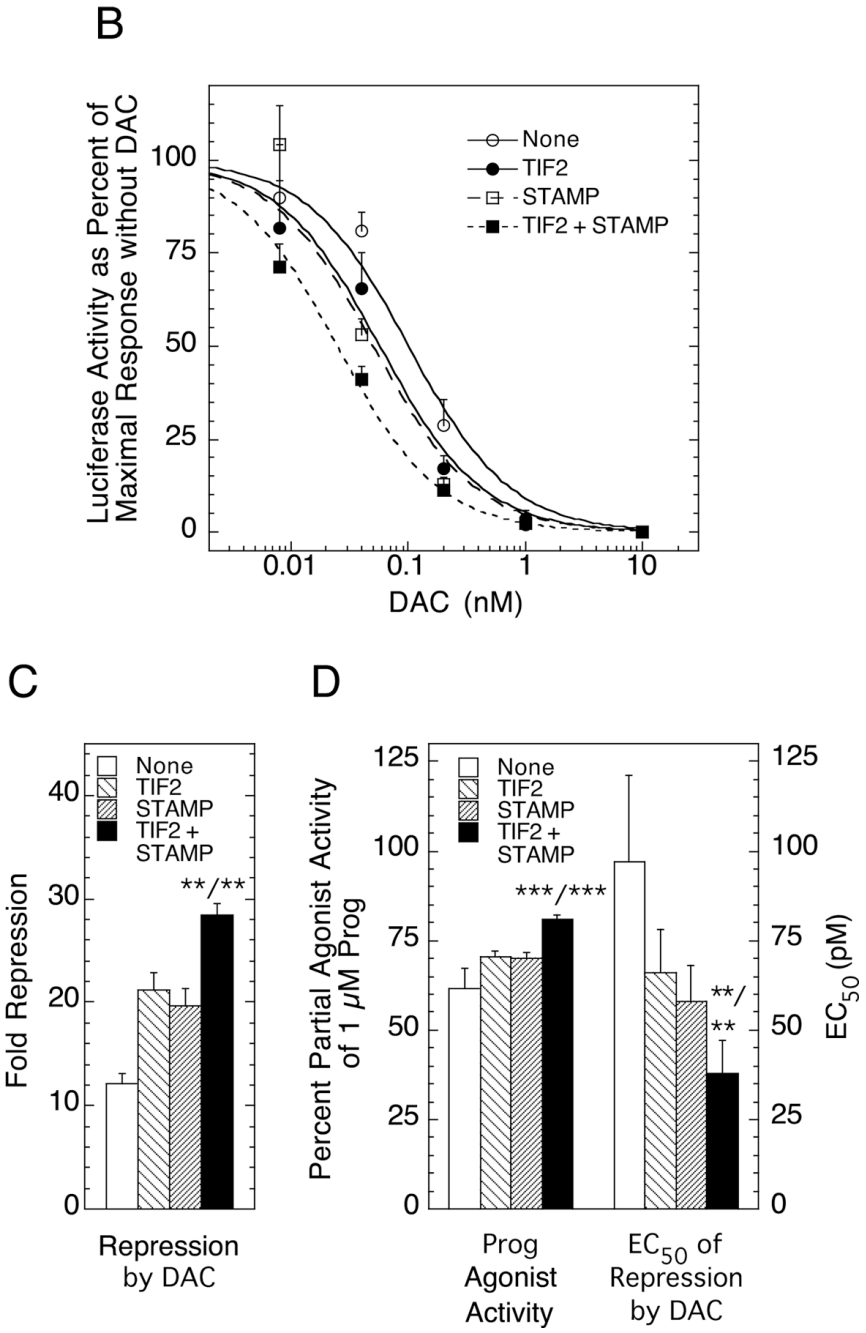


Fig. 4. Activity of DAC in GR-controlled repression

(A) Structures of Dex vs. DAC. (B) Dose-response curve for repression by GR-DAC complexes with and without TIF2 and/or STAMP. Experiment, work-up, and plotting of data for one representative experiment are precisely the same as for Fig. 3A except that DAC is the agonist steroid used. The values of (C) fold repression (V_{max}) and (D) percent partial agonist activity of 1 μM of the antiglucocorticoid Prog (left half of figure) and EC_{50} (right half of figure) from 5 independent experiments as in B with DAC, or with 1 μM Prog, are averaged (± S.E.M.) and plotted. Asterisks on either side of slash above the data for TIF2 + STAMP refer to the significance compared to GR + TIF2 (left of slash) and to GR + STAMP (right of slash). ** $P < 0.01$, *** $P < 0.003$ (t-test and paired t-test).

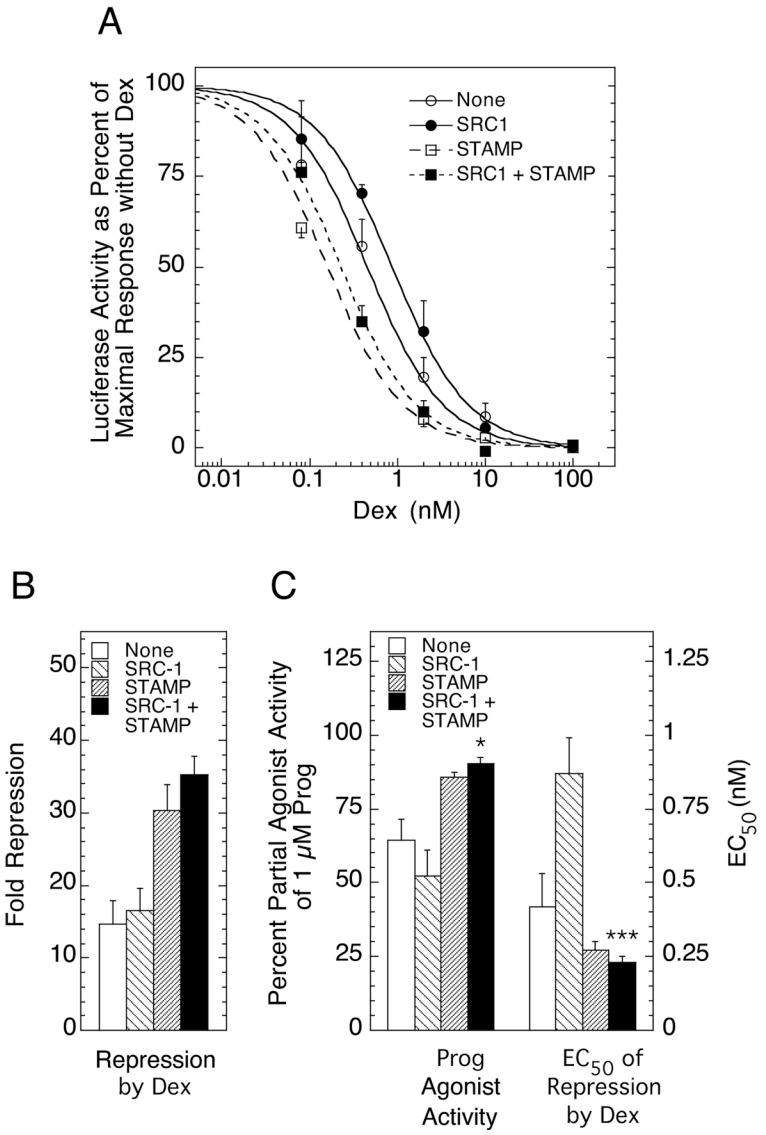


Fig. 5. Cooperation of STAMP with SRC-1 in modulating properties of GR-mediated gene repression

(A) Dose-response curve for repression by GR-Dex complexes with and without SRC-1 and/or STAMP. Experiment, work-up, and plotting of data for a representative experiment are precisely the same as for Fig. 3A except that 40 ng of SRC-1a and 80 ng of HA-STAMP plasmids are used with Dex. The values of (B) fold repression (V_{max}) and (C) percent partial agonist activity of 1 μM of the antiglucocorticoid Prog (left half of figure) and EC_{50} (right half of figure) from 5–6 independent experiments as in A with Dex, or with 1 μM Prog, are averaged (± S.E.M.) and plotted. Asterisks above the data for SRC-1 + STAMP refer to the significance compared to the theoretical values calculated from those of GR + SRC-1 and of GR + STAMP (see Materials and Methods for calculations). * $P < 0.05$, *** $P = 0.0002$ (t-test). The fold repression with SRC-1 + STAMP is greater than that for SRC-1 ($P = 0.0012$, t-test), but not STAMP, alone.

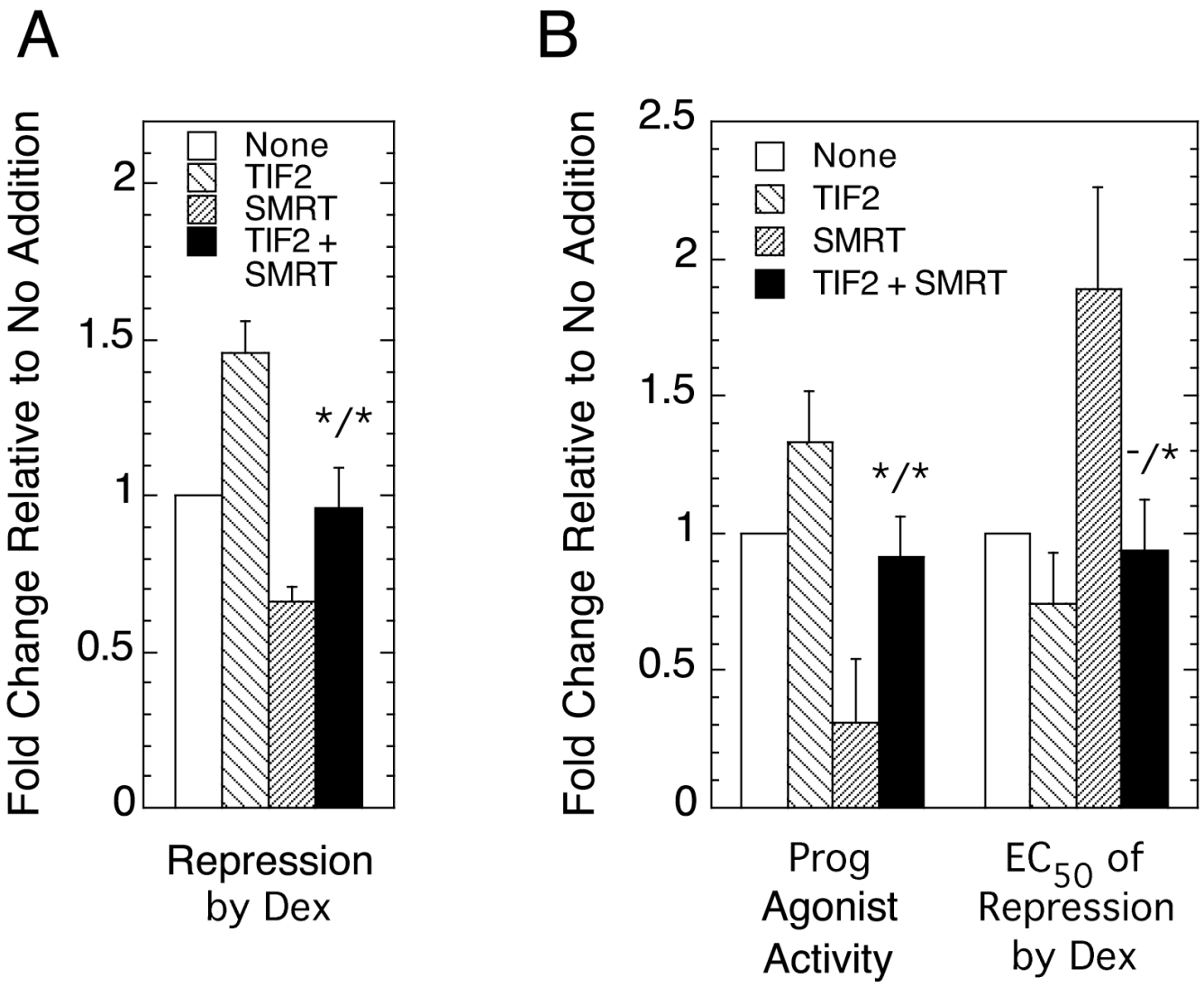


Fig. 6. Competitive inhibition of TIF2 and SMRT for altering the properties of GR-regulated gene repression

Experiment and work-up are precisely the same as for Fig. 3 except that 20 ng of TIF2 and 20 ng of SMRT plasmids were used with Dex or 1 μM Prog. The average change (± S.E.M.) relative to the control with no added factor for (A) fold repression (V_{max}) and (B) percent partial agonist activity of 1 μM of the antiglucocorticoid Prog (left half of figure) and EC_{50} (right half of figure) from 5–6 independent experiments is plotted. Asterisks on either side of the slash above the data for TIF2 + SMRT refer to the significance compared to GR + TIF2 (left of slash) and to GR + SMRT (right of slash). ¯P > 0.05, * P < 0.05 (t-test and paired t-test).

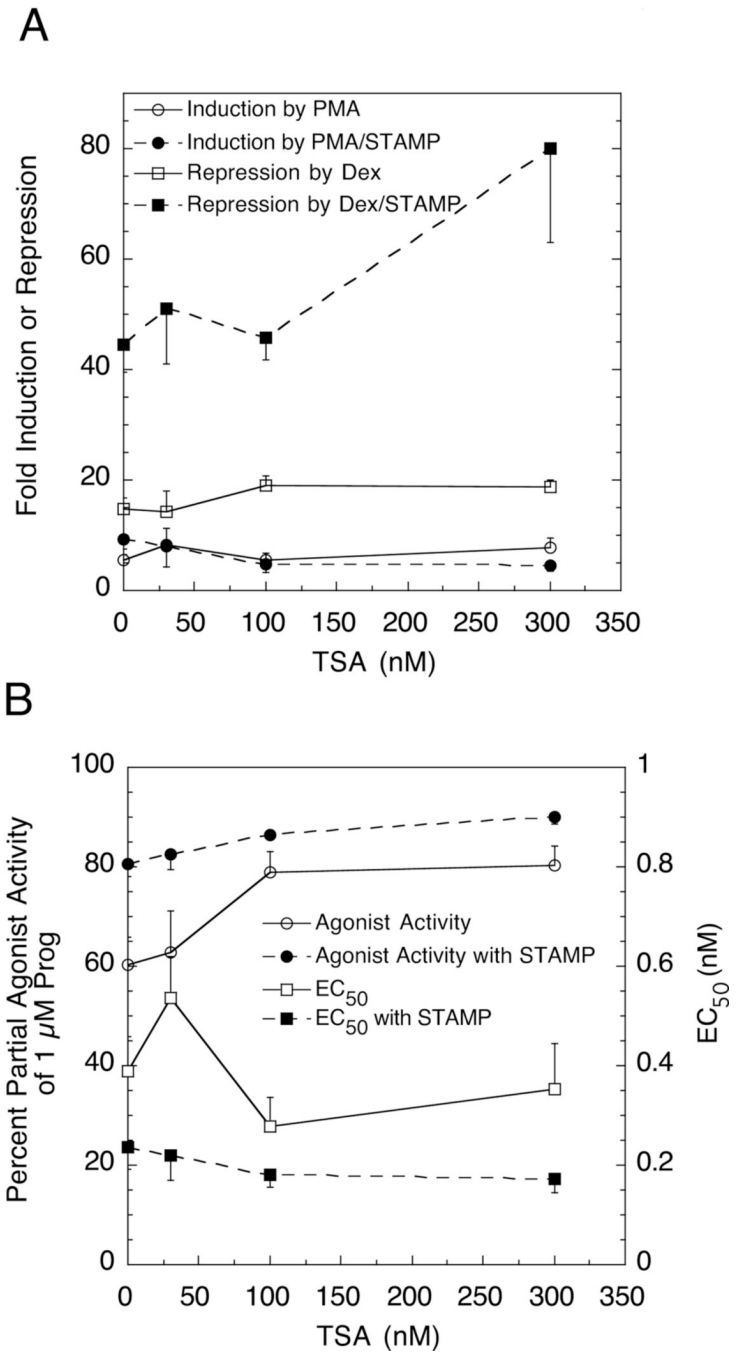


Fig. 7. Effect of TSA on properties of GR-mediated gene repression with and without STAMP

Experiment and work-up are precisely the same as for Fig. 3 except that the indicated concentrations of TSA (added with steroid) ± 80 ng HA-STAMP plasmid were used with Dex or 1 μM Prog. The (A) fold induction by PMA and fold repression by Dex (V_{max}) and (B) the percent partial agonist activity of 1 μM Prog, and EC_{50} for Dex from 5 (with 30 nM TSA) or 7 independent experiments are averaged (± S.E.M.) and plotted.

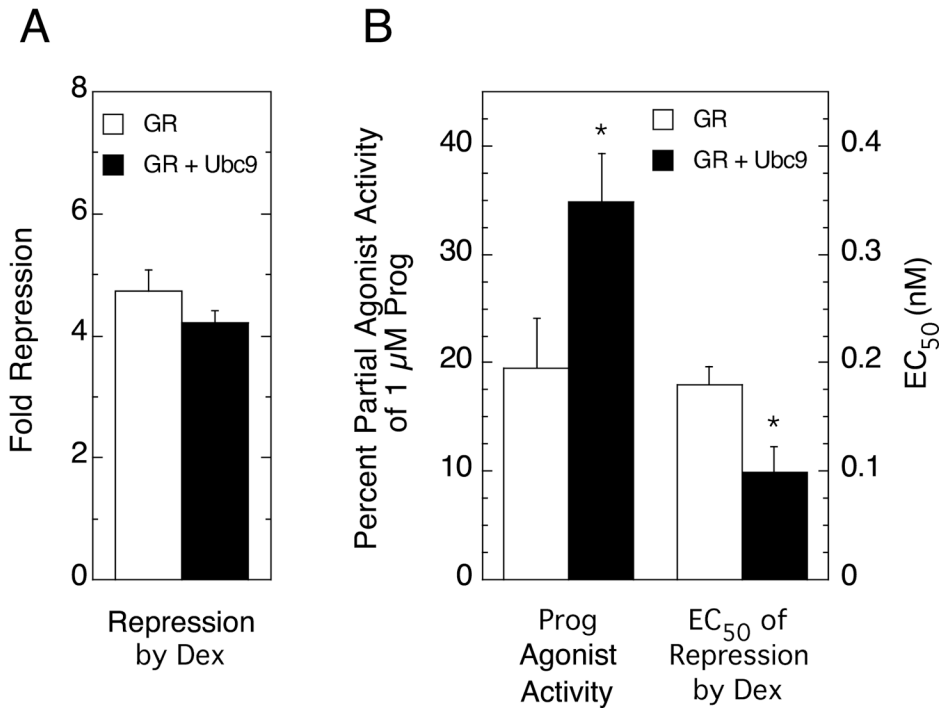


Fig. 8. Modulation of GR-controlled repression by Ubc9

Experiment and work-up are precisely the same as for Fig. 3 except that Ubc9 plasmid (100 ng) is added where indicated with Dex or 1 μM Prog. The (A) fold repression by Dex (V_{max}) and (B) percent partial agonist activity of 1 μM of the antiglucocorticoid Prog (left half of figure) and EC_{50} (right half of figure) from 5–6 independent experiments are averaged (± S.E.M.) and plotted. * $P < 0.05$ (t-test).

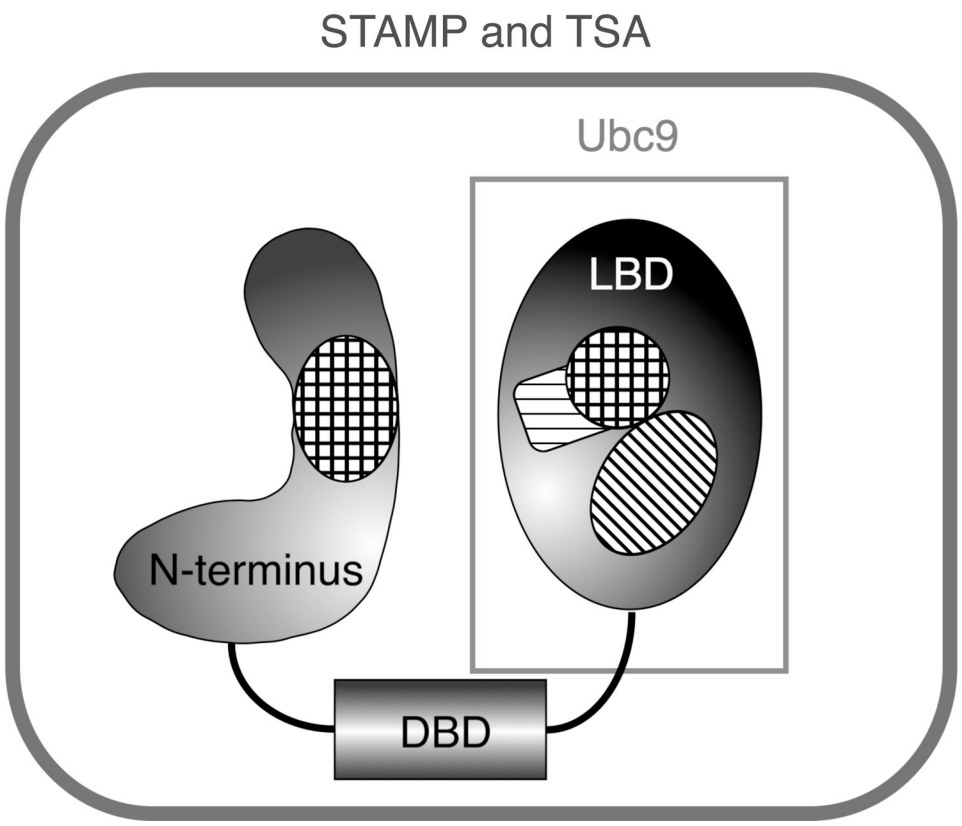


Fig. 9. Model of GR regions probed by modulatory factors/agents during gene repression and activation

The cartoon depicts the three domains of GR (N-terminus, DBD, and LBD) joined by linker segments. The steroids (DAC vs. Dex) most acutely affect the region in the LBD indicated by the oval filled with thick down-slanting lines. The shape with thin horizontal lines indicates that portion of the SMRT interaction site that does not overlap with the binding site for coactivators TIF2 and SRC-1 in the LBD. Completely overlapping binding sites of SMRT and coactivators in the LBD and N-terminus are indicated by cross-hatched ovals. Ubc9 contacts the LBD and linker in a currently unlocalized region(s) indicated by the rectangle surrounding the LBD and linker. Productive STAMP interactions require sequences of the entire GR protein, as designated by the shape encompassing the entire GR. TSA affects proteins that may associate with multiple regions of GR, also designated by the shape encompassing the entire GR. See text for further details.

Table 1

Modulation of induction properties of GR-DAC complexes

A)	Fold change with 10 vs. 0.1 ng GR plasmid in		
Conditions	**V_{max}**	**Fold Induction**	**EC_{50}**
GR + DAC	7.04 ± 0.66	4.18 ± 0.43	0.10 ± 0.02
	(0.0008)	(0.063†)	(<0.0001)

B)	Fold change with added Ubc9 plasmid in		
Conditions	**V_{max}**	**Fold Induction**	**EC_{50}**
Low (0.1 ng) GR + DAC	4.68 ± 0.63	1.00 ± 0.15	0.82 ± 0.14
	(0.0021)		(0.26)
High (10 ng) GR + DAC	1.03 ± 0.07	0.16 ± 0.01	0.083 ± 0.014
		(<0.0001)	(<0.0001)
High (10 ng) GR + Dex	1.13 ± 0.11	0.37 ± 0.17	0.103 ± 0.017
		(0.13†)	(<0.0001)

Average changes (calculated as 10 ng GR/0.1 ng GR and "plus Ubc9"/"no Ubc9") in V_{max}, fold induction, and EC_{50} for DAC and Dex induction of a GREtkLUC reporter in transiently transfected CV-1 cells from N = 4–6 independent experiments such as in Fig. 2 were determined as described in Materials and Methods. The changes in V_{max} and fold induction with different GR concentrations without (A) and with (B) Ubc9 are not the same because the basal level activity is higher with added GR and/or Ubc9. P values for statistical significance are in parentheses

(† = Wilcoxon test).