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Ligand-selective transactivation and transrepression via the glucocorticoid receptor: Role of cofactor interaction☆

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ABSTRACT

The mechanisms that determine ligand-selective transcriptional responses by the glucocorticoid receptor (GR) are not fully understood. Using a wide panel of GR ligands, we investigated the relationships between the potency and maximal response for transactivation via a glucocorticoid response element (GRE) and transrepression via both nuclear factor κB (NFκB) and activator protein-1 (AP-1) sites, relative binding affinity for the GR, as well as interaction with both coactivators and corepressors. The results showed ligand-selective differences in potency and efficacy for each promoter, as well as for a particular ligand between the three promoters. Ligand potency correlated with relative affinity for the GR for agonists and partial agonists in transactivation but not for transrepression. Maximal response was unrelated to relative affinity of ligand for GR for both transactivation and transrepression. A good and significant correlation between full length coactivator binding in two-hybrid assays and efficacy as well as potency of different receptor–steroid complexes for both transactivation and transrepression supports a major role for coactivator recruitment in determination of ligand-selective transcriptional activity. Furthermore, ligand-selective GR binding to GRIP-1, as determined by both two-hybrid and DNA pull down assays, correlated positively with ligand-selective efficacy for transactivation of both a synthetic GRE reporter with expressed GR as well as of an endogenous gene via endogenous GR. The receptor interacting domain of the corepressor SMRT exhibited strong interaction with both agonists and partial agonists, similar to the results for coactivators, suggesting a possible role for SMRT in activation of transcription. However, there was no correlation between ligand affinity for the GR and cofactor interaction. These results provide strong quantitative biochemical support for a model in which GR-mediated ligand-selective differential interaction with GRIP-1, SRC-1A, NCoR and SMRT is a major determinant of ligand-selective and promoter-specific differences in potency and efficacy, for both transactivation and transrepression.

Abbreviations: TA, transactivation; TR, transrepression; DEX, dexamethasone; cort, cortisol; pred, prednisolone; prog, progesterone; MPA, medroxyprogesterone acetate; NET, norethindrone acetate; CpdA, compound A; ald, aldosterone; GRE, glucocorticoid response element; NFκB, nuclear factor κB; AP-1, activator protein-1; RBA, relative binding affinity; GRIP-1, glucocorticoid receptor interacting protein-1; SRC-1, steroid–receptor coactivator; NCoR, nuclear receptor corepressor; SMRT, silencing mediator of retinal and thyroid hormone receptor; RID, receptor interacting domain; NSC, non-silencing control.

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1. Introduction

The glucocorticoid receptor (GR) is a ligand-activated transcription factor that regulates transcription of many target genes via several mechanisms. These include transactivation via binding of liganded-GR to consensus glucocorticoid response elements (GREs) as well as transrepression via tethering mechanisms where the GR interferes with other transcription factors such as activator protein-1 (AP-1) and nuclear factor κB (NFκB) (Hayashi et al., 2004). A wide range of ligands bind to the GR resulting in a range of transcriptional responses for both transactivation and transrepression in a ligand-, promoter-, and cell-specific manner. The liganded-GR can interact with many components of the transcriptional machinery, including coactivators, corepressors, chromatin remodeling proteins, components of the mediator complex as well as RNA polymerase II and components of the basal transcriptional machinery (Kumar and Thompson, 2005). However, the extent to which these interactions contribute to ligand-selective biological responses has not been determined for steroid receptors. The GR and its endogenous ligand play a key role in many physiological processes and synthetic glucocorticoids are extensively used in treating several diseases. It is thus important to understand the biochemical basis for ligand-selective transcription via the GR, with a view to developing new drugs with fewer side-effects (Rhen and Cidlowski, 2005).

It is generally accepted that ligand-selective maximal responses by steroid receptors are due to the induction of ligand-specific conformations of the liganded receptor, exposing different interacting surfaces and thus resulting in differential recruitment of coregulators. There is substantial evidence that different ligands induce different conformations in the ligand-binding domains of steroid receptors (Shiau et al., 2002; Kauppi et al., 2003; Padron et al., 2007; Kroe et al., 2007). Different ligands have also been shown to result in differential coactivator and corepressor recruitment for the androgen receptor (AR) (Kang et al., 2004; Baek et al., 2006; Yoon and Wong, 2006; Ozers et al., 2007), estrogen receptor (ER) (An et al., 2001; Jaber et al., 2006; Peterson et al., 2007), progesterone receptor (PR) (Wang and Simons, 2005; Wang et al., 2007; Liu et al., 2002), mineralocorticoid receptor (MR) (Hultman et al., 2005) and GR (Kroe et al., 2007; Wang and Simons, 2005; Wang et al., 2007, 2004b; Coghlan et al., 2003; Garside et al., 2004; Cho et al., 2005a; Miner et al., 2007; Tao et al., 2008). Strong evidence exists for a steroid-dependent cofactor binding model whereby the transcriptional response of a steroid receptor to agonists, partial agonists and antagonists is linked to the recruitment of coactivator, mixed coactivator/corepressor and corepressor complexes, respectively (Kang et al., 2004). Several studies concur with parts of the above model (Ozers et al., 2007; Jaber et al., 2006; Wang and Simons, 2005; Wang et al., 2007, 2004b; Liu et al., 2002; Hultman et al., 2005; Garside et al., 2004; Cho et al., 2005a; Miner et al., 2007; Tao et al., 2008; Miner, 2002). However, others refute aspects of this model (Baek et al., 2006; Yoon and Wong, 2006; Peterson et al., 2007; Wang et al., 2007; Hultman et al., 2005). For example, in support of the model it has been shown that agonist-bound GR binds GRIP-1 to a greater extent than partial agonist-bound GR (Cho et al., 2005a) and agonist-bound PR binds SRC-1 and not SMRT, whereas antagonist-bound PR binds both SRC-1 and SMRT (Liu et al., 2002). However, others studies that refute the model show that the differential response to an agonist versus a selective modulator or partial agonist via the AR and MR is not due to differential extents of coactivator recruitment (Baek et al., 2006; Hultman et al., 2005).

A critical evaluation of the literature reveals that there is little quantitative evidence for the model with no studies showing a direct correlation between the extent of recruitment of coregulators by a liganded-steroid receptor and the potency (ligand concentration for half maximal response) or efficacy (maximal response) for transactivation. In addition, most of the above studies use too few ligands (two to four ligands) to establish a general conclusion. Since most researchers have investigated the model in the context of transactivation, very little is known about the effects of ligand on differential cofactor recruitment by steroid receptors in the context of transrepression (An et al., 2001; Garside et al., 2004; Rogatsky et al., 2001, 2002; Wu et al., 2004; He and Simons, 2007; Sun et al., 2008). This paper investigates the quantitative relationship between the interaction of the liganded-GR with coactivators and corepressors, the affinity of ligand for receptor, plus the efficacy and potency of the transcriptional effect for both transactivation and transrepression, for a wide panel of 11 ligands.

2. Materials and methods

2.1. Cell lines and test compounds

COS-1 and U2OS cells (ATCC, Manassas, VA) were cultured in high glucose (1 g/ml) Dulbecco's Modified Eagles Medium (Sigma–Aldrich, South Africa) supplemented with 10% (v/v) fetal calf serum (Delta Bioproducts, South Africa), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen, Paisley, UK) at 37 °C in a 5% CO_2 incubator. Dexamethasone ((11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione), cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione-17-hydroxycorticosterone), prednisolone (1,4-pregnadiene-11β,17α,21-triol-3,20-dione), progesterone (4-pregnene-3,20-dione), MPA (6α-methyl-17αhydroxyprogesterone acetate), NET-A (norethisterone-17-acetate), aldosterone (11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al), and RU486 (mifepristone, 11β-(4-dimethyl amino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one) were purchased from Sigma–Aldrich, South Africa. Compound A (2(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride) was synthesized as previously described (Louw et al., 1997). DO6 and AL438 were a generous gift from Dr J. Miner (Ligand Pharmaceuticals, San Diego, USA), however due to availability limits experiments with DO6 and AL438 could not be performed at final concentration higher than 1 μM. [^{3}H] dexamethasone (86 Ci/mmol) for competition binding assays was purchased from Amersham, South Africa. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma–Aldrich, South Africa.

2.2. Plasmids

The pTAT-GRE-E1b-luc plasmid containing two copies of the GRE from the *TAT* gene has been described previously (Sui et al., 1999). The human GR pCMV was a gift from Dr M.J .Garabedian (New York University, USA) and the pCMV-β-Gal plasmid was obtained from Dr G. Hageman (University of Ghent, Belgium). The 5× NFκB-luc and 7× AP-1-luc plasmids as well as pFR which contains five repeats of the GAL4 binding element fused upstream of a basic TATA promoter and the Luciferase reporter, were from Stratagene (Houston, TX, USA). VP-16-GR, GAL-TIF2.4 (containing amino acids 624–1010 of TIF2), GAL-GRIP-1 (containing full length GRIP-1) have been described previously (Wang et al., 2004b, 2007). The GAL-NCoR-RID (amino acids 1944–2453) and GAL-SMRT-RID (amino acids 982–1495) plasmids were obtained from Mitch Lazar (University of Pennsylvania, Pennsylvania). The plasmid pGAL-SRC-1A (containing full length SRC-1A) was a gift from Bert O'Malley (Baylor College of Medicine, Houston, TX). The plasmids pHA-GRIP (containing full length GRIP-1) and GAL-TIF2.4 were obtained from Michael R. Stallcup (USC, Los Angeles) while pSVL-GR was obtained from Keith Yamamoto (UCSF, San Francisco).

2.3. Whole cell binding assays

Competitive whole cell binding assays were performed as previously described (Koubovec et al., 2005) with minor modifications. Briefly, COS-1 cells were seeded into 24-well tissue culture plates at 5×10^4 cells/well. On day two COS-1 cells were transiently transfected with 0.25 μg HA-hGR-pCMV/well using FuGENE 6 (Roche, South Africa) according to the manufacturer's instructions. On day three the cells were washed twice with prewarmed PBS and incubated with DMEM containing 20 nM [^{3}H] DEX (86 Ci/mmol) and varying concentrations of unlabeled steroids for 90 min at 37 °C. Thereafter cells were placed on ice and washed three times for 15 min with ice cold PBS containing 0.2% (w/v) BSA. Cells were lysed with 200 μl Reporter lysis buffer (Promega, Madison, WI, USA) and binding was determined by scintillation counting. Binding data were analysed using GraphPad Prism software with non-linear regression and one-site competition binding options. Non-specific binding was about 20–25% of total binding. Relative binding affinities (RBAs) were calculated from the IC_{50} values in nM expressed as % of the IC_{50} in nM for DEX.

2.4. Luciferase reporter assays

For transactivation assays COS-1 cells were seeded into 10 cm dishes. The next day the cells were transfected with 10 μg HA-hGR-pCMV and 3.75 μg pTAT-GRE-E1b-luc using FuGENE 6 (Roche, South Africa). On the morning of day three the cells were replated into 24-well dishes at a density of 7×10^4 cells/well. This approach was used to guarantee that each well received equal amounts of transfected cells. In the evening the cells were stimulated with different compounds for 16 h. Thereafter the cells were washed twice with PBS and lysed in 50 μl of Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). Luciferase activity was normalised to protein content per well as determined by standard Bradford assay.

For transrepression assays COS-1 cells were seeded into 24-well plates at a density of 5×10^4 cells/well. The following day the cells were transfected with 0.125 μg of HA-hGR-pCMV, 0.25 μg of 5× NFκB-luc or 7× AP-1-luc plasmids and 0.025 μg of pCMV-β-Gal using FuGENE 6. Twenty-four hours after transfection the cells were washed with PBS and incubated with serum-free medium containing 20 ng/ml PMA and different concentrations of the GR ligands for 16 h. Luciferase activity in the lysate was measured as described above. The values obtained were normalised to expression of β-galactosidase to normalise for transfection efficiency between wells, which was measured using the GalactoStar Assay Kit from Tropix (Bedford, MA, USA). Both transactivation and transrepression data were analysed using GraphPad Prism software with non-linear regression and sigmoidal dose response options.

2.5. Real time PCR

U2OS cells were plated in 12-well plates at a density of 2.5×10^5 cells/well. After 24 h the medium was replaced with serum-free DMEM and after 2 h of serum starvation, the different GR ligands were added for 2 h. The final concentration of each ligand was calculated to give greater than 97% GR occupancy, based on the RBA. The cells were then washed with PBS, and RNA was extracted using Tri Reagent (Sigma–Aldrich, South Africa). RNA was reverse transcribed with Oligo-dT priming, using the Transcriptor First Strand cDNA synthesis kit (Promega, Madison, WI), and an equal volume of each cDNA synthesis reaction was used as template for real time PCR, using the Sensimix dT Kit (Quantace, London). Quantitative PCR was carried out using QuantiTect primers (Qiagen, Germanstown, MD) for Glucocorticoid induced leucine zipper (GILZ). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalisation (F: 5′ TGAACGGGAAGCTCACTGG 3′; R: 5′ ATTCGTTGTCATACCAGG 3′). Standard curves were used to determine the efficiency of each primer set, and the relative expression of GILZ in each sample was calculated according to the Pfaffl mathematical model (Pfaffl, 2001).

2.6. siRNA

U2OS cells were plated in 12-well plates at a density of 0.8×10^5 cells/well. After 24 h, cells were transfected with 10 nM siRNA, directed against either the GR (GR6), or a validated non-silencing control sequence (NSC) (Qiagen, Germanstown, MD), using HiPerfect transfection reagent (Qiagen, Germanstown, MD). Cells were left for 48 h, before being treated with GR ligands as above. RNA was then harvested and subjected to real time PCR as described above, or alternatively total protein was extracted and analysed by Western blot to check GR levels in each experiment.

2.7. Western blot analysis

Cells were washed once with PBS, harvested in SDS sample buffer, boiled for 5 min and separated on an 8% polyacrylamide gel by SDS–PAGE. Proteins were transferred to Hybond ECL membrane (Amersham Biosciences, UK) and probed with antibodies against GR (H300) (Santa Cruz, CA) or against beta tubulin (Sigma–Aldrich, South Africa), which was used as a loading control. Blots were developed using Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire).

2.8. Mammalian two-hybrid assay

U2OS cells were plated into 24-well plates at a density of 5×10^4 cells/well and transfected with 0.1 μg VP-16-GR, 0.1 μg of either GAL-TIF2.4, GAL-GRIP-1, GAL-SRC-1A, GAL-NCOR-RID or GAL-SMRT-RID and 0.2 μg pFR. To control for transfection efficiency the pCMV-β-GAL plasmid (0.025 μg) was cotransfected. The following day the cells were washed with PBS and incubated with serum-free medium containing 10^{-5} M of the inducing compounds. After 24 h the cells were washed and lysed in 50 μl Reporter lysis buffer (Promega, Madison, WI, USA) and luciferase and β-galactosidase activities were determined as described above.

2.9. ABCD assay

The avidin–biotin-complex-DNA (ABCD) assay was performed as previously described (Cho et al., 2005a) with some modifications. In brief, cytosols were prepared from COS-1 cells overexpressing wt GR or GRIP-1. Cytosols containing liganded-GR complexes were activated by heating at 20 °C for 30 min, followed by incubation with biotinylated DNA containing a single GRE immobilized on streptavidin–agarose beads. GRIP-1 cytosols were then added followed by centrifugation and assessment of binding of proteins by Western blotting. Biotinylated sense and anti-sense oligonucleotides were obtained from Invitrogen/Life Technologies and streptavidin–agarose beads were obtained from Sigma. Cell cytosols plus or minus GR were prepared in TAPS buffer from COS-7 cells transfected with carrier DNA or pSVL-rGR, respectively (Cho et al., 2005a). Whole cell extracts plus or minus HA-GRIP-1 were prepared using Cytobuster (Novagen) according to the manufacturer's instructions from COS-7 cells transfected with carrier DNA or pHA-GRIP-1, respectively. Western blotting was performed as previously described using the BuGR-2 anti-GR antibody (Affinity Bioreagents, Golden, CO) and anti-HA antibody for HA-GRIP-1 detection (HA-probe, F7) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and signals were quantified as previously described (Cho et al., 2005a).

2.10. Statistical analysis

Statistical analyses were carried out using GraphPad Prism software, using one-way analysis of variance with either Bonferroni or Dunnett post-tests. Correlation analyses were performed according to Pearson. Statistical significance of differences is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

3. Results

Using a wide panel of eleven GR ligands in parrallel this study investigates for the first time, using a systematic and quantitative approach, the relationship between the potency (EC_{50}) and maximal response (efficacy) of transcriptional activity, the relative binding affinity for the GR, and the apparent affinity for cofactor interaction. We investigated transactivation via GREs and transrepression via both NFκB and AP-1 sites, as well as both coactivator (full length SRC-1A and full length GRIP-1) and corepressor (NCoR-RID and SMRT-RID) interaction. Experiments were performed where possible with saturating concentrations of compounds to eliminate effects due to differential fractional occupancy of the GR attributable to varying affinities of ligands for the GR. Importantly, RBA, transactivation and transrepression experiments were performed in COS-1 cells (deficient in GR and other steroid receptors) in the absence and presence of expressed GR, in order to establish a requirement for the GR, since many of the ligands also bind to other steroid receptors which are found to varying extents in many other cell types.

The panel of eleven GR ligands was chosen to include available endogenous and synthetic steroidal and non-steroidal agonists, partial agonists, selective modulators and antagonists (see supplementary figure for structures). Most of the ligands are well described in the literature, with some exceptions. D06 is a synthetic non-steroidal ligand that binds to the GR with a low affinity, has been shown to antagonize the effects of DEX via the GR, has no known agonist activity and does not induce DNA binding by GR *in vitro* or *in vivo* (Miner et al., 2003). AL438 is a potent, synthetic, non-steroidal agonist for selective promoters and a partial agonist for other promoters, in both GR-mediated transactivation and transrepression (Coghlan et al., 2003). CpdA, a synthetic non-steroidal analogue of a plant derivative has been previously shown to compete with DEX for binding to the GR and to repress NFκB-mediated but not AP-1-mediated gene expression in the presence of the GR (De Bosscher et al., 2005) and to be a potential anti-inflammatory drug (Dewint et al., 2008). MPA and NET-A are steroidal synthetic progestins used in contraception and hormone replacement therapy and have also been previously shown to compete with DEX for binding to the GR (Koubovec et al., 2005). NET-A can antagonize the trancriptional activity of DEX via the GR (data not shown) while MPA is an agonist or partial agonist (Koubovec et al., 2005).

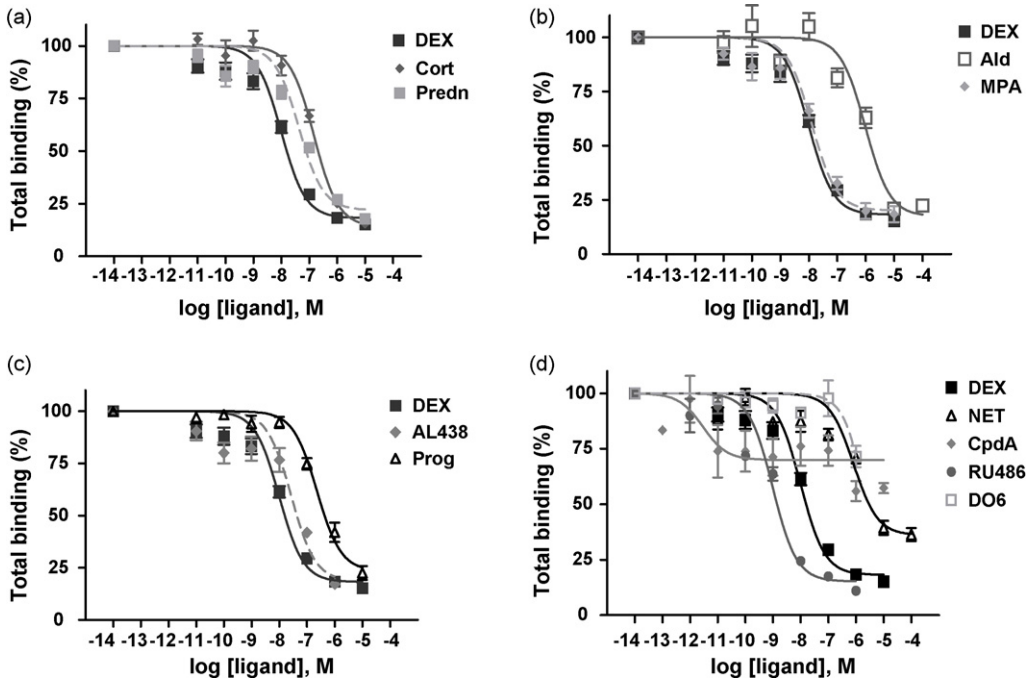

Fig. 1. Competition with [^{3}H] DEX for binding to the GR. COS-1 cells were transiently transfected with HA-hGR-pCMV and incubated with 20 nM [^{3}H] DEX in the absence and presence of increasing concentrations of the eleven different test compounds. Competition for binding is illustrated by the percent of [^{3}H] DEX bound to the hGR. Results shown are combined curves from pooled data of two to three independent experiments, each performed in triplicate (± S.E.M.). D06 bound very weakly to the GR and due to availability limits it was not possible to add at concentrations greater than 1 μM. (a) "Full agonists", (b) "Full/Partial agonists", (c) "Partial agonists" and (d) "Dissociated ligands" and "Antagonists". Note that DEX is included in all panels as a reference.

3.1. Ligand binding

Relative binding affinities for the GR of the panel of ligands were determined by competitive whole cell binding assays in COS-1 cells transiently transfected with the human GR. Most of the compounds exhibited binding curves consistent with competitive binding to a single site, compared with DEX for binding to this site, and can be considered as true GR ligands (Fig. 1). The compounds exhibited a wide range of relative affinities for the GR compared to DEX (Table 1). For some of the low affinity ligands it was not possible to obtain accurate RBAs due to incomplete curves, in which case RBAs were estimated using Graphpad Prism, although Ki's have been previously reported for these ligands i.e. 210 nM for D06 (Miner et al., 2003) and 270 nM for NET-A (Koubovec et al., 2005). Interestingly CpdA displayed an atypical binding curve in these cells, unlike that seen in other cells (De Bosscher et al., 2005), suggesting an unusual mode of binding to the GR.

Table 1
Potencies for transactivation and transrepression as well as relative binding affinities of the ligands for the GR expressed as EC_{50} or IC_{50} in nM.

Ligand	GRE EC_{50}	NFκB EC_{50}	AP-1 EC_{50}	Binding IC_{50}
DEX	0.23 ± 0.12	0.005 ± 0.002	0.003 ± 0.002	14 ± 4
Cort	16 ± 12	7 ± 0.7	2 ± 1	152 ± 9
Pred	0.1 ± 0.06	0.05 ± 0.09	0.002 ± 0.002	68 ± 25
Ald	138 ± 31	27 ± 14	18 ± 9	1130 ± 324
MPA	2 ± 0.4	0.7 ± 0.1	0.0005 ± 0.0002	19 ± 3
AL438	8 ± 3	13 ± 5	0.05 ± 0	61 ± 13
Prog	1688 ± 497	384 ± 249	0.004 ± 0.004	274 ± 77
RU486	na	0.008 ± 0.005	0.004 ± 0.006	1 ± 0.4
CpdA	na	1543[a]	na	0.003 ± 0.004
NET	na	na	na	1688 ± 300[a]
DO6	na	na	na	6500[a]

EC_{50} values for potencies of transactivation and transrepression as well as IC_{50} values for binding affinities as determined in transiently transfected COS-1 cells are given in nM. na = no activity. Values shown for TA and TR were obtained from three to five independent experiments, each performed in triplicate. Values shown for binding were obtained from two to three independent experiments, each performed in triplicate. The values were obtained separately from curves of the individual experiments and the mean ± S.E.M. calculated from several experiments.

[a] Estimated by GraphPad Prism due to incomplete curve.

3.2. Ligand-selective transactivation and transrepression potency and efficacy

To determine potency and efficacy for transactivation (TA) on a GRE-containing promoter via the liganded-GR, COS-1 cells were transiently transfected with a luciferase reporter plasmid linked downstream of the tyrosine aminotransferase (TAT) GRE, as well as a human GR expression plasmid. Similarly, for transrepression (TR) COS-1 cells were transiently transfected with luciferase reporter plasmids linked downstream of either five copies of an NFκB site or seven copies of an AP-1 site, both in the presence of the GR expression vector. Dose response curves were plotted (Fig. 2), and EC_{50}s (ligand concentration required for half maximal response or potency) in nM (Table 1) and maximal responses (efficacies) relative to DEX (Table 2) were determined.

The compounds exhibited a wide range of efficacies and potencies. For the purposes of this study, we will henceforth refer to a ligand, for the particular gene and cell system being investigated, as a full agonist if it induces the maximal possible response and as a partial agonist if it induces a response less than maximal. Given the margin of experimental error in the system, we will define full agonist activity for this study as efficacy ≥85% and partial agonist activity as efficacy <85% as compared to DEX. We will define an antagonist as a compound that binds to the receptor, induces no response on its own and has been shown to antagonize the transcriptional effects of DEX. For both TA and TR, DEX, cort and pred exhibited full agonist activity, AL438 and prog exhibited partial agonist activity, while D06 and NET-A exhibited no activity. Some

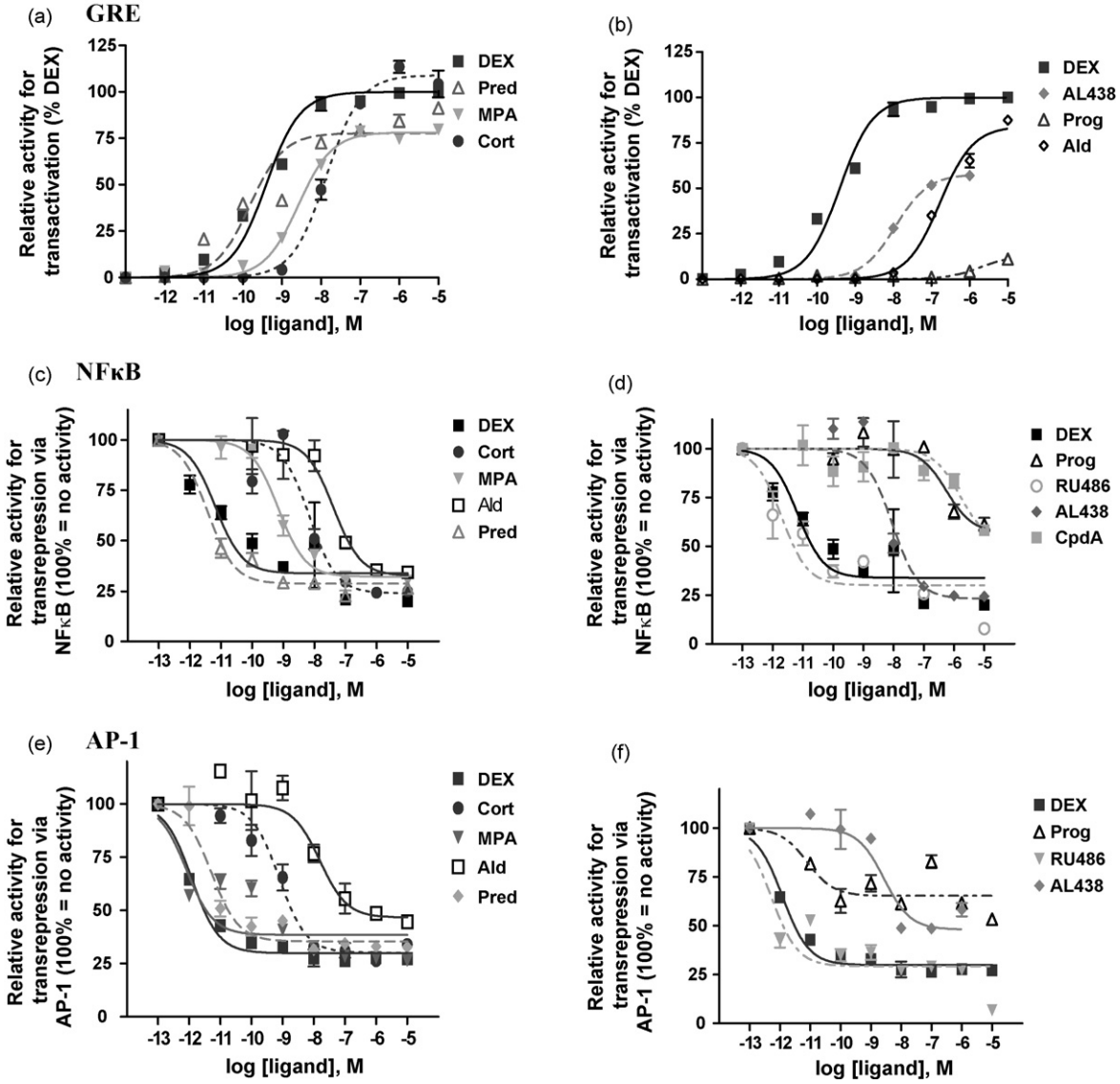

Fig. 2. Relative activity for transactivation and transrepression via the GR. COS-1 cells were transiently transfected with HA-hGR-pCMV and either pTAT-GRE-E1b-luc (a) and (b) NFκB-luc (c) and (d) or AP-1-luc (e) and (f). The cells were incubated in absence and presence of increasing concentrations of the eleven different test compounds as described in materials and methods, thereafter the lysates were tested for luciferase activity. For transrepression, cells were stimulated with PMA before adding ligand. Only curves of ligands that transactivate or transrepress are shown. One representative experiment for each compound performed in triplicate (± S.E.M.) is shown, unless no activity was observed at 10 μM compound relative to background.

compounds i.e. RU486 and CpdA exhibited no agonist activity on at least one promoter while exhibiting full or partial agonist activity on at least one other promoter, and will be referred to as dissociated glucocorticoids. RU486 exhibited no agonist activity for TA and MPA behaved as a partial agonist for TA, while both compounds exhibited full agonist activity for TR via both NFκB and AP-1 sites (Tables 1 and 2). Interestingly, although efficacies for the agonists DEX, cort and pred are similar on the three synthetic promoters, there are several differences for the partial agonists. For example, prog exhibits 18%, 30% and 61% efficacy relative to DEX on the GRE, NFκB and AP-1 promoters, respectively (Table 2). AL438 appears to be less efficacious on the AP-1 than on the NFκB promoter (58% vs. 84%), while MPA is more efficacious for TR via AP-1 than TA (98% vs. 73%) (Table 2). Experiments performed in the absence of transfected GR revealed that the responses obtained were due to the presence of the GR (not shown). These results thus reveal ligand-selective differences in efficacy on a single promoter as well as between promoters for some ligands, in the same cell system via the same receptor.

When comparing EC_{50}s (defined as potencies) between promoters, a general trend can be detected where for a particular ligand, the potency for TA via a GRE is less than the potency for TR via NFκB which is in turn less than that for AP-1 (Table 1) (e.g. for MPA the EC_{50}s (nM) for GRE:NFκB:AP-1 are 2:0,7:0,0005). In addition, the differences in potency between the three promoters for a particular ligand differ dramatically for different ligands. For example, DEX has a 46-fold greater EC_{50} for TA than for TR via NFκB (0.23 nm vs. 0.005 nM) while for AL438 they are similar (8 nM vs. 13 nM). Similarly, while several full agonists for TR have a similar EC_{50} for TR via NFκB and AP-1 (7 nM and 2 nM, respectively for cortisol; 27 nM vs. 18 nM respectively for ald), MPA, AL438 and prog exhibit much greater potency for TR via AP-1 than via NFκB (e.g. 0.7 nM vs. 0.0005 nM, respectively for MPA) (Table 1). These differences cannot be explained by varying levels of expressed proteins since

Table 2
Relative efficacies for transactivation and transrepression via the GR and ligand classification.

Ligand	GRE Rel. efficacy	NFκB Rel. efficacy	AP-1 Rel. efficacy	Ligand type
DEX	100	100	100	Full agonist
Cort	101 ± 16	89 ± 9	104 ± 25	Full agonist
Pred	90 ± 11	102 ± 25	88 ± 4	Full agonist
Ald	91 ± 11	83 ± 26	82 ± 19	Full agonist or partial agonist
MPA	73 ± 5	87 ± 19	98 ± 7	Full agonist or partial agonist
AL438	67 ± 12	84 ± 3	58 ± 3	Partial agonist
Prog	18 ± 15	30 ± 6	61 ± 11	Partial agonist
RU486	na	92 ± 6	91 ± 23	Dissociated
CpdA	na	29 ± 5	na	Dissociated
NET	na	na	na	Antagonist
DO6	na	na	na	Antagonist

Relative efficacies for transactivation via pTAT-GRE-E1b-luc and for transrepression via either NFκB-luc or AP-1-luc reporter constructs, were determined in transiently transfected COS-1 cells. Relative efficacies were calculated from the maximal responses. Values were obtained from the individual curves from three to five independent experiments, each performed in triplicate, where after the mean ± S.E.M. was calculated for each ligand. Values are expressed as a % relative to DEX activity, where the difference from the top to the bottom of the DEX curve was taken as 100%. For this study, the compounds are grouped as "full agonist" when this activity was observed for all three promoters, "full agonist or partial agonist" when the activity is either that of full agonist or partial agonist on the three promoters, "partial agonist" when the activity is that of a partial agonist on all three promoters, "dissociated" when the ligand showed no activity on at least one promoter with partial or full activity on at least one other promoter and as "antagonist" when the compound exhibited no activity on any of the three promoters. na = no activity. Full agonist activity is defined here as ≥85%, and partial agonist as <85%, relative to 100% activity of DEX.

each independent experiment was performed with the whole panel of ligands in parallel. These results thus reveal ligand-selective differences in potency on a single promoter as well as between promoters for some ligands, in the same cell type, via the same receptor.

To compare the results using the synthetic reporter assays and expressed GR with those using an endogenous GRE-containing promoter with endogenous GR, real time PCR was used to measure the relative efficacies of eight of the ligands for transactivation of the *GILZ* gene in U2OS cells (Fig. 3a). DEX treatment resulted in approximately 6-fold upregulation of GILZ after 2 h, which is in line with published data for this gene (Chen et al., 2006). Prog, NET-A, and RU486 did not result in transcriptional activation of the gene. In order to confirm a requirement for the GR in transactivation of GILZ, endogenous GR expression was knocked down by siRNA. Western blot analysis confirmed that transfection of siRNA for the GR resulted in approximately 75% reduction in endogenous GR protein levels compared to transfection of non-silencing control RNA (Fig. 3b and c). Upregulation of GILZ mRNA was lost when GR was knocked down (Fig. 3d), confirming that the ligand-dependent upregulation was due to the presence of GR. A positive correlation was found between the efficacy for GILZ transactivation and reporter assay results on the synthetic GRE-luc construct ($r^2 = 0.60$, $P = 0.0253$) (not shown), supporting the physiological relevance of the results on synthetic promoter reporter genes with expressed GR.

3.3. Relationship between affinity, potency and efficacy for transactivation and transrepression

It is interesting to examine the relationship between affinity, potency and efficacy for both transactivation and transrepression in the COS-1 cells (Table 3). Those agonists and partial agonists with high affinity could be expected to have high potency, since a greater percentage of receptor would be occupied by the ligand with the higher affinity, at a given concentration of ligand. This is indeed observed for the agonists and partial agonists for TA via the synthetic GRE (Fig. 4a, Table 3). Interestingly, it does not hold for TR via either NFκB or AP-1. The significance of this is unclear but suggests that steps downstream of steroid binding to receptor, that differ for TR versus TA, can alter the EC_{50} (Tao et al., 2008; Kim et al., 2006). However, it is clear that for agonists and partial agonists, RBA does not correlate with efficacy for either TA (Fig. 4b) or TR (Table 3).

3.4. Ligand-selective interaction of the GR with coactivators

To determine the capability of the different compounds to facilitate interaction between the liganded-GR and the coactivators GRIP-1, TIF2.4, and SRC-1A, the mammalian two-hybrid assay was performed in U2OS cells transiently transfected with VP-16-hGR plus a luciferase reporter plasmid containing GAL4 binding sites, in the presence of expression vectors for the cofactors expressed as chimeric proteins linked to the GAL4 DNA-binding domain (Fig. 5). Since the magnitude of gene expression in two-hybrid assays is determined by the affinity of the two interacting proteins for each other (SuwwandeFelipe et al., 2004), these results can be taken as a measure of apparent affinity of the liganded-GR for the coregulator protein. These experiments were done in U2OS cells rather than in COS-1 cells, due to better reproducibility of the experiments in U2OS cells (not shown).

What is immediately evident in Fig. 5 is that all the coactivators interacted with the GR in a ligand-selective manner. Experiments in the absence of transfected GR showed that the interactions were GR-dependent (not shown). GRIP-1 interacted more strongly with the GR in the presence of agonists for TA, as opposed to partial agonists, which in turn lead to stronger interactions than those ligands showing no TA activity (Fig. 5a). This correlation is clearly evident when comparing the relative GRIP-1 interaction with TA efficacy by correlation analysis (Table 4, Fig. 6). A good and significant correlation was obtained between GRIP-1 interaction and efficacy of TA ($r^2 = 0.75$, $P < 0.001$) as well as TR via NFκB sites ($r^2 = 0.68$, $P < 0.01$). A less good, but still significant correlation was obtained between GRIP-1-GR interaction and TR via AP-1 sites ($r^2 = 0.48$, $P < 0.05$)

Table 3
Correlation coefficients for efficacies and potencies of transactivation and transrepression with relative binding affinities.

RBA	GRE		NFκB		AP-1	
	Efficacy	Potency	Efficacy	Potency	Efficacy	Potency
All ligands	0.10	0.10	0.08	0.18	0.08	0.10
Agonists and partial agonists	0.11	0.38**	0.05	0.18	0.03	0.11

Relative efficacies and potencies shown in Tables 1 and 2 were correlated to the respective relative binding affinities (RBAs) using Pearson correlations. The correlation scatter plots for RBA versus potency and RBA versus efficacy for the GRE are shown in Fig. 4a and b, respectively. The table shows Pearson r^2 values for "all ligands" and "agonists and partial agonists" only, for all three promoter constructs. Note the group of compounds included in the analysis for "agonist and partial agonists" was particular to each promoter, since some compounds for example acted as an agonist on one promoter but showed no activity on another. Note for potency, the value–log [EC_{50}] nM was used for correlation analysis.

** Statistical significance of differences indicates $P < 0.01$.

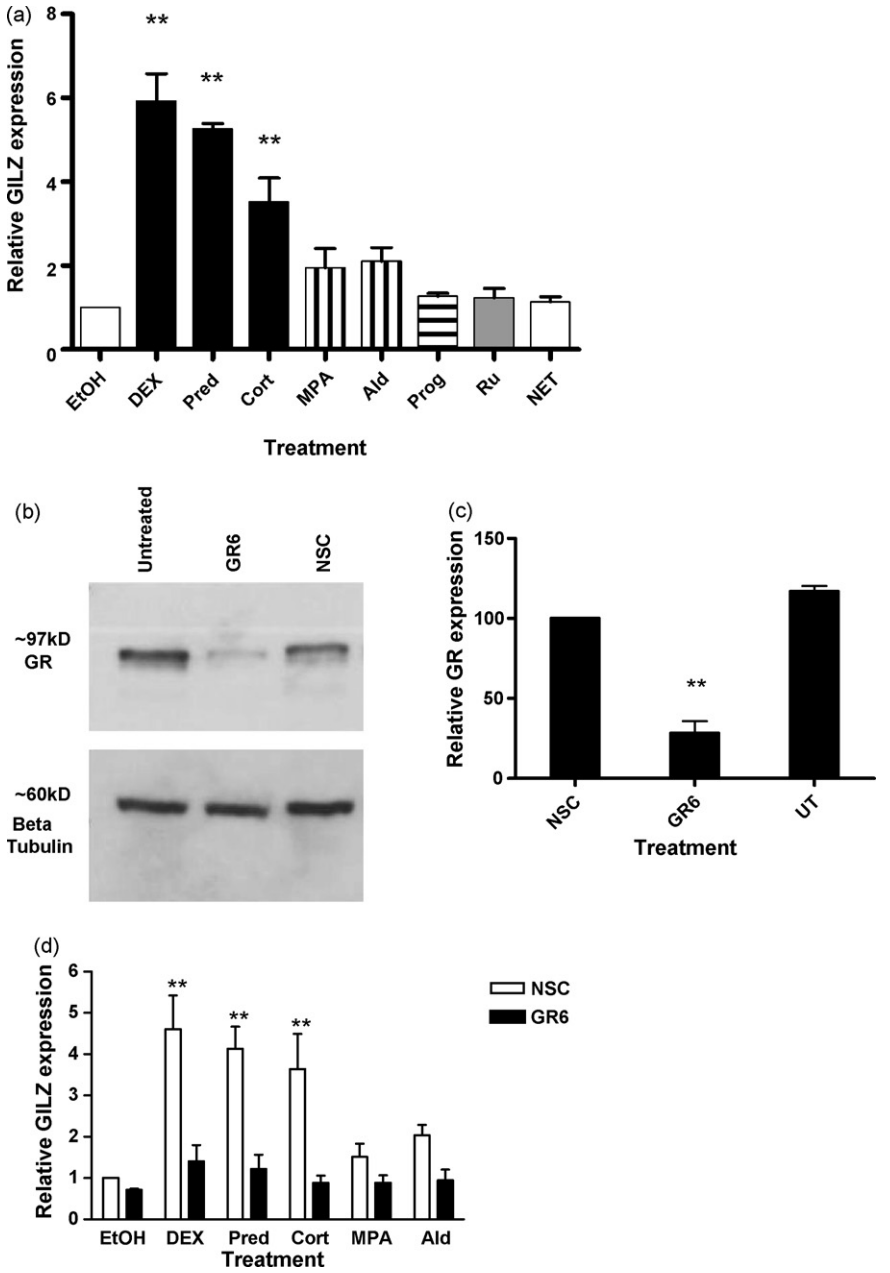

Fig. 3. Regulation of endogenous *GILZ* gene by GR ligands. (a) U2OS cells were serum starved for 2 h prior to treatment with saturating concentrations of each ligand for 2 h. Concentrations of ligands were as follows: 100 nM DEX, 1 μM pred, 1 μM cort, 100 nM MPA, 10 μM ald, 1 μM prog, 100 nM RU486, 10 μM NET. RNA was harvested whereafter cDNA was synthesized and then subjected to real time PCR using primer sets specific for GILZ and GAPDH. Levels of GILZ transcripts were normalised to GAPDH levels for each sample. The graph shows pooled results of three independent experiments ± S.E.M. The bars are shaded to indicate type of ligand as defined in Table 2 as either always full agonists (solid black), full agonists or partial agonists (vertical stripes), always partial agonists (horizontal stripes), dissociated glucocorticoids (grey) or always antagonists (white). (b) U2OS cells were transiently transfected with 10 nM siRNA encoding GR (GR6) or 10 nM non-silencing control siRNA (NSC), or left untreated (UT) for 48 h. Total protein was harvested and GR expression was analysed by Western blot, relative to beta tubulin. (c) GR expression from three independent experiments was quantified relative to beta tubulin using AlphaEase densitometry software. Graph shows mean ± S.E.M. Statistically significant difference to NSC ($P < 0.01$) is denoted by **. (d) U2OS cells were transiently transfected with 10 nM siRNA encoding GR (GR6) or 10 nM non-silencing control RNA (NSC). After 48 h cells were serum-starved and treated with saturating concentrations of GR ligand for 2 h. RNA was then harvested, and reverse transcribed. GILZ expression was quantified by real time PCR, and normalised to GAPDH expression for each sample. Results from three independent experiments were pooled and graph shows mean ± S.E.M. Statistically significant difference to NSC, EtOH ($P < 0.01$) is denoted by **.

(Table 4). Similarly, a good ($r^2 > 0.47$) and significant ($P < 0.05$) correlation was obtained for GRIP-1 interaction and potency of TA and TR via AP-1 and NFκB sites (Table 4). Taken together this suggests that ligand-selective binding of liganded-GR to GRIP-1 is a major determinant for the potency and maximal biological response for TA via synthetic GREs (Table 4, Fig. 6) as well as TR via both NFκB and AP-1 (Table 4). Similar results were obtained with TIF2.4, a fragment of TIF2, the human homologue of GRIP-1, containing the receptor interaction domains (Fig. 5b) (Ding et al., 1998; He et al., 2002). In support of the physiological relevance of these results, a positive correlation was also found between the ligand-dependent efficacy for endogenous GILZ transactivation (Fig. 3) using a smaller panel of eight ligands, and ligand-dependent GRIP-1 recruitment in the two-hybrid assay ($r^2 = 0.87$, $P < 0.001$) (not shown).

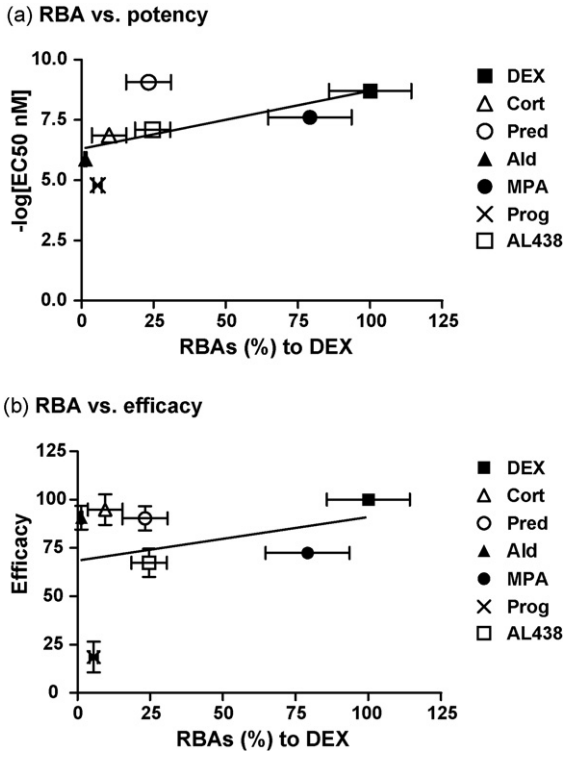

Fig. 4. Correlation analysis for RBA versus potency and efficacy on the synthetic GRE-reporter A correlation analysis was performed for only agonists and partial agonists for transactivation on the GRE reporter for RBA versus potency (a) and RBA versus efficacy (b) using Pearson correlations. The r^2 values are shown in Table 3. Note that RU486 is not shown in this graph because it fell off the scale with a value for RBA of 1294. Note for potency, the value $-\log[EC_{50}]$ nM was used for correlation analysis.

Interaction of the GR with SRC-1A also revealed a ligand-selective pattern and also a good and significant correlation with TA and TR efficacy (GRE: $r^2 = 0.75$, $P < 0.001$; NFκB: $r^2 = 0.64$, $P < 0.01$; AP-1: $r^2 = 0.44$, $P < 0.05$), as well as potency of TA and TR via NFκB and AP-1 sites (GRE: $r^2 = 0.65$, $P < 0.01$; NFκB: $r^2 = 0.56$, $P < 0.01$; AP-1: $r^2 = 0.60$, $P < 0.01$). However the SRC-1A results revealed some distinct differences as compared to GRIP-1 (Table 4, Fig. 5). SRC-1A appeared to show less discrimination between agonists and partial agonists than GRIP-1, since it interacted to a similar extent with the GR bound to some of the partial agonists (i.e. prog and AL438) as bound to the full agonists (DEX, cort and pred), while ald (a full agonist in TA but a partial agonist in TR) exhibited a greater apparent affinity for SRC-1A than all the other agonists, unlike that seen for GRIP-1. No correlation existed between affinity of the GR for ligand and interaction with coactivators (not shown).

Table 4
Correlation coeffcients for efficacies of transactivation and transrepression with co-factor interaction.

Two-hybrid assay	GRE		NFκB		AP-1	
	Efficacy	Potency	Efficacy	Potency	Efficacy	Potency
GRIP-1	0.75***	0.77***	0.68**	0.58**	0.48*	0.48*
TIF2.4	0.95***	0.76***	0.60**	0.43*	0.42*	0.28
SRC-1A	0.75***	0.65**	0.64**	0.56**	0.44*	0.60**
NCoR-RID	0.05	0.00	0.47*	0.60**	0.37*	0.37*
SMRT-RID	0.74***	0.54**	0.78***	0.65**	0.25	0.50*
ABCD assay						
GRIP-1	0.402*	0.46*	0.28	0.30	0.12	0.20

Relative efficacies for the three promoter constructs were determined as described for Table 2 and correlated to the respective relative ability of the GR-ligand complex to recruit the co-factors GRIP-1, TIF2.4, SRC-1A, NCoR-RID and SMRT-RID using Pearson correlations. Results shown are Pearson r^2 values for all ligands.

* Statistical significance of differences indicates $P < 0.05$.

** Statistical significance of differences indicates $P < 0.01$.

*** Statistical significance of differences indicates $P < 0.001$.

3.5. Ligand-selective interaction of the GR with corepressors

To determine the relative affinities of the liganded-GR for the corepressors NCoR-RID and SMRT-RID, a similar strategy as for coactivators using the mammalian two-hybrid assay in U2OS cells was used. Fig. 5d and e shows that both NCoR-RID and SMRT-RID exhibit ligand-selective interaction with the GR. Recruitment of NCoR-RID correlates positively and significantly with potency and efficacy for TR via both NFκB and AP-1 sites, but not with TA (Table 4). Similarly, interaction with SMRT-RID correlates positively and significantly with potency and efficacy for TR via NFκB, but only with potency and not efficacy via AP-1, suggesting some differences in the mechanisms of TR. Interestingly, while NCoR-RID interaction does not correlate with TA potency or efficacy, SMRT-RID interaction exhibits a good and significant correlation with efficacy ($r^2 = 0.74$, $P < 0.001$) and potency ($r^2 = 0.54$, $P < 0.01$) for TA (Table 4).

SMRT-RID exhibited a similar pattern of ligand-selective interaction to that of both GRIP-1 and NCoR-RID, with some exceptions (Fig. 5e). RU486-GR exhibited the strongest interaction with NCoR-RID (Fig. 5d) in contrast to results with GRIP-1, SRC-1A and SMRT-RID. However, the rest of the ligands exhibited a very similar pattern relative to each other as that obtained for GRIP-1, with the ligands acting as agonists for TA also giving a signal above background for NCoR-RID interaction, albeit to a lower extent than RU486 (Fig. 5d). Unlike the result obtained for NCoR-RID, the RU486-GR interacted weaker than DEX-GR with SMRT-RID. In addition, AL438-GR interacted with SMRT-RID to a similar extent as DEX-GR, unlike AL438-GR interaction with GRIP-1. Interestingly NET-A and D06, which are antagonists for both TA and TR (Table 2), failed to interact with corepressors. Furthermore, with the exception of RU486, we did not observe an increased interaction of the corepressors with the partial agonist-bound or dissociated ligand-bound GR as compared to agonist-bound GR. No correlation was obtained for ligand affinity for the GR and interaction with corepressors (data not shown).

3.6. Recruitment of GRIP-1 by DNA-bound liganded-GR

In order to assess the role of the native DNA-bound liganded-GR in ligand-selective recruitment of a coactivator in a cell-free system, by comparison with the interaction in the whole cell in the two-hybrid assay, the ABCD pull down assay (Cho et al., 2005a) was performed with native GR, HA-tagged GRIP-1 (full length protein) and biotinylated DNA containing a single GRE immobilized on Streptavidin–agarose beads. The results showed clear ligand-selective binding of GRIP-1 to the liganded-GR-GRE complex (Fig. 7). No binding of GRIP-1 was detected in the absence of ligand (Fig. 7), in the absence of GR and in the absence of GRIP-1 (not shown). The *in vitro* binding of GR to the GRE was, however, ligand-independent, as shown by the approximately constant levels of GR for the different compounds (Fig. 7a and c). This is consistent with previous reports of ligand-independent *in vitro* activation of GR for *in vitro* DNA binding (Cho et al., 2005a). A comparison of these ABCD results with the results obtained for GRIP-1 in the two-hybrid assay revealed that the pattern of ligand-selective GRIP-1 interaction (Fig. 7b and d) was similar, with a general trend of agonists for TA bound to GR showing the greatest interaction, partial agonists for TA bound to GR showing weaker interaction and the

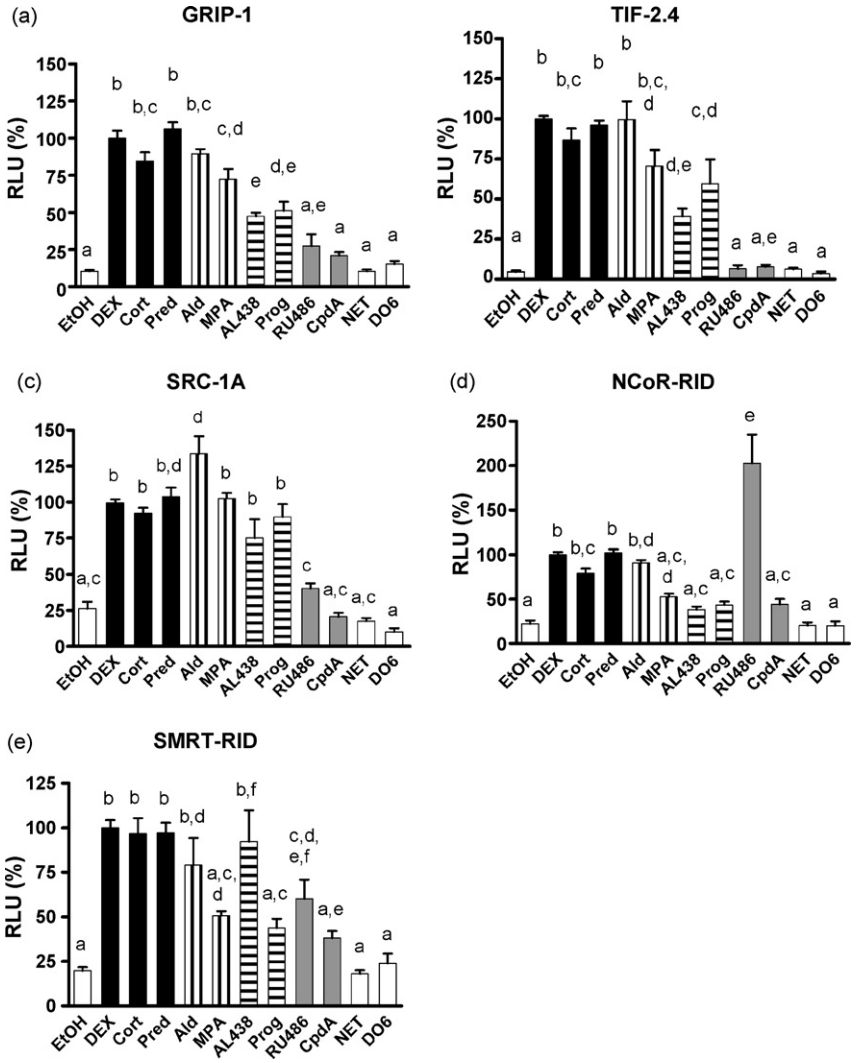

Fig. 5. Ligand-selective interaction of the GR with cofactors in a mammalian two-hybrid assay For mammalian two-hybrid assays U2OS cells were transiently transfected with VP-16-GR, pFR-luc and either GAL-GRIP (a), GAL-TIF2.4 (b), GAL-SRC-1A (c), GAL-NCoR-RID (d) or GAL-SMRT-RID (e). The cells were stimulated with 10 μM of the different ligands and luciferase activity was determined. The bars are shaded to indicate type of ligand as described in the legend to Fig. 3. Results shown are pooled data from four to six independent experiments each performed in duplicate. The mean values (±S.E.M.) for the ligands were expressed as % relative to DEX, which was taken as 100%. Statistical analysis was performed with Bonferroni's multiple comparison test. The letters a, b, c etc. indicate statistical significance where values with the same letter are not statistically significantly different from each other.

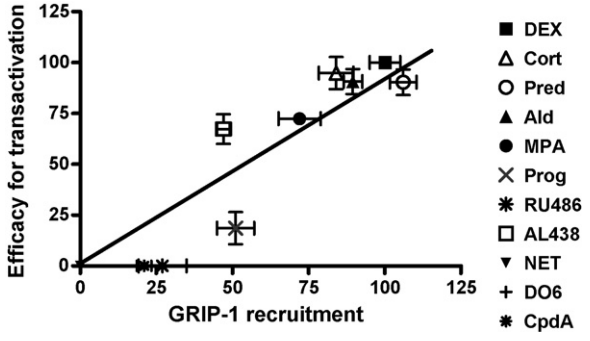

Fig. 6. Correlation analysis for efficacy of transactivation versus GR–GRIP-1 interaction. Relative efficacies of transactivation on the synthetic *GRE*-reporter gene in COS-1 cells (Table 2) were compared to relative GRIP-1 interaction for all the ligands as determined by mammalian two-hybrid assay (Fig. 5a) using Pearson correlations. Results showed $P < 0.001$ and $r^2 = 0.75$ (Table 4).

ligands showing no agonist activity for TA showing no interaction. However, some interesting differences were observed. The ABCD assay appears to discriminate sharply between different TA agonists, with DEX-GR exhibiting approximately double the amount of recruitment as compared to pred-GR, which in turn recruits more GRIP-1 than cort-GR. Additionally, AL438, a partial agonist for TA, facilitated greater recruitment by the liganded-GR of GRIP-1 than the full agonist cortisol, unlike that seen in the two-hybrid assay. Furthermore, while in the two-hybrid assay some of the compounds exhibiting no TA activity (i.e. CpdA and RU486) still resulted in a small signal above background, in the ABCD assay this was not the case. The significance of these differences is unclear since they are not reflected in agonist biological activity for TA for DEX, pred and cort, but are for the compounds with no TA agonist activity.

Correlation analysis revealed a significant but weak correlation between GRIP-1 recruitment by the ABCD assay and TA efficacy ($r^2 = 0.402$, $P < 0.05$) and potency ($r^2 = 0.46$, $P < 0.005$) on the synthetic GRE. (Table 4). Interestingly we have found that the relative efficacy of a smaller panel of eight of the ligands for transactivation of the endogenous *GILZ* gene by real time PCR correlated well

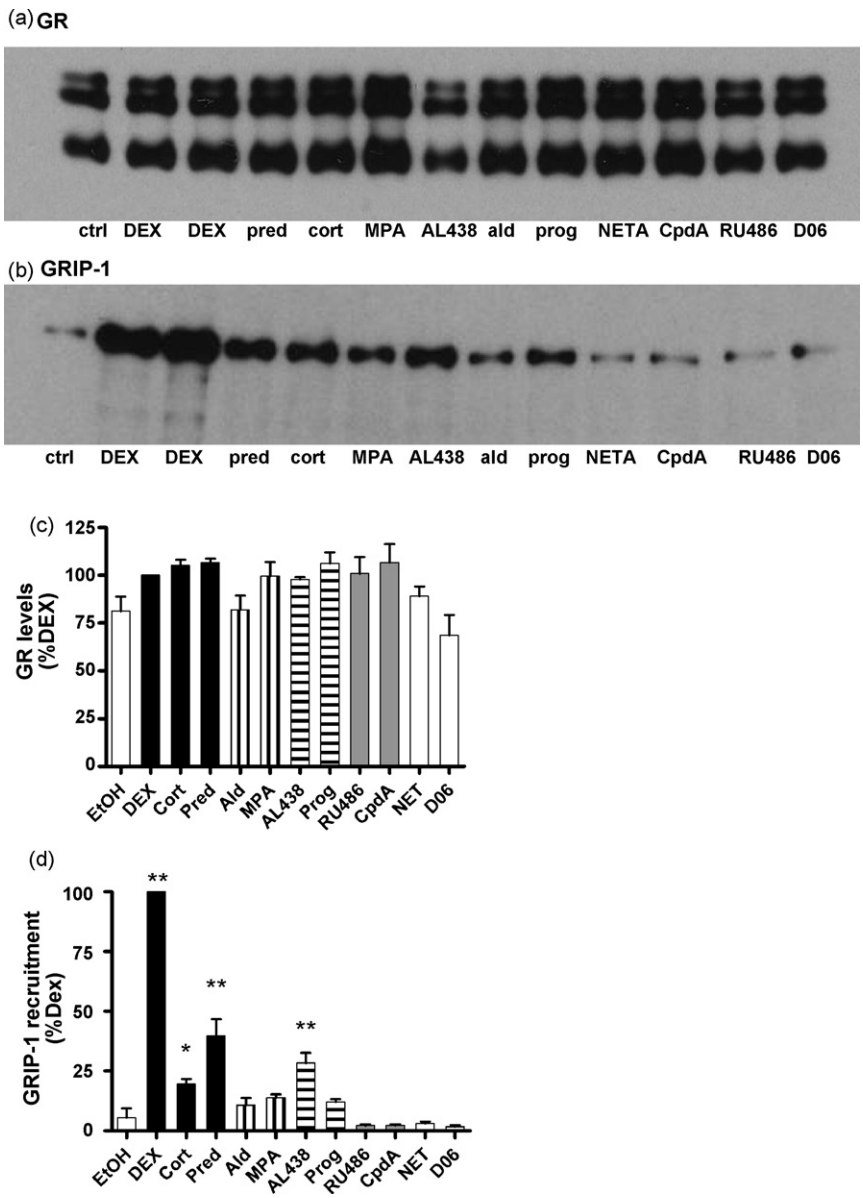

Fig. 7. Ligand-selective GRIP-1 binding to DNA-bound GR *in vitro*. COS-7 cytosols (30 μl) containing expressed GR prebound with ethanol with or without 10 μM different ligands (as shown in figs) were incubated with biotinylated GRE oligonucleotides attached to Streptavidin beads followed by COS-7 cytosols (30 μl) containing over-expressed HA-GRIP-1. DNA-bound GR or HA-GRIP-1 were separated by SDS–PAGE and visualized by Western blotting with anti-GR (a) or anti-HA antibodies (b). The amount of GR or GRIP-1 was quantified by laser densitometry and the results for the GR (c) and HA-GRIP-1 (d) from three independent experiments (average ± S.E.M.) were plotted. The bars in (c) and (d) are shaded to indicate type of ligand as described in the legend for Fig. 5. Statistical significance of differences is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

and significantly ($r^2 = 0.70$, $P = 0.01$) with the results obtained in the ABCD assay (data not shown). There was no significant correlation with TR efficacy or potency on NFκB or AP-1 synthetic promoters for the ABCD assay.

4. Discussion

4.1. Ligand affinity for GR and potency and efficacy of transactivation and transrepression

Our panel of ligands exhibited a wide range of affinities for the GR as well as efficacies and potencies of transactivation and transrepression on three different synthetic promoters. These results show, for the first time in a systematic approach with binding and transcriptional activity being determined in the same system for a wide panel of ligands, that the efficacy of a ligand for both TA and TR is unrelated to its relative affinity for the receptor. This is consistent with a model whereby the particular conformation induced in the GR by a particular ligand is unrelated to the affinity of the ligand for the GR. We find that the affinity of agonists and partial agonists for the GR correlates with potency for TA via a GRE, but not for TR via both NFκB and AP-1. The significance of this is unclear but suggests that steps downstream of steroid binding to receptor, which differs for TR versus TA, can alter the EC_{50} (Tao et al., 2008; Kim et al., 2006). The results showed ligand-selective differences in efficacy and potency on one promoter and between promoters for some ligands, in the same cell system via the same receptor. This suggests that critical interacting partners of the liganded-GR differ for transcriptional regulation at the three promoters, which are affected differently by the nature of the ligand.

These results on reporter genes also support recent microarray data showing ligand- and gene-selective differences between transactivation and transrepression of endogenous genes via the GR (Wang et al., 2006) that underscore basic mechanistic differences in the two general modes of GR action. However, for a particular GR ligand, the opposite effect of gene induction versus repression on a GRE- versus an AP-1-containing promoter appears to result from interactions that are downstream of ligand and cofactor binding to the GR (Sun et al., 2008). The current data establish that for GR-signalling ligand- and gene-selective differences do not require the presence of chromatin and can be achieved in the context of minimal synthetic promoters in the absence of chromatin. Furthermore, our findings that transactivation via an endogenous GRE also correlates positively with transactivation via the synthetic GRE-luc construct, as well as with GRIP-1 recruitment in a two-hybrid assay, as well as on liganded-GR-bound DNA *in vitro*, are consistent with the above. The promoter-specific differences in the rank order of potencies and efficacies of several ligands via the synthetic GRE, AP-1 and NFκB promoters may have important implications for anti-inflammatory drug design where it may be advantageous to selectively target endogenous genes (Rhen and Cidlowski, 2005; Adcock et al., 1999).

4.2. Role of coactivators and corepressors in ligand-selective GR-mediated transactivation

As detailed in Section 1, a critical evaluation of the results in the literature reveals that there is very little quantitative evidence showing a direct correlation between the extent of recruitment of coactivators or corepressors by a liganded-steroid receptor and the potency or efficacy for transactivation. The few studies that do address this question are limited in that they do not quantitatively compare the interactions with cofactors with the transactivation responses and in that they use too few ligands to allow for general conclusions to be drawn with confidence. Additionally, most studies on this topic were done with cofactor peptides (for example Kroe et al., 2007; Ozers et al., 2007; Hultman et al., 2005; Miner et al., 2007), which might not truly reflect the interaction of the full length endogenous cofactors with the GR. Despite several reports in the literature to the contrary (see Section 1), it appears to be generally accepted that agonist-liganded receptor interacts with coactivators but are not able to interact with corepressors, that antagonist-liganded receptor interacts with corepressors but not with coactivators and that partial agonist-liganded receptor can interact with both coactivators and corepressors, while interacting more with corepressors than agonist-receptor (Kang et al., 2004). Our results in this systematic study with eleven GR ligands support some but refute other aspects of the above model.

In support of this model, our results clearly show that apparent affinity of agonist-GR for full length coactivators GRIP-1 and SRC-1A is much greater than that for antagonist-receptor, while for partial agonists the apparent affinity relative to agonists varies depending on ligand, nature of coactivator, and binding to DNA. We do, however, observe that GR liganded to several of the partial agonists recruit similar levels or even more coactivator than GR liganded with agonist. Nevertheless our results suggest that coactivator recruitment is a major determinant for the potency and maximal biological response for transactivation via GREs for the GR. These results are consistent with other reports of coactivators increasing the potency and efficacy of liganded-GR (Wang and Simons, 2005; Szapary et al., 1999; Chen et al., 2000; Cho et al., 2005b; Szapary et al., 2008), MR (Wang et al., 2004a), and PR (Wang et al., 2007; Szapary et al., 2008; Giannoukos et al., 2001) for transactivation (Simons, 2008). Interestingly, recent studies with GR mutants suggest that the activity of recruited coactivators is sensitive to the combination of both bound agonist steroid and receptor structure (Tao et al., 2008).

A comparison between our ABCD and two-hybrid results both done with full length GRIP-1 suggests that binding to a GRE changes the relative affinity of agonist– and antagonist–GR complexes for GRIP-1, as well as for the partial agonist AL438. Furthermore, we find that GRIP-1, TIF2.4, SRC-1A and SMRT-RID cofactor interaction with the GR all correlate positively and significantly with potency of the ligands for transactivation. This suggests that no other factors or processes are playing a major determining role in the transcriptional response. Furthermore, the correlation between both ligand efficacy and potency for transactivation and apparent affinity of liganded receptor for SMRT-RID but not NCoR-RID, strongly suggests a role for SMRT-RID but not NCoR-RID in increasing transactivation via GREs in the current system. This is consistent with a previous report showing that over-expressed full length SMRT, but not NCoR, can slightly increase the potency for DEX and increase the maximal response of a partial agonist via the GR on a *GRE*-reporter gene in 1470.2 cells (Song et al., 2001), while the opposite effect was observed with SMRT-RID and the GR in a different system using CV-1 cells (Wang et al., 2004b; Szapary et al., 1999). Although over-expressed NCoR has been shown to decrease the potency for DEX and decrease the maximal response of a partial agonist via the GR on a *GRE*-reporter gene in a cell-selective manner (Song et al., 2001), we do not detect a negative correlation between interaction with NCoR-RID and transactivation potency and efficacy for the panel of all ligands, or for only agonists and partial agonists (Table 4), suggesting that repressive effects of NCoR-RID on inhibiting transactivation may be highly selective for specific ligands such as RU486 for the GR (Fig. 5d). It is, however, possible that the full length corepressors may exhibit different interactions to the truncated RIDs.

Whether agonist–steroid receptor complexes recruit corepressors appears to be controversial. Our results showing that the GR bound to agonists DEX, cort and pred, for transactivation and transrepression, recruit the corepressors NCoR-RID and SMRT-RID with similar or greater apparent affinities than most of the partial agonists in our system, do not support the model outlined above. This is consistent with other reports that agonist-bound AR recruits both SMRT (Yoon and Wong, 2006), and NCoR (Yoon and Wong, 2006; Cheng et al., 2002) and is also consistent with results for the GR and PR (Wang and Simons, 2005; Wang et al., 2007, 2004b). Furthermore, although the interaction of NCoR-RID with agonist-GR is much less than that of RU486-GR, (RU486 is an antagonist for transactivation in our system), consistent with the model, similar levels of SMRT-RID are recruited by the GR liganded to both DEX and RU486, which is not consistent with the model. We do show that partial agonists and selective modulators recruit both coactivators and corepressors, consistent with the model, albeit with varying apparent affinities for different coactivators and corepressors. Our finding that interaction of SMRT-RID correlates broadly with potency and efficacy for transactivation suggests that SMRT may participate in transcriptional activation depending on the promoter context, consistent with the recent report that SMRT is required for the full agonist activity of ERα (Peterson et al., 2007). It would be interesting to test this hypothesis for the GR by ChIP analysis with full length native proteins on endogenous genes.

The antagonists for both TA and TR in our system (DO6, NET-A) do not recruit any coregulator above background levels. The finding that these antagonists failed to interact with either coactivators or corepressors is not consistent with the above model which assumes that antagonism arises due to failure to interact with coactivators with a concomitant preference for corepressor interaction. A similar lack of both coactivator and corepressor recruitment was found

for the MR antagonist eplerinone (Hultman et al., 2005). This suggests that one mode of antagonism for the GR may be simply due to passive antagonism, as proposed for the ER (Shiau et al., 2002). This could occur via stabilization of an inactive conformation of helix 12 of the liganded receptor such that it is unable to recruit either coactivators or corepressors.

There appears to be some controversy in the literature as to whether antagonists liganded to steroid receptors can recruit coactivators. In this study, for GR liganded with antagonists for TA, we did not detect any interaction above background with GRIP-1, SRC-1A or TIF2.4. Consistent with this result, it has been shown that the ER bound to the antagonist ICI 182780 does not recruit SRC-1 (Jaber et al., 2006), and that the MR does not bind coactivator peptides when complexed with antagonists (Hultman et al., 2005). However, others have reported that antagonist-bound GR does recruit GRIP-1 (Wang et al., 2004b; He and Simons, 2007) and that antagonist (RU486)-bound PR recruits SRC-1 (Liu et al., 2002). Wang et al. (2004b) refer to all compounds that bind GR and act as partial agonists, such as progesterone and RU486, as antagonists or antiglucocorticoids. They also note that the amount of partial agonist activity varies with experimental conditions, even with the same cell and reporter system. This differs from the operational definition used in the present study and that used in some other studies. Thus, some of the above controversy may result from differences in nomenclature and experimental design or to receptor-specific or promoter-specific effects.

4.3. Role of coactivators and corepressors in ligand-selective GR-mediated transrepression

The role of coactivator and corepressor recruitment in TR via the GR is unclear. Experiments with GR mutants and coactivator receptor-interacting domains suggest that, unlike for TA, coactivator interaction by the liganded-GR is not required for TR via NFκB (Wu et al., 2004). In this study RU486 acted as an agonist for TR and yet did not recruit coactivator peptides *in vitro*. However, other studies have identified GRIP-1/TIF2 as a key component of transcriptional repression by the GR via AP-1 on collagenase-3 and synthetic promoters (Rogatsky et al., 2001, 2002; He and Simons, 2007; Sun et al., 2008) and via NFκB on the *IL*-8 gene (Rogatsky et al., 2002). Rogatsky et al. (2002) proposed that cofactors such as GRIP-1 can act to further repress gene transcription by agonist-GR complexes without changes in histone acetylation or p300 levels (Rogatsky et al., 2001), and that these coregulators have separable activator and repressor domains (Rogatsky et al., 2002). Interestingly GRIP-1 potentiated repression of agonist-GR but not antagonist (RU486)-GR via AP-1 on the collagenase-3 promoter, suggesting that different mechanisms are involved for DEX versus RU486-mediated TR in that system (Rogatsky et al., 2001). Our results showing a correlation between ligand-selective GRIP-1 and SRC-1A interaction with the GR and TR efficacy and potency are consistent with the above results of others (Rogatsky et al., 2001, 2002; He and Simons, 2007; Sun et al., 2008) and suggest a key role for these coactivators in ligand-selective transrepression, in which context they could both act to cause greater repression of transcription. This would also be consistent with recent results showing a requirement for GRIP-1 recruitment for ER-mediated transrepression (Cvoro et al., 2006). Interestingly, we observed a significant and positive correlation between apparent affinity of liganded-GR for SMRT-RID and NCoR-RID and maximal activity and potency for transrepression via NFκB sites, as well as for potency via AP-1 sites, suggesting that both NCoR and SMRT can also play a key role in transrepression by agonists and partial agonists for the GR.

4.4. Ligand-dependent preference for GRIP-1 versus SRC-1A by the GR

Our results show that the relative preference of liganded-GR for GRIP-1 versus SRC-1A is clearly dependent on the ligand. Previous investigators have shown that on the MMTV promoter, the PR liganded to progesterone interacts preferentially with SRC-1 rather than GRIP-1, while the GR liganded to DEX interacts preferentially with GRIP-1 rather than SRC-1 (Li et al., 2003). Similarly, the MR also exhibits cofactor preferences when liganded to aldosterone, showing interaction with SRC-1–4a peptides but not with GRIP-1 peptides (Hultman et al., 2005). Our findings show that the GR cofactor selectivity shifts towards a preference for SRC-1A over GRIP-1 when liganded to progesterone and MPA, and shows a striking increase in preference for SRC-1A when liganded to aldosterone. This suggests that the ligand plays a role in cofactor selectivity independent of the receptor, within the MR, PR and GR family. This would be consistent with crystal structure studies suggesting that the GR and PR share a common mechanism of coactivator selectivity (Bledsoe et al., 2002).

In summary, the current data show that GR bound to agonists, antagonists and selective modulators interacts to different extents with different coregulators. Taken together, our results provide strong biochemical support for the idea that ligand-selective differential interaction of the GR with coregulators like GRIP-1, SRC-1A, NCoR and SMRT could be the biochemical basis for ligand-selective and promoter-specific differences in potency and maximal transcriptional response for transactivation via GREs as well as transrepression via AP-1 and NFκB tethering mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2008.10.008.

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