

Original article

The glucocorticoid receptor β isoform can mediate transcriptional repression by recruiting histone deacetylases

Audrey Kelly, PhD,^{a*} Holly Bowen, PhD,^{a*} Young-Koo Jee, MD,^{b*} Nabila Mahfiche, BSc,^a Cecilia Soh, PhD,^a Tak Lee, MD, DSc,^a Catherine Hawrylowicz, PhD,^a and Paul Lavender, PhD^a London, United Kingdom, and Cheonan, Korea

Background: The glucocorticoid receptor (GR) is able to participate in regulation of transcription by a variety of mechanisms, one of which involves DNA binding and recruitment of regulatory cofactors. The best-studied forms of the receptor are the 777-amino-acid α and the 742-amino-acid β variants. The β isoform, which does not bind cortisol in human subjects, has been proposed to be a dominant-negative inhibitor of the transcriptional activation-competent GR α isoform.

Objective: GR α has roles in both transcriptional activation and repression. We wished to determine the influence of GR β on genes that are normally transcriptionally repressed by glucocorticoids. We studied *IL5* and *IL13*, which both contribute to the asthmatic phenotype.

Methods: We used transient transfection systems and coimmunoprecipitation experiments to determine whether GR β has repressive activity on the promoters of the human *IL5* and *IL13* genes.

Results: GR β is able to act as a transcriptional repressor of cytokine genes and mediates its function through the recruitment of histone deacetylase complexes.

Conclusion: GR α and GR β act in a similar manner on *IL5* and *IL13* promoters, serving to repress transcription.

In this circumstance GR β does not act as a dominant-negative inhibitor of GR α .

Clinical implications: Our data suggest that if GR β contributes toward glucocorticoid resistance by acting as a dominant-negative inhibitor of GR α function, then its influence does not

lie at genes such as *IL5* and *IL13*. GR β might have a more important role at genes that are normally activated by glucocorticoids. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Cytokine gene regulation, glucocorticoid receptor, transcription

The α and β splice variants of the glucocorticoid receptor (GR) were first described in 1985,¹ followed in 1991 by description of the human genomic DNA sequence.² GR α and GR β are the best-studied variants of GR and are 777 and 742 amino acids long, respectively. The variants are generated by use of 2 splice acceptor sites within the ninth exon of the gene. The α and β forms share a message encoded by 8 common exons being translated to 727 amino acids but vary thereafter. The α isoform adds 50 amino acids after the splice site. These amino acids contribute to the ligand-binding domain of the receptor, the role of which in human subjects is the binding of cortisol.³ Ligand binding is a prerequisite for nuclear relocalization, coactivator recruitment, and transcriptional activation activity in response to cortisol. The β isoform adds only 15 amino acids and does not permit cortisol binding. This isoform has been suggested to be a negative regulator of genes that are activated by glucocorticoids.⁴⁻⁶

Recently, it has become clear that both alternative transcription initiation sites and alternative translation site use within the GR message has greatly expanded the possible repertoire of protein products from a single gene.⁷ This ability to either include or exclude exons might alter the topology of the protein, the affinity or specificity of DNA binding, the ability to recruit ligand, the capacity for cofactor interaction, and the capacity to act as either a repressor or an activator. Lu and Cidlowski⁷ also reported that GR isoforms, which vary by virtue of distinct translation-initiation site use, regulate distinct populations of target genes, despite having identical DNA-binding and ligand-binding domains, underlining the influence of the amino terminus of GR on transcriptional regulation. GR β has recently been shown to regulate upward of 5000 genes in the absence of both ligand and GR α , with broadly similar numbers being activated or repressed. It has been proposed that RU486 is a ligand for this isoform; however, RU486 acts to diminish both transcriptional activation and repression mediated by GR β .⁸

From ^aKings College London, Medical Research Council/Asthma UK Centre in Allergic Mechanisms of Asthma, Guy's Hospital, London, and ^bthe Division of Allergy and Pulmonary Medicine, College of Medicine, Dankook University, Cheonan.

*These authors contributed equally to this work.

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Reprint requests: Paul Lavender, PhD, Medical Research Council/Asthma UK Centre in Allergic Mechanisms of Asthma, 5th Floor Thomas Guy House, Guy's Hospital, London SE1 9RT, United Kingdom. E-mail: paul.lavender@kcl.ac.uk.

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Abbreviations used

AP-1: Activator protein 1
 CAT: Chloramphenicol acetyl transferase
 GR: Glucocorticoid receptor
 GRE: Glucocorticoid response element
 HDAC: Histone deacetylase
 NF-AT: Nuclear factor of activated T cells
 TSA: Trichostatin A

Because GR isoforms can perform both transcriptional activating and repressing functions, an unanswered question is the following: How is the decision to recruit either transcriptional coactivators or repressors made? Previous data have suggested that genes that are repressed by glucocorticoids do not contain classical glucocorticoid response elements (GREs), and the GR either binds to atypical response elements, such as negative GREs (nGREs), or tethers to DNA through interactions with other transcription factors.⁹⁻¹¹ It is possible that the recruitment of the GR to DNA through either route imparts on the GR a distinct conformation, which is consistent with the ability to discriminate corepressors from coactivators.

The capacity of GR β to regulate transcription in the absence of ligand clearly implies that at least a fraction of GR β is located within the nucleus. Although some reports suggest that GR β is constitutively intranuclear, others demonstrate its presence in both nuclear and cytoplasmic compartments. This difference in partitioning might be cell type specific or potentially related to disease status.¹² GR β has been suggested to be the most abundant GR isoform in neutrophils¹³ and to be upregulated in a variety of inflammatory conditions.¹⁴⁻¹⁷ Nuclear localization might permit it to participate in transcriptional regulation in the absence of ligand, whereas GR α remains sequestered in the cytoplasm. GR β has been suggested to be upregulated in the asthmatic lung, and in some individuals the ability of glucocorticoids to repress cytokine transcription is lost, although the mechanism of this steroid resistance is unresolved. Study of these patients with steroid-resistant asthma might provide important clues concerning the progression of asthma; however, it is first important to understand the mechanism of GR β function in cytokine gene transcription. In this study we have assessed the influence of GR β on genes that are normally repressed by glucocorticoids and sought to dissect the mechanism of this effect.

METHODS**Cell lines and culture conditions**

HEK293 cells were grown in Dulbecco modified Eagle medium, and HeLa cells were grown in minimal essential medium, both supplemented with 10% FCS (Sigma, Poole, United Kingdom) that had been charcoal stripped, L-glutamine (2 mmol/L), penicillin (100 IU/mL), and streptomycin (100 μ g/mL) at 37°C and 5% CO₂ in humidified air. Dexamethasone (Sigma) was stored at a concentration of 10⁻² mol/L in ethanol further diluted in the appropriate culture medium and added to cells to give a final concentration of 10⁻⁷ mol/L.

Trichostatin A (TSA; Sigma) was used at a final concentration of 33 $\times$ 10⁻⁹ mol/L.

Plasmids

Most of the plasmids used in this study have been previously described: p-130-+35.*IL5*. chloramphenicol acetyl transferase (CAT) and p-225-+50.*IL13*.CAT,¹⁰ pcDNA3.Flag GR α ,¹¹ CMV.Flag.histone deacetylase (HDAC) 1,¹⁸ and CMV.GATA-3.Flag.¹⁹ CMV.Flag GR β was generated by means of PCR of hGR β from CD4⁺ cDNA by using the sense oligonucleotide (5'agcttagatcc ATGGACTACAAAGACGATGACGACAAGGGTACCATGGAC TCCAAAGAATCATTAAGTCC) and the antisense oligonucleotide (5'acgtgtagccTCAGATTAATGTGTGAGATGTGC). The sense oligonucleotide also encodes a Flag epitope after an initiating methionine residue (underlined) and before and in frame with the amino terminal of GR (GR-initiating methionine codon is shown in bold). The amplicon was digested with *Bam*HI and cloned into the same site in vector pcDNA3.1. All plasmid inserts were fully sequenced with BigDye chemistry (GE Healthcare) and an Applied Biosystems 377 automated sequencer (Applied Biosystems, Foster City, Calif).

Transfections

HEK293 and HeLa cells were transfected by means of calcium phosphate precipitation, as previously described.²⁰ For HeLa cells, 2 μ g of reporter plasmid was used along with the same amount of expression vectors for GATA-3. One microgram of CMV GR α , GR β , or HDAC1 was used in transfections. Plasmid transfections were equilibrated with an empty CMV expression vector. For HDAC inhibition assays, TSA was added to cell cultures at a final concentration of 33 nM, where indicated.

Biotinylated oligo affinity pulldown

To allow binding of protein to the oligonucleotides, FLAG-purified GR α and GR β prepared from transfected 293 cells was incubated with 0.6 μ mol/L biotinylated oligonucleotides and 10 μ g Poly (deoxyinosine-deoxycytidine) (Amersham Biosciences, Piscataway, NJ) in binding buffer (20 mmol/L Tris [pH 7.5] and 150 mmol/L NaCl). Sense strand oligonucleotides were 5' biotinylated, and antisense oligonucleotides were unbiotinylated. The GRE corresponding to -187 to -161 from the mouse mammary tumor virus long terminal repeat was used as a positive control for GR binding and has the following sequence: 5' GATCGTTTATGGTTA CAACTGTTCTTAAACA.²¹ The *IL-5* -130 to -90 oligo used was as follows: 5'ctagTAAGATATAAGGCATTGGAAACATTT AGTTTCACGATATGC. DNA-bound proteins were collected with streptavidin sepharose (Amersham Biosciences) for 1 hour, washed 4 times with PBS, and resuspended in sample loading buffer. Western blots probed with anti-pan-GR antibody were performed as described below.

Immunoprecipitations

Immunoprecipitations were performed as previously described¹¹ with modifications. HEK293 cells were transfected with either CMV.hGR α or CMV.hGR β and CMV.Flag.HDAC1 or CMV.Flag.GATA-3 as a negative control. Whole-cell lysates were prepared in IPH buffer (50 mmol/L Tris.Cl [pH 8.0], 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP40, and 1 mmol/L phenylmethylsulfonyl fluoride), and Flag-tagged proteins were immunoprecipitated by means of incubation with Flag-agarose beads (Sigma) at 4°C. Immunoprecipitates were captured by means of centrifugation, and beads were washed 4 times with IPH containing 600 mmol/L NaCl, with thorough vortexing of samples at each wash. Immunoprecipitates were then resuspended and electrophoresed

through 8% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, blocked in TBS/Tween/4% milk, and then probed with anti-GR antisera (Transduction Laboratories) or anti-Flag (Sigma). The location of immunoreactive complexes was revealed by means of ECL (Amersham) and autoradiography. Antibodies used were as follows: mouse anti-GATA-3 anti-sera (HG3-31; Santa Cruz Biotechnology, Santa Cruz, Calif), mouse anti-GR (Transduction Laboratories 611227) and rabbit anti-GR β (Abcam 3581), and appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG [sc-2005] and goat anti-rabbit IgG [sc-2004, Santa Cruz Biotechnology]). These were visualized with ECL reagent (Amersham, Chalfont St Giles, United Kingdom).

Statistical analysis

Statistics were performed with the Student *t* test. Error bars represent SEMs in all cases.

RESULTS

GR β is a repressor of both the *IL5* and *IL13* promoters

We have previously determined that repression of the *IL5* and *IL13* genes by glucocorticoids mapped to nuclear factor of activated T cells (NF-AT)/activator protein 1 (AP-1) sites within the proximal promoter¹⁰ and that repression was brought about, at least in part, by competition of the activation mediated by the NF-AT/AP-1 complex. HeLa cells that had been grown in media containing charcoal-stripped serum were transiently transfected with *IL5*- and *IL13*-driven reporter constructs to address the influence of the β isoform on transcriptional regulation through these sites. Stripped serum was used to remove steroid from the culture medium that might bind to the endogenous GR of the HeLa cells and mask the influence of GR β . Transcriptional activation of these reporter constructs was achieved by means of coexpression of GATA-3. The GATA-3 response elements of both *IL5* and *IL13* are distinct from the site bound by GR. We have previously shown that upregulation in this manner renders the promoters particularly sensitive to glucocorticoids (Fig 1)¹⁰; consequently, this method was chosen to investigate a transcriptional influence of GR β . Equal quantities of an empty expression vector or vectors containing GR α or GR β cDNAs were coexpressed along with GATA-3 (GATA-3, GATA-3 plus GR α , and GATA-3 plus GR β , respectively; Fig 1). Fig 1 shows that in the absence of ligand, both promoters are significantly repressed by the GR β isoform, although, as previously observed, the background activity of the *IL5* construct was lower than that of *IL13*, and repression was more pronounced.¹⁰ As controls, we treated GATA-3-transfected cells with 10^{-7} mol/L dexamethasone in the absence or presence of additional GR α or with GR α in the absence of ligand. As expected, treatments with GR α or ligand alone caused partial repression, whereas in combination they permitted full repression of the promoter. These data confirm a role for GR β in transcriptional repression.

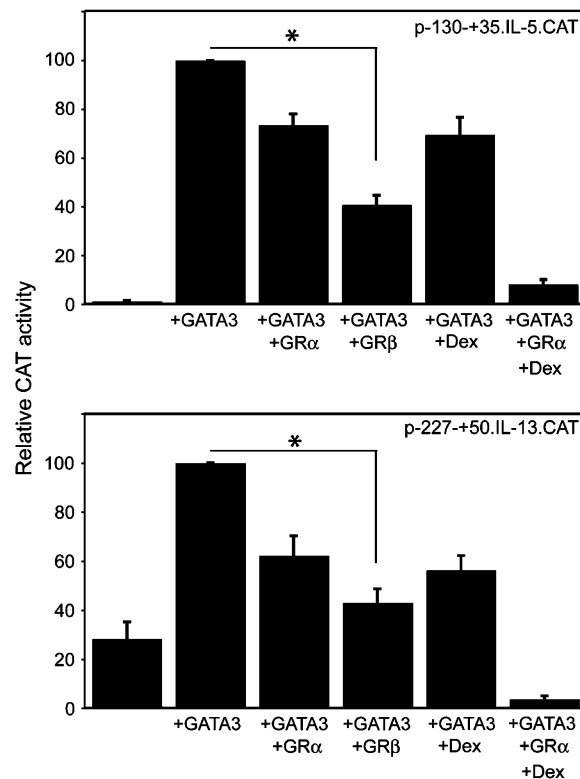


FIG 1. GR β can mediate transcriptional repression of *IL5* and *IL13* promoters. CAT activity was measured in extracts from HeLa cells cotransfected with the cytokine promoter constructs, along with plasmids encoding GATA-3, GR α , or GR β , as indicated. Results are the mean of at least 4 independent experiments. Error bars indicate SEMs. **P* < .001. Dex, Dexamethasone.

GR β binds to the *IL5* NF-AT/AP-1 response element *in vitro*

The mechanism by which GR mediates transcriptional repression is complex and in some circumstances involves recruitment of GR to DNA.²² We had previously demonstrated that GR α was able to bind to consensus NFAT/AP-1 sites within the proximal promoter of *IL5*. Biotinylated DNA pulldown assays were performed to determine whether purified GR β might have a similar activity. Affinity-purified GR α and GR β were incubated with either a consensus biotinylated GRE or *IL5* target. DNA-bound proteins were collected after incubation with streptavidin agarose. After washing, proteins were electrophoresed through PAGE gels and blotted onto nitrocellulose before Western blotting with anti-GR antiserum. Both GR α and GR β displayed similar DNA-binding activity in this assay (Fig 2), being able to bind to either a consensus GRE or an *IL5* target. These DNA-binding data were not entirely surprising because the 2 proteins have an identical DNA-binding domain and differ at the extreme carboxy terminus. This variant C-terminus does, however, render on GR β the capacity to regulate its own distinct set of target genes with only a fraction of these being shared with GR α .⁸

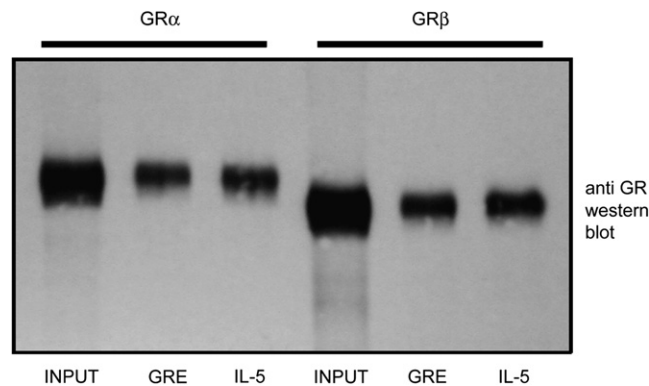


FIG 2. GR α and GR β bind DNA to the IL-5 NF-AT/AP-1 response element *in vitro* with equal affinity. A biotinylated DNA pulldown assay showing the ability of GR α and GR β to bind to a consensus GRE or the IL-5 NF-AT/AP-1 site is presented. Equivalent DNA-binding capacity was displayed by either GR α (lanes 2 and 3) or GR β (lanes 5 and 6).

Repression of *IL5* and *IL13* by GR β is mediated by HDACs

GR α is known to indirectly recruit proteins that have the ability to modify nucleosomal histone tails, particularly the amino termini of the core histones H3 and H4. We previously demonstrated that the repressive activity of GR α at the *IL5* promoter involved HDAC recruitment. Two sets of experiments were undertaken to determine whether GR β acted in a similar manner to GR α . First, we demonstrated that TSA was able to relieve GR β -mediated transcriptional repression of GATA-3-activated reporter constructs driven by both the *IL5* (−130+35, IL-5.CAT) and *IL13* (−227+50, IL-13.CAT) promoters (Fig 3). These data are similar to the results obtained previously showing relief of GR α -mediated transcriptional repression by TSA.¹⁰ The data imply that HDACs are involved in the repression of *IL5* and *IL13* by GR β . This was formally tested by overexpression of HDAC1 along with GATA-3 and GR β and measuring CAT activity from *IL5*- and *IL13*-driven reporters (Fig 4). HDAC1 was able to augment GR β -mediated repression when overexpressed in transient transfection experiments with both *IL5* and *IL13* reporter constructs. Taken together, the data from these 2 sets of experiments suggest that both GR α and GR β can promote active repression of target promoters, and this activity is facilitated by corepressor recruitment.

GR β interacts with HDAC1

It was important to demonstrate that GR β was able to interact with HDACs in a similar way to GR α to formally link GR β to HDAC recruitment. GR β and GR α were overexpressed in combination with Flag-tagged HDAC1 or Flag-tagged GATA-3 in HEK293 cells, and whole-cell extracts were prepared from each group. Western blots of these extracts are shown in Fig E1 in the Online Repository at www.jacionline.org. Protein expression was checked by means of Western blotting, which revealed similar expression levels of GR β (Fig 5, A, lanes 1 and 4), GR α (Fig 5, A, lanes 2 and 5), Flag HDAC1

(Fig 5, B, lanes 1-3), and Flag GATA-3 (Fig 5, B, lanes 4-6). Proteins from whole-cell extracts of these transfected cells were immunoprecipitated with anti-Flag antisera and washed stringently before migration to SDS-PAGE gels. Western blotting was performed with anti-GR antibodies. GR β and GR α immunoreactivity was present in Flag immunoprecipitates from Flag HDAC1-expressing cells (Fig 5, C, lanes 1 and 2, respectively) but absent in Flag immunoprecipitates from cells expressing the negative control, Flag.GATA-3 (Fig 5, C, lanes 4 and 5). These data show that GR β , like GR α , forms a stable complex with HDACs.

DISCUSSION

GR β expression has been proposed to be upregulated in T cells from asthmatic patients,^{14,16} and it has been suggested that it might function as a dominant-negative inhibitor of glucocorticoid-regulated genes to participate in steroid resistance. Data supporting this suggestion involved analysis of promoters that were activated by glucocorticoids. Coexpression of GR β led to a reduction in activation potential of GR,²³ and it was suggested that this inhibition of activation might be caused by the formation of nonfunctional GR α /GR β heterodimers or by non-signaling GR β homodimers. In T cells the expression of GR β relative to GR α is low, which has raised questions as to how GR β can be a dominant-negative inhibitor. In other cell types, such as neutrophils, however, GR β has been suggested to be the more abundant isoform,¹³ and this has led to a suggestion that changing the ratios of the subtypes might contribute to variation in responsiveness to glucocorticoids.²⁴ Indeed, it is clear that expression of different GR isoforms varies with cell type (see Fig E1, B, in the Online Repository at www.jacionline.org).⁷ It remains to be ascertained as to what levels of GR β expression are required to influence expression of GR-regulated genes. Clearly, however, GR β has its own distinct set of target genes.⁸

A feature of T_H2 cells is their capacity to express IL-4, IL-5, and IL-13 on activation.²⁵ These cytokines have

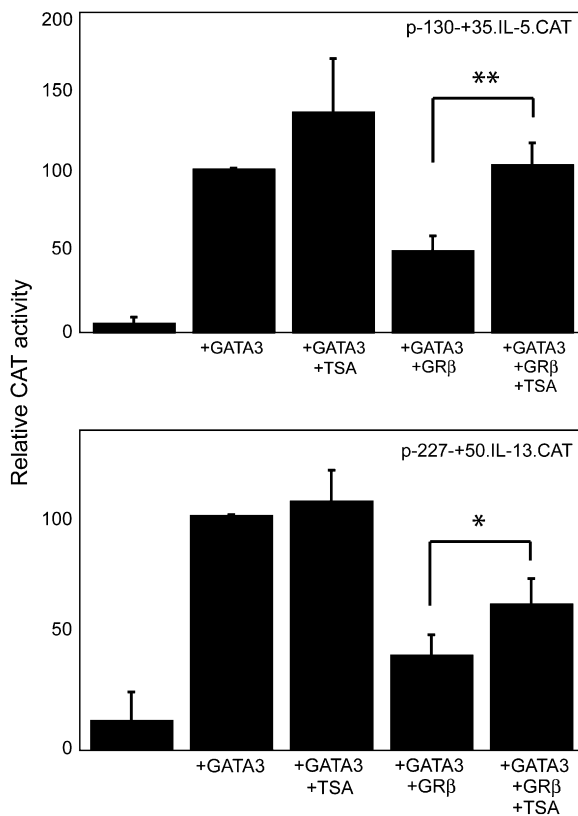


FIG 3. Repression by GR β is mediated by HDACs. CAT activity was measured in extracts from HeLa cells cotransfected with the cytokine promoter constructs, along with plasmids encoding GATA-3 and GR β , as indicated. Cells were treated with or without TSA (33×10^{-9} M). Results are the mean of 4 experiments, with error bars representing SEs. ** $P < .01$, * $P < .05$.

been proved to be involved in a variety of events that contribute to the asthma phenotype, and each of them is potentially transcriptionally repressed by glucocorticoids. This repression is also complex, with components of transcription, signaling, and receptor shuttling and turnover. Analysis of transcriptional repression can be influenced by each of these mechanisms.

The GR is known to actively shuttle between nucleus and cytoplasm. GR β partitioning differs between cell types, although a body of data suggested that it is predominantly intranuclear, first because it is not retained in the cytoplasm associated with the chaperone complex and second because unliganded receptor are thought to display delayed nuclear export. The GR is targeted for degradation after ligand administration²⁶; therefore transcriptional events modulated by the GR must take place relatively rapidly before the GR itself is removed. Genes such as Fos and Jun, which are involved in mediating gene expression during T-cell activation, are also normally both rapidly induced and inactivated; consequently, this suggests that both activatory and repressive pathways have a limited temporal functional life, which must be kept in tight concert. In inflammatory conditions in which T cells are in a more activated state, normal turnover of

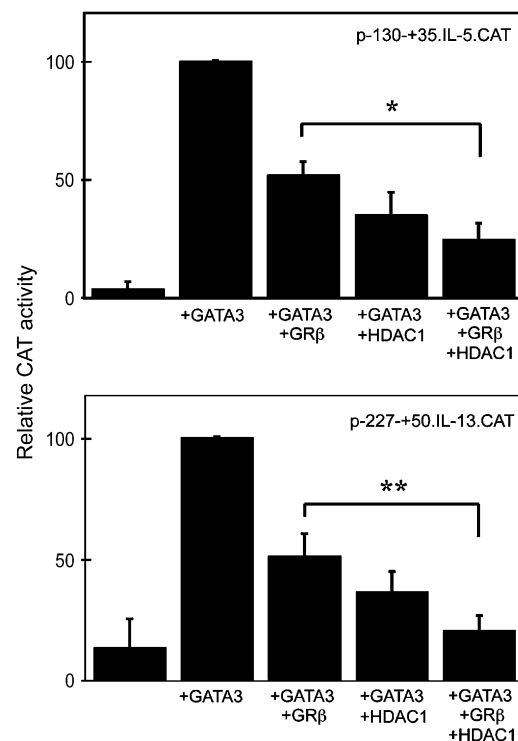


FIG 4. HDAC overexpression augments repression by GR β . HeLa cells were transiently transfected with *IL5*- or *IL13*-driven reporters and GATA-3, along with GR β and HDAC1, where indicated. Data are expressed as CAT activity relative to the GATA-3-treated samples and are the mean of a minimum of 4 independent experiments. Error bars indicate SEMs. * $P < .05$; ** $P < .01$.

GRs on ligand administration might leave the cells without the capacity to repress continued AP-1-mediated signaling. This might equally be true of other activatory pathways that might be repressed by GRs, such as nuclear factor κ B activation.

Paradoxically, there must be a mechanism to accommodate genes that are either activated or repressed by glucocorticoids within the same cell. As an example of this, cytokine genes, such as *IL5*, *IL13*, and *IL10*, can be expressed by a single T cell, and each is transcriptionally activated by AP-1 complexes. The GR is able to very efficiently repress transcription of the first 2, at least in part, by targeting AP-1 proteins,¹⁰ yet *IL10* expression is induced by cortisol,^{27,28} despite the potential for sequestration of AP-1 components and inhibition of c-Jun N-terminal kinase activity, which is involved in repression of *IL5* and *IL13*. This indicates that for any gene, regulatory activities must have a rank order of importance, and the result is a sum of all potential positive and negative activities that impinge on it.

The association with certain cofactors might act to stabilize a pool of GRs to facilitate continued participation in regulatory events. To support this, we found that the complex containing GRs and HDACs was particularly stable. Stevens et al²⁹ compared the homology of GRs with the peroxisomal proliferator-activated receptor α and suggested that a domain of GRs (amino acids 567-

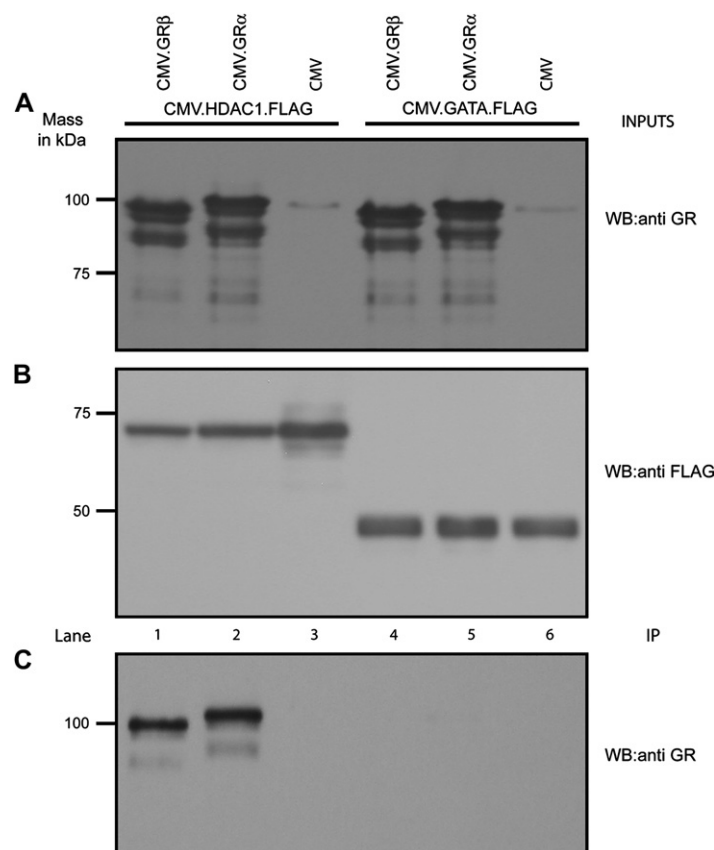


FIG 5. GR β forms a complex with HDAC1. HEK293 cells were transiently transfected with either CMV.Flag.HDAC1 or CMV.Flag.GATA-3, as indicated, in the presence or absence of either CMV.GR α or CMV.GR β . **A** and **B** show Western blots of inputs to demonstrate equivalent levels of expression. **C**, Proteins were immunoprecipitated with anti-Flag. agarose and were electrophoresed and probed with antibodies directed to the GR.

600) showed conserved amino acids with the peroxisomal proliferator-activated receptor domain known to bind to the nuclear receptor corepressor/silencing mediator of retinoid and thyroid hormone receptors corepressors. They went on to demonstrate that the ligand-binding domain of the GRs was sufficient to interact with the corepressors in a mammalian 2-hybrid assay. The complex formation observed with either GR β and GR α with HDACs suggested that both might participate in transcriptional repression. Direct assays of this capacity, use of HDAC overexpression, and treatment of cells with TSA all supported the assertion. By way of contrast, if GR β were to be a dominant-negative inhibitor of GR α on genes normally repressed by glucocorticoids, we would have expected none of these findings.

Both Bilodeau et al³⁰ and Ito et al³¹ have proposed circumstances in which HDACs are either mislocalized or present in reduced quantities. Clearly, this would have the consequence of reducing transcriptional repressive capacity, contributing to the phenotype of steroid resistance. It remains to be determined whether HDAC association might also be involved in regulating GR turnover, but the functional association between activatory

signals, such as AP-1, and the GR is in tight balance, and perturbation of 1 or more components might render normal regulatory controls ineffectual.

Our data suggest that should GR β be expressed at sufficiently high levels in T cells, it might contribute to transcriptional repression of cytokine genes rather than acting as an inhibitor of this role. These findings suggest therefore that if GR β is to play a role in the establishment of steroid resistance, its influence is more likely to be at the level of genes that are normally activated by GRs.

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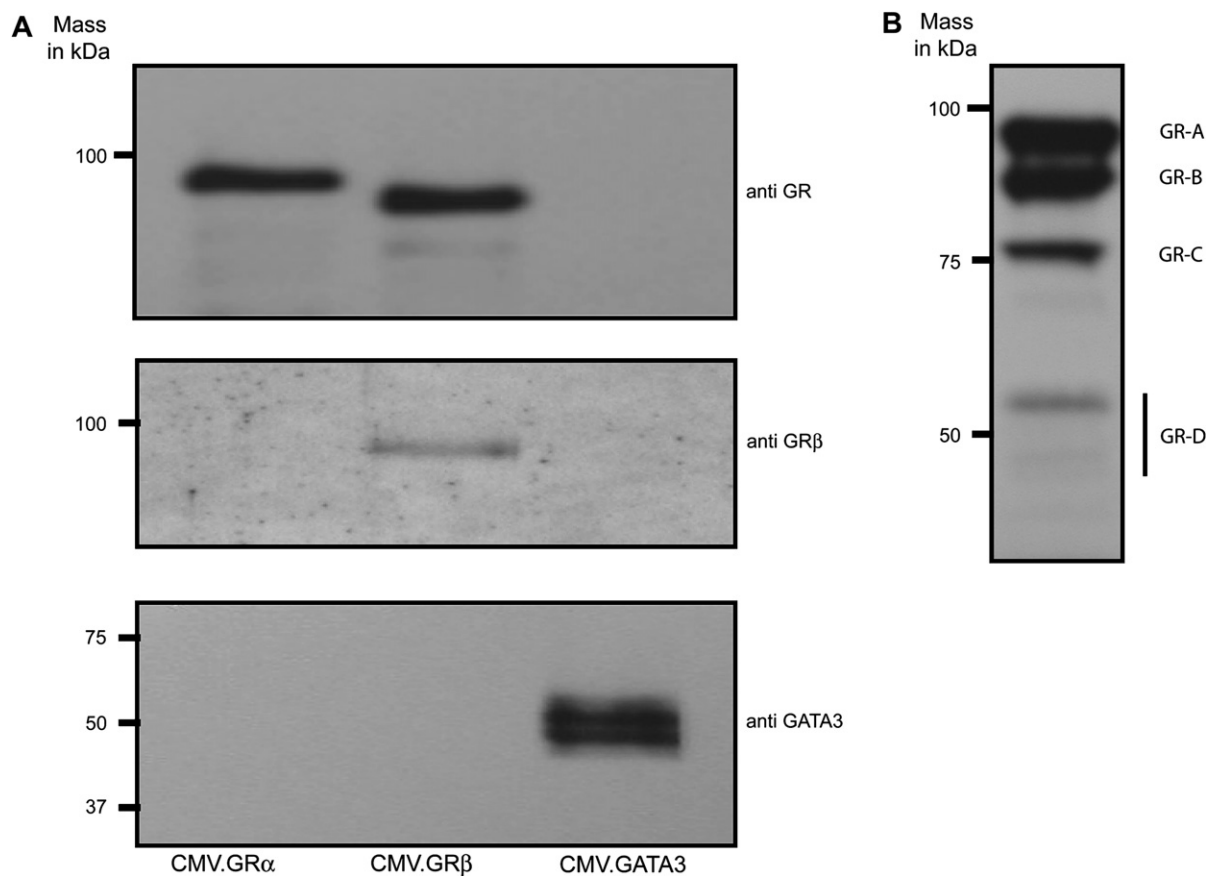


FIG E1. A, Western blotting of extracts from GR α - and GR β -overexpressing 293 cells. Cells were transfected with either GR α , GR β , or GATA-3, as indicated. Western blots of cell lysates were probed with anti-GR, anti-GR β , or anti-GATA-3 antisera. **B**, Western blot of cell lysate from HeLa cells showing expression of GR isoforms.