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Interaction of glucocorticoid receptor isoforms with transcription factors AP-1 and NF-κB: lack of effect of glucocorticoid receptor β

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Abstract

Glucocorticoids act through the glucocorticoid receptor (GR) to enhance or repress transcription of glucocorticoid responsive genes depending on the promoter context and cellular background. The human GR primary transcript is alternatively spliced resulting in hGRα and hGRβ isoforms. Transactivation and transrepression are mediated by hGRα and while it has been demonstrated that hGRβ, can act as a dominant negative inhibitor of hGRα mediated transactivation, its effects on transrepression are not known. To investigate hGRβ actions, we used GR-deficient COS-7 and HEK-293 cells. When hGRα (0.5 μg 10^6 cells^{-1}) was transfected into COS-7 cells dexamethasone (150 nM) inhibited TNFα (80 U ml^{-1}) effects on a NF-κB responsive reporter gene by 40%. There was no evidence of a dominant negative effect when hGRβ (1–10 μg) was co-transfected with hGRα up to ratios of 10:1. Similarly hGRβ had no effect on hGRα inhibition of a phorbol ester stimulated Ap-1-responsive reporter gene in COS-7 or HEK-293 cells. In comparison, an apparent dominant negative effect of hGRβ on hGRα-mediated transactivation was found to be attributable to non-specific transcriptional squelching in COS-7 cells. In summary, the potential for hGRβ, to act as a dominant negative inhibitor of hGRα-mediated transactivation remains controversial, but our data suggest that hGRβ, was unable to act as a dominant negative inhibitor of either hGRα-mediated transrepression or transactivation in these promoter and cell contexts. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Glucocorticoid receptor; Isoform; Squelching; GRβ; AP-1; NF-κB

1. Introduction

Glucocorticoids exert potent anti-inflammatory and immunosuppressive effects when administered at pharmacological doses and are a major component of therapy for severe inflammatory and autoimmune diseases, such as asthma and rheumatoid arthritis. The majority of these actions are mediated through the ligand-dependent form of the glucocorticoid receptor, hGRα[1], which acts to down-regulate expression of many of the chemokine, cytokine and adhesion molecule genes, required for coordination of the various cellular and humoral components contributing to immunity. The glucocorticoid receptor down-regulates gene expression primarily by antagonising (transrepressing) the actions of transcription factors normally required for the expression of a particular gene. Activator protein 1 (AP-1) and nuclear factor of kappa B cells (NF-κB) are transcription factors which bind to response elements found in the regulatory regions of many cytokine and chemokine genes and their transrepression by hGRα has been extensively studied.

Transactivation by glucocorticoids involves binding of GR homodimers to DNA elements, GREs (glucocorticoid response elements), comprising palindromically arranged half-sites (Beato et al., 1989), although in

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some target genes, the response element may comprise series of direct repeats of the GRE half-site, for example as in the mouse mammary tumour virus long terminal repeat (MMTV LTR; Chalepakis et al., 1988). In contrast to this direct GR-DNA interaction in transactivation, the mechanism of transrepression is generally more complex, and frequently involves protein-protein interactions. The GR is known to interact physically with AP-1 (Schüle et al., 1990), NF-κB (Wigler et al., 1994; McKay and Cidlowski, 1998) and the cAMP response element binding protein, CREB (Imai et al., 1993).

At least two hGR isoforms (hGRα and hGRβ) arise by alternative splicing of the GR gene (Hollenberg et al., 1985; Bamberger et al., 1995; Oakley et al., 1996). The hGRα is the form that mediates ligand-induced regulation of glucocorticoid-responsive genes. Comparison of hGRα with the hGRβ splice variant demonstrates that both receptors contain amino acids 1–727, corresponding to exons 1–8 of the GR gene, and then diverge with the sequence of hGRα continuing for an additional 50 amino acids, while hGRβ is truncated after 15 unique residues. This truncation of the C-terminus of hGRβ results in loss of an effective ligand-binding domain (Encio and Detera-Wadleigh, 1991). Information on the physiological role of hGRβ is limited, but its expression has been demonstrated in a variety of cell types (Oakley et al., 1997). It has been proposed that hGRβ acts as an inhibitor of GRα transcriptional activity, and may therefore act to modulate cell sensitivity to glucocorticoids in vivo. Immunoprecipitation and immunoblotting experiments indicate that hGRβ can associate with hsp90 (de Castro et al., 1996; Hecht et al., 1997), and also that it can be found in the nucleus (Oakley et al., 1997). It remains unclear, however, how hGRβ is able to dissociate from hsp90 and translocate to the nucleus in the absence of ligand binding.

Recent work has shown that hGRβ can antagonise the effects of hGRα in CV-1 cells and COS-7 cells in a concentration-dependent manner, acting as a dominant negative inhibitor of glucocorticoid-induced transactivation (Oakley et al., 1996; Bamberger et al., 1997a). There is also evidence that hGRβ can inhibit transactivation mediated by the mineralocorticoid receptor (Bamberger et al., 1997a). However, there is very limited data on the effect of the hGRβ isoform on glucocorticoid-induced transrepression, which is of major physiological and pharmacological relevance. Bamberger et al. (1997b) have shown that hGRβ did not antagonise the repressive effects of hGRα at an interleukin-2 promoter-construct. However, in view of the fact that a number of different mechanisms of transrepression exist we studied the interactions between hGRβ and hGRα on a range of transiently expressed glucocorticoid-regulated gene promoter elements in a number of different cell lines.

We present data showing that hGRβ is unable to inhibit the activity of AP-1 or NF-κB. The expression of hGRβ has no effect on the ability of hGRα to mediate transrepression of either AP-1 or NF-κB activity. Furthermore, our data suggest that in the systems we have used the apparent dominant negative actions of hGRβ on hGRα-mediated transactivation may be explained by non-specific squelching.

2. Materials and methods

2.1. Materials

All cell culture reagents and tissue culture-treated plastics were obtained from Life Technologies (Paisley, UK). Rabbit anti-human glucocorticoid receptor antibody PA510 was purchased from Cambridge Bioscience (Cambridge, UK). All other reagents except where noted were from Sigma (Poole, UK).

2.2. Cell culture of COS-7, HeLa and HEK-293 cells

COS-7 African green monkey kidney, HeLa and human embryonic kidney 293 cells were grown in complete growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum, 2 mM L-glutamine, 1% (v/v) nonessential amino acids and 50 μg ml^{-1} penicillin-streptomycin) and passaged after treatment with trypsin-EDTA every 3–4 days to maintain sub-confluent cultures.

2.3. Reporter gene constructs

Reporter gene constructs containing the lac z α subunit, encoding β-Gal activity, were constructed by ligating oligonucleotides containing transcription factor consensus binding site sequences into a plasmid containing the β-Gal gene under the control of the β-globin minimal promoter. Oligonucleotides containing three tandem repeated, consensus NREs (GGGGACTTTCC), four tandem repeated TREs (GTGACTAA) or two tandem repeated GREs (GAACACAGTGTTCT) were used to generate the constructs pNRE3Gal, pTRE4Gal and pGRE2Gal, respectively. The region −469 to −69 of the MMTV LTR was amplified by PCR and cloned into the same reporter plasmid backbone to generate pMMTVGal.

2.4. Human glucocorticoid receptor expression constructs

Human GRα cDNA was isolated by PCR from human placental cDNA using modified 5′ primer, containing *Kpn*I and the 'Kozak' defined translational initiation sequence (CCA/GCCATG), and a 3′ primer

containing an *Xho*I restriction site. The resulting PCR product contained the Kozak sequence upstream of hGRα cDNA sequence 133–2466, and was cloned into the *Kpn*I and *Xho*I restriction sites of expression vector pcDNA3 (R&D Systems Europe Ltd, Abingdon, UK) to generate pcDNA3hGRα. The *Cla*I-*Xho*I fragment from pRSVhGRβ (kindly provided by Professor R. Evans) was used to replace *Cla*I-*Xho*I fragment of pcDNA3hGRα to generate the human GRβ expression cassette, pcDNA3hGRβ. A control plasmid, paC373, was used to maintain constant amounts of DNA in each transfection. Plasmid pCMVluc, containing the luciferase gene under constitutive control of the CMV promoter was kindly donated by Dr M. Berwaer. Plasmid pGreenLantern was purchased from Life Technologies (Paisley, UK).

2.5. Transient transfections

Twenty four hours prior to transfection cells were seeded at 2×10^5 cells ml^{-1} in complete growth medium. Cells were transfected by the method of calcium phosphate using 25 μg DNA per 1 ml of precipitate in a 10 cm dish as previously described (Wigler et al., 1994). Typically, 10–15 μg of reporter construct was co-transfected with 0.5 μg pcDNA3hGRα, pcDNA3hGRβ or pcDNA3 and including 2 μg pGreenLantern or pCMVluc to control for transfection efficiency. Constant amounts of DNA were maintained between transfections using paC373. Six hours post-transfection cells were osmotically shocked for 90 s with 15% glycerol (v/v) in DMEM, washed three times in PBS and media replaced with DMEM supplemented with 0.5% bovine serum. Cells were harvested after 16–20 h by trypsin-EDTA treatment and seeded at 10^5 cells $well^{-1}$ in 96-well microtitre plates in phenol red-free DMEM supplemented with 0.5% bovine serum in the presence of treatments for a further 20–24 h. Similarly 10^5 cells were plated in 24-well microtitre plates for luciferase and 10^5 cells dispensed into LP-10 tubes for GFP analysis. Expression of β-galactosidase was induced using TNFα (80 U ml^{-1}) or TPA (50 ng ml^{-1}). Dexamethasone was used at final concentrations of 10^{-7} to 10^{-10} M.

2.6. β-Galactosidase reporter gene assay

β-Galactosidase (β-Gal) activity was measured using the chromogenic substrate chlorophenolred-β-D-galactopyranoside (CPRG, Boehringer, UK) as previously described (Dixon et al., 1997). Briefly, 100 μl of CPRG cocktail (consisting of 7 μl 50 mM CPRG, 7 μl 10 × -Z-buffer (600 mM Na_2HPO_4, 400 mM NaH_2PO_4, 100 mM KCl, 10 mM $MgSO_4 \cdot 7H_2O$, 500 mM *p*-mercaptoethanol, adjusted to pH 7.0 with 1 M NaOH), 1 μl 20% (w/v) SDS and 85 μl H_2O) added directly to each well. β-Gal activity was measured as absorbency at 570 nm after incubation at 37°C. Incubation times varied depending upon reporter gene construct used, but was typically 30 min to 3 h. To correct for differences in transfection effciency β-galactosidase activity was corrected for luciferase or green fluorescent protein activity.

2.7. Luciferase reporter gene assay

Firefly luciferase activity in cell lysates was measured in a Berthold luminometer (Lumat LB 9501). Cell culture media was aspirated and cells lysed in 250 μl lysis buffer (25 mM TrisPO_4 (pH 7.8), 15% (v/v) glycerol, 5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 1 mM EDTA (pH 8.0), 0.0025% (w/v) BSA) at room temperature on shaking plafform. Luciferase activity measured for 5 s in 180 μl cell lysate after addition of substrate buffer (830 μM ATP, 300 μM D-luciferin, 66% (v/v) lysis buffer).

2.8. Green fluorescent protein FACS analysis

GFP expression was analysed by fluorescence-activated cell sorting (FACS) in Beckton Dickinson FAC Scan. Percentage of cells expressing GFP was used to measure transfection efficiency and correct β-Gal activity accordingly.

2.9. Western blot analysis

Cells were lysed in 200 μl lysis buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1% (v/v) and Triton X-100, 1 mM PMSF in isopropanol added immediately prior to use). Cells were chilled on ice for 20 min prior to centrifugation at 12 000 × *g* for 10 min at 4°C. Proteins were separated on 8–19% Tris-glycine polyacrylamide gel, transferred to polyvinylidene difluoride transfer membrane. Blots were blocked for 90 min in blocking buffer (5% (w/v) BSA, 150 mM NaCl, 10 mM Tris (pH 8.0), 0.1% (v/v) Tween-20) at room temperature. Blots were washed with wash buffer (150 mM NaCl, 10 mM Tris (pH 8.0), 0.1% Tween-20) and incubated with rabbit anti-human glucocorticoid receptor antibody PA-510 diluted 1:2000 in blocking buffer overnight at 4°C. Blots were washed and then incubated with goat anti-rabbit IgG-horse radish peroxidase conjugate diluted 1:2500 in blocking buffer for 1 h at room temperature, before washing and visualisation by enhanced chemiluminescence (ECL) detection (Amersham, UK).

Antibody PA-510 has been shown to react with both the inactivated and activated form of the human GR (Srivastava and Thompson, 1990). Produced after immunization of rabbit with 26 amino acid synthetic peptide whose sequence is from the N-terminal region of the human GR. (A_{150}PTEKEFPKTHSDVSSEQHLKGQ TG_{175})

3. Results

3.1. Human GRα, but not hGRβ can repress AP-1 and NF-κB activity

In COS-7 cells TPA (50 ng ml^{-1}) induced reporter gene activity 18-fold from the AP-1-responsive construct, pTRE4Gal (Fig. 1a). Co-transfection of human GRα (0.01 μg 10^6 $cells^{-1}$) transferred glucocorticoid-responsiveness to these cells, resulting in a dexamethasone induced, dose-dependent decrease in β-galactosidase activity, with 100 nM dexamethasone inhibiting reporter gene transcription by 58% (Fig. 1b). However, human GRβ did not exert any transrepressive effects on TPA-stimulated reporter gene activity when transfected at the same concentration (Fig. 1b).

Similar results were observed in COS-7 cells co-transfected with a NF-κB response element construct, pNRE3Gal, and either hGRα or hGRβ. Recombinant human TNFα (80 U ml^{-1}) stimulated reporter gene activity was repressed by 40% in cells transfected with hGRα, but not in cells transfected with hGRβ (Fig. 2).

In another GR deficient cell line, HEK-293, co-transfected hGRα, but not GRβ was able to mediate transrepression of AP-1 or NF-κB activity in response to dexamethasone (Figs. 1c,d and 2c,d).

3.2. Confirmation of hGRβ expression from pcDNA3hGRβ

We confirmed that hGRβ protein was expressed in these cells after transfection with pcDNA3hGRβ as

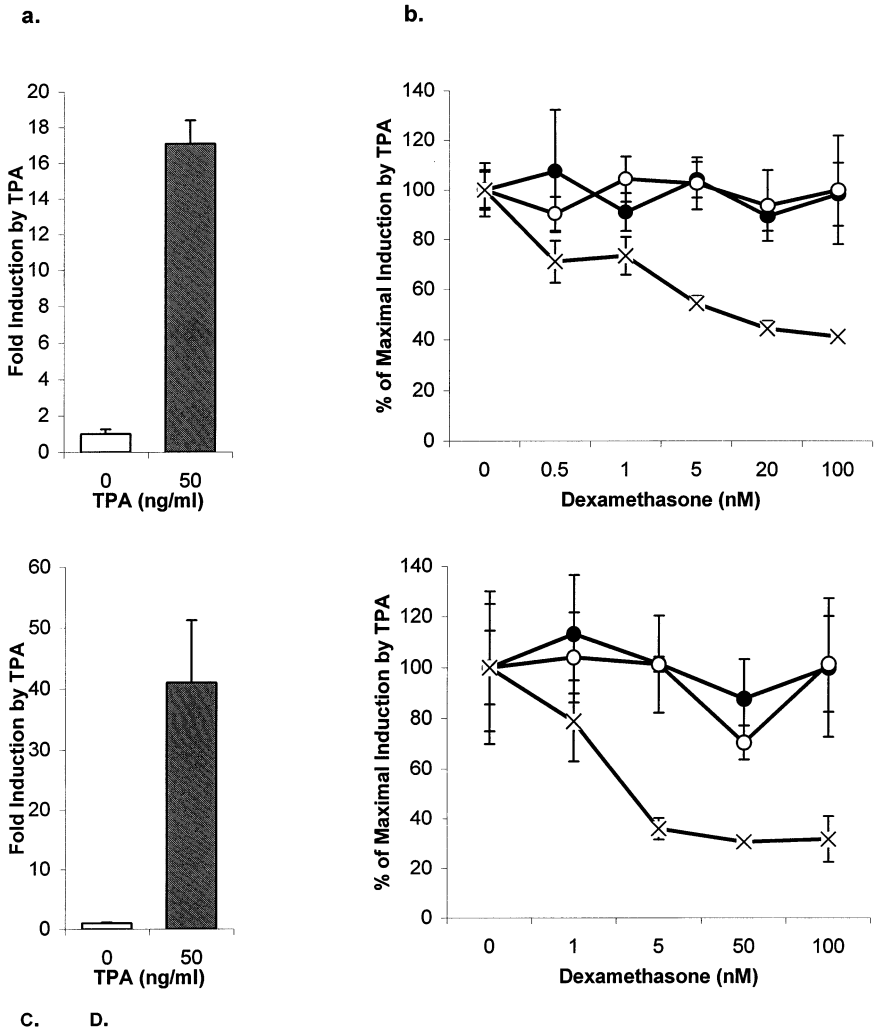


Fig. 1. Transrepression of AP-1 activity by hGRα, but not hGRβ. COS-7 (a) and HEK-293 (c) cells were transfected with phorbol ester-responsive reporter construct (pTRE4Gal). β-Galactosidase activity was measured in untreated cells (open bars) and cells stimulated with 50 ng ml^{-1} TPA (hatched bars). COS-7 (b) and HEK-293 cells (d) were co-transfected with pTRE4Gal (4 μg) and 0.5 μg of pcDNA3 (○), pcDNA3hGRα (X) or pcDNA3hGRβ (●). Cells were stimulated with 50 ng ml^{-1} TPA and a range of dexamethasone concentrations (0–100 nM). The data are plotted as the mean of triplicate samples (±1 SD) and represent the fold induction above unstimulated activity (a and c) and the repression of TPA-induced β-Gal activity as a percentage of the maximal TPA induction (b and d). β-Galactosidase activity was corrected for GFP activity. The data are representative of three (a and b) and two (c and d) independent experiments.

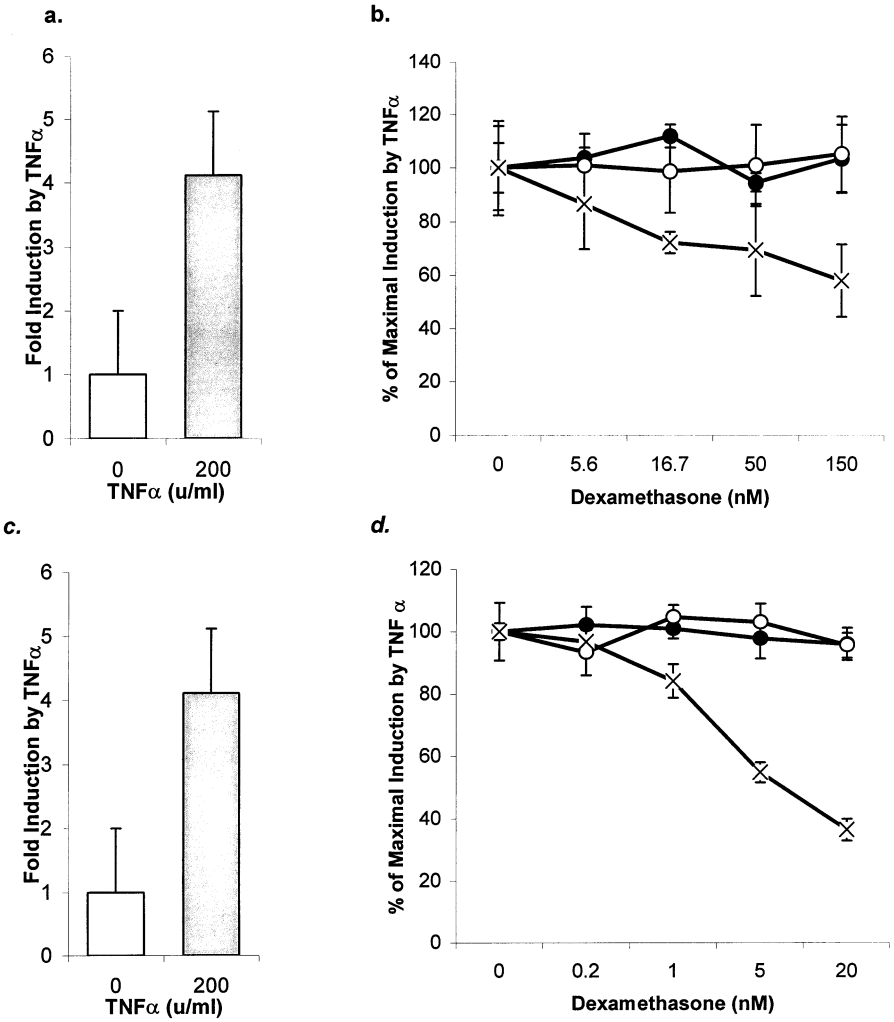


Fig. 2. Transrepression of NF-κB activity by hGRα, but not hGRβ. COS-7 (a) and HEK-293 (c) cells were transfected with NF-κB-regulated reporter construct (pNRE3Gal). β-Galactosidase activity was measured in untreated cells (open bars) and cells stimulated with 80 U ml^{-1} TNFα (stippled bars). COS-7 (b) and HEK-293 cells (d) were co-transfected with pNRE3Gal (15 μg) and 0.5 μg of pcDNA3 (○), pcDNA3hGRα (X) or pcDNA3hGRβ (●). Cells were stimulated with 80 U ml^{-1} TNFα and a range of dexamethasone concentrations (0–150 nM). The data are plotted as the mean of triplicate samples (±1 SD) and represent the fold induction above unstimulated activity (a and c) and the repression of TNFα-induced β-Galactosidase activity as a percentage of the maximal TNFα induction (b and d). β-Galactosidase activity was corrected for GFP activity. The data are representative of three independent experiments.

demonstrated by western immunoblot. The quantity of a 97 KDa protein, consistent with the predicted size for hGRβ, was demonstrated to increase with increasing amounts of transfected pcDNA3hGRβ (Fig. 3). Interestingly, we were able to detect significant levels of immunoreactive material in HEK-293 mocktransfected cellular extracts, associated with hGRα protein expression. These data suggest that HEK-293 cells do express hGRα, contrary to published data (van de Stolpe et al., 1994). The observation of limited amounts of hGRα protein in these cells fit with experiments where restrained transcriptional activity was apparent in HEK-293 cells transfected with pMMTVGal but not pGRE2Gal (Brogan, I.J., Davis, J.R.E., White, A., unpublished data).

3.3. Expression of hGRβ has no effect on the ability of hGRα to transrepress AP-1 or NF-κB activity

To examine the effect of hGRβ on hGRα-mediated transrepression of AP-1 activity, COS-7 cells were transfected with a constant amount of pTRE4Gal and hGRα and increasing concentrations of hGRβ to give ratios of hGRβ: hGRα in the range (1:1–17:1). Co-expression of hGRβ with hGRα had no further effect on AP-1 activity, nor did hGRβ antagonise the ability of hGRα to mediate transrepression of AP-1 activity (Fig. 4).

Similarly, hGRβ over-expressed in ratios of 1:1–10:1 relative to hGRα did not increase transrepression of NF-κB activity and did not prevent hGRα-mediated transrepression at NF-κB responsive elements (Fig. 5).

Similar results were obtained in HeLa and HEK-293 cells transfected with reporter constructs regulated by AP-1 or NF-κB. hGRβ failed to modulate the actions of the endogenous or exogenous GR on either transcription factor in either cell line (data not shown).

3.4. Human GRβ fails to act as a dominant negative inhibitor of hGRα-mediated transactivation

In COS-7 cells transfected with a positively regulated GRE (pGRE2Gal) cotransfection of hGRα (50 ng 10^6

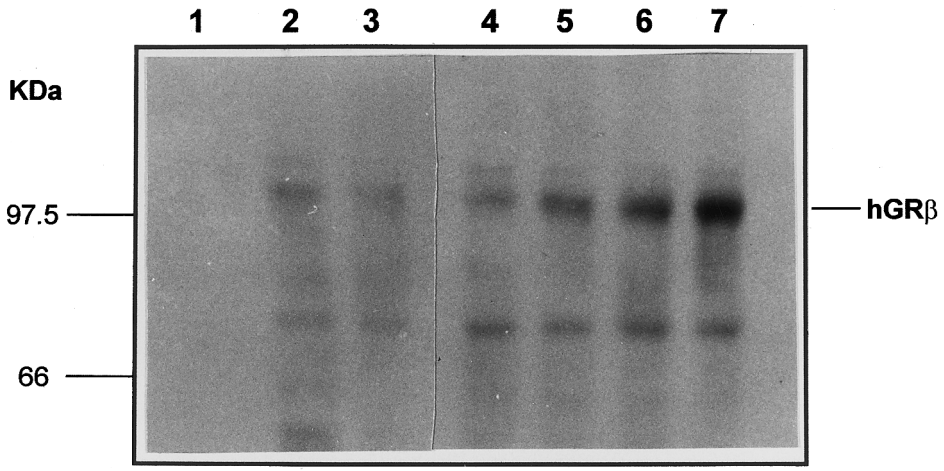


Fig. 3. Expression of hGRβ in transfected cells. Cells were transfected with increasing amounts (0.5–15 μg) of pcDNA3hGRβ. Each transfection was standardised to 20 μg with paC373. Whole cell protein was isolated and analysed by Western immunoblot. Lane 1: 6 μl high molecular weight Rainbow markers (Amersham RPN 756). Lane 2: 75 μg mock transfected HeLa cell protein extracts. Lane 3: 75 μg mock transfected HEK293 cell protein extracts. Lanes 4–7: 75 μg HEK293 cell protein extracts from cells transfected with 0.5, 2.5, 5 and 15 μg pcDNA3hGRβ. Data represents Western blot analysis of a single experiment and is representative of three individual experiments.

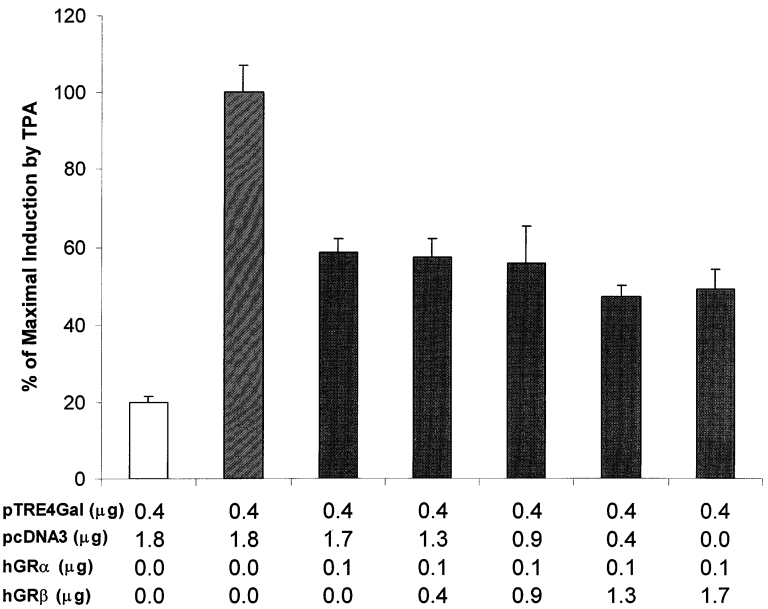


Fig. 4. Expression of hGRβ has no effect on the ability of hGRα to transrepress AP-1 activity. COS-7 cells were co-transfected with pTRE4Gal (0.4 μg) and pcDNA3hGRα (0.1 μg) and a range of pcDNA3hGRβ (0.4–1.7 μg). The total amount of pcDNA3-containing vector was maintained at 1.8 μg with pcDNA3 (0.4–1.8 μg). β-Galactosidase activity was measured in untreated cells (open bars), cells stimulated with 20 ng ml^{-1} TPA (hatched bars), cells co-treated with 20 ng ml^{-1} TPA and 150 nM dexamethasone (filled bars). The data are plotted as the mean of triplicate samples ($\pm$ 1 SD) and represent the repression of TPA-induced β-Galactosidase activity as a percentage of the maximal TPA induction corrected for luciferase activity. The data are representative of four independent experiments.

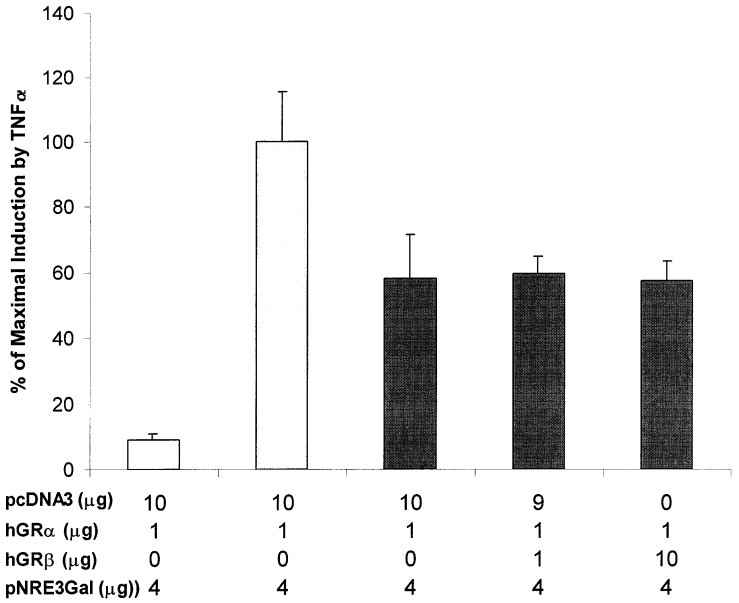


Fig. 5. Expression of hGRβ has no effect on the ability of hGRα to transrepress NF-κB activity. COS-7 cells were co-transfected with pNRE3Gal (4 μg) and pcDNA3hGRα (1 μg) and a range of pcDNA3hGRβ (1–10 μg). The total amount of pcDNA3-containing vector was maintained at 11 μg with pcDNA3 (1–10 μg). β-Galactosidase activity was measured in untreated cells (open bars), cells stimulated with 80 U ml^{-1} TNFα (stippled bars), cells co-treated with 80 U ml^{-1} TNFα and 150 nM DEX (filled bars). The data are plotted as the mean of triplicate samples (± 1 SD) and represent the repression of TNFα-induced β-Galactosidase activity as a percentage of the maximal TNFα induction corrected for luciferase activity. The data are representative of four independent experiments.

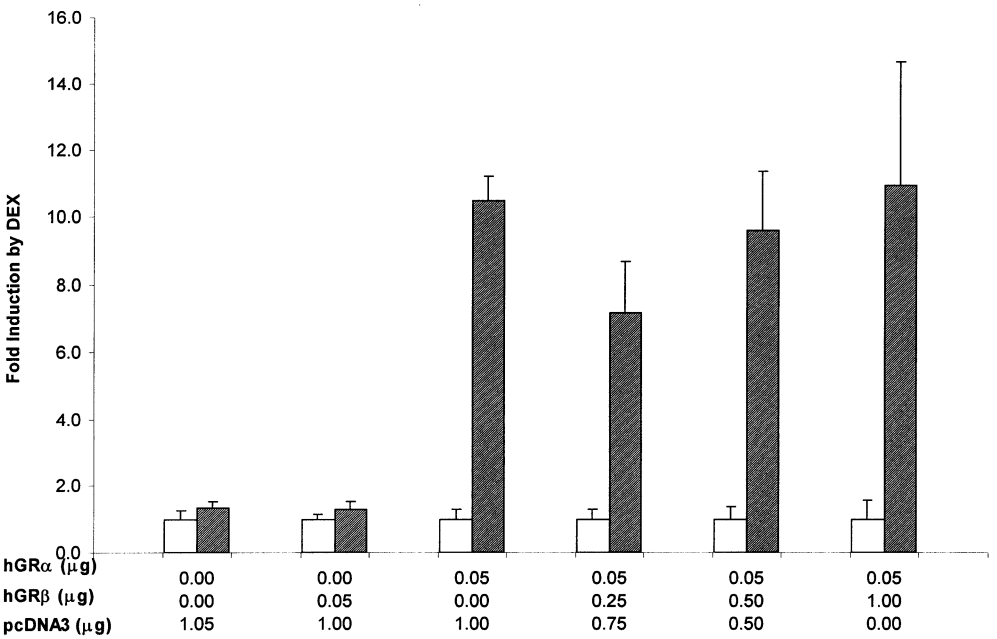


Fig. 6. Effect of maintaining constant promoter levels on dominant negative activity of hGRβ. COS-7 cells were co-transfected with pGRE2Gal (15 μg) and pcDNA3hGRα (0.05 μg) and a range of pcDNA3hGRβ (0.05–1 μg). The total amount of pcDNA3-containing vector was maintained at 1.05 μg with pcDNA3 (0.5–1.05 μg). β-Galactosidase activity was measured in untreated cells (open bars) and cells treated with 150 nM dexamethasone (hatched bars). The data are represented as fold induction above unstimulated activity, corrected for luciferase activity and plotted as the mean of triplicate samples (± 1 SD). The data are representative of five independent experiments.

$cells^{-1}$) caused a 10.5-fold induction following treatment with 150 nM DEX (Fig. 6). As expected GRβ, when transfected into COS-7 cells, was unable to mediate transactivation at the GRE (pGRE2Gal) over a range of DEX concentrations (5–450 nM; data not shown). Human GRβ (50–1000 ng 10^6 $cells^{-1}$) was then co-transfected with hGRα (50 ng 10^6 $cells^{-1}$) and pGRE2Gal. The transcriptional activity of hGRα was

unaffected by hGRβ expression at ratios up to 20:1 (Fig. 6).

However, in COS-7 cells transfected with ratios of hGRβ: hGRα of 1:1–20:1, there was a significant DNA dose-dependent repression of hGRα-mediated transactivation (data not shown). This appeared similar to the dominant negative activity previously described for hGRβ. Since the total amount of pcDNA3 vector (pcDNA3, pcDNA3hGRα, and pcDNA3hGRβ) ranged from 0.5–5.0 μg 10^6 cells^{-1}, and therefore was not constant, we investigated if this could contribute to transcriptional squelching in this transfection system. hGRβ (0.5–5 μg 10^6 cells^{-1}) was co-transfected with the constitutively active, glucocorticoid non-responsive reporter gene constructs, pRSVGal (0.25 μg 10^6 cells^{-1}) and pCMVGal (0.25 μg 10^6 cells^{-1}). The hGRβ expression vector, pRSVhGRβ caused a dose-dependent inhibition of pCMVGal activity over the range 0.5–5 μg 10^6 cells^{-1}, with a 71 ± 16% inhibition at 5 μg 10^6 cells^{-1} (Fig. 7a). A similar dose-dependent inhibition was observed with the pcDNA3hGRβ expression vector with a 59 ± 22.4% inhibition at 5 μg 10^6 cells^{-1} (Fig. 7b). This was attributed to squelching since the empty expression vector, pcDNA3, repressed pCMVGal activity by 75 ± 18.4% in the absence of GRβ cDNA (Fig. 7c). This inhibition was not increased further by the addition of pcDNA3hGRβ when the total amount of pcDNA3-containing DNA was kept constant (Fig. 7c).

4. Discussion

The mechanisms by which hGRα mediates transrepression have been the focus of intensive study, but remain incompletely resolved. Furthermore, the role of nonligand binding variants of the GR, such as hGRβ, in gene transrepression has not been fully elucidated. We have presented data which indicate that hGRβ does not mediate ligand-dependent or -independent transrepression of either AP-1 or NF-κB activity in COS-7, HEK-293 or HeLa cells, suggesting that hGRβ does not contribute to GR-mediated transrepression. Furthermore, we demonstrate that hGRβ is unable to act as a dominant negative inhibitor of hGRα-mediated transrepression of AP-1 or NF-κB activity in COS-7 cells. Therefore, even if expressed in excess of hGRα, hGRβ is unlikely to counteract the effects of hGRα-mediated glucocorticoid suppression. The failure of hGRβ to antagonise the repressive effects of hGRα when interacting with other transcription factors is similar to the findings of Bamberger et al. (1997b) who studied an interleukin-2 promoter reporter in lymphocytes.

Published data concerning the relative ratios of hGRβ and hGRα protein expression and the sub-cellular and tissue distribution of hGRβ are currently conflicting (de Castro et al., 1996; Hecht et al., 1997). It appears that hGRβ protein is expressed ubiquitously with hGRα, but overall at lower levels (Hecht et al., 1997; Oakley et al., 1997). Immunocytochemical evidence, however, indicates that whereas hGRβ protein expression may be lower than that of hGRα at the whole tissue level, it may be more highly expressed in individual specific cell types within a tissue (Oakley et al., 1997). Analysis of hGRβ mRNA expression in human bronchial epithelium and alveolar macrophages indicates that hGRβ is expressed at lower levels than its ligand-binding counterpart. Furthermore, down-regulation of GR gene expression following treatment with budesonide did not alter the ratio

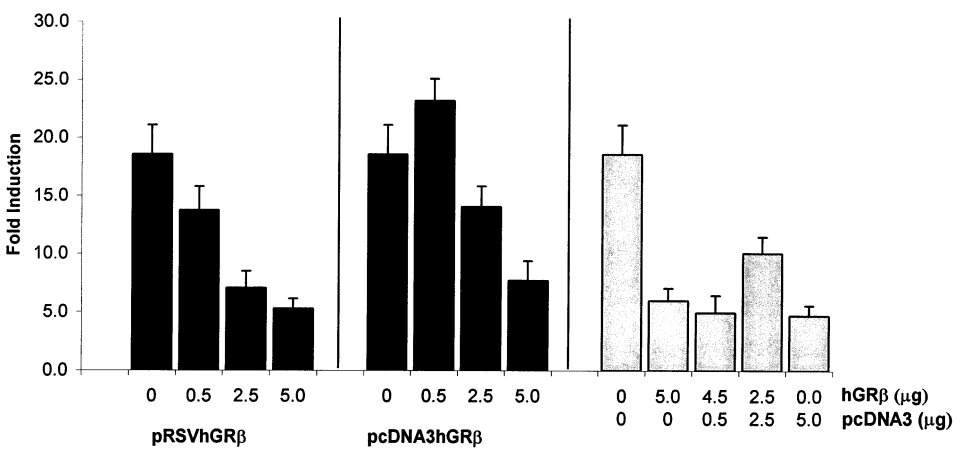


Fig. 7. Effect of hGRβ expression on a constitutively active, glucocorticoid-unresponsive promoter. COS-7 cells were co-transfected with pCMVGal (0.25 μg 10^6 cells^{-1}) and a range of pRSVhGRβ (a) or pcDNA3hGRβ (b) concentrations (0–5 μg 10^6 cells^{-1}) as indicated. (c) COS-7 cells were co-transfected with pCMVGal (0.25 μg 10^6 cells^{-1}), pcDNA3 (0–5 μg 10^6 cells^{-1}) and a range of pcDNA3hGRβ concentrations (0–5 μg 10^6 cells^{-1}) as indicated to maintain pcDNA3 concentration of 5 μg 10^6 cells^{-1}. β-Gal activity was measured in untreated cells and cells treated with 0.5–150 nM dexamethasone (data not shown). Values for unstimulated cells are shown. The data are represented as fold induction above activity in control (pac373) transfected cells, corrected for luciferase activity and plotted as the mean of triplicate samples (± 1 SD). The data are representative of three independent experiments.

of α and β mRNA levels (Korn et al., 1997). Interestingly, another group failed to find conserved hGRβ or hGRβ-like sequences in the mouse GR gene, suggesting that this splice variant may not be evolutionarily conserved (Otto et al., 1997).

The inability of hGRβ to modulate the transrepressive actions of hGRα led us to examine the dominant negative actions of hGRβ on hGRα-mediated transactivation in our system. Two groups have previously reported that hGRβ is able to act as a dominant negative repressor of hGRα-mediated transactivation at the MMTV promoter in COS-7 cells (Oakley et al., 1996; Bamberger et al., 1997a) and at palindromic GREs in COS-7 and HeLa cells (Oakley et al., 1996). However, while our results from studies with pRSVhGRβ in COS-7 and HeLa cells (data not shown) appear similar to these observations, we present data which indicates that in our system the apparent dominant negative actions of hGRβ can be accounted for by non-specific 'squelching' associated with the promoter sequences driving hGRβ cDNA expression.

In our studies with constitutively active reporter genes (pRSVGal and pCMVGal) strong, DNA-dependent squelching of reporter gene activity was observed with both hGRβ expression constructs and also with the empty expression vector, pcDNA3. When the total amount of pcDNA3-derived vector DNA was kept constant, we observed no dominant negative activity. In agreement with our findings, Hecht et al. (1997) also failed to observe a dominant negative action of hGRβ on hGRα-mediated transactivation, when they studied COS-7 cells transfected with the secreted placental alkaline phosphatase gene linked to the MMTV promoter.

The discrepancy between these reports regarding hGRβ action is hard to explain. It has been suggested that hGRβ may have a higher turnover rate than hGRα, and that the time post-transfection at which reporter gene activity is measured may be important to whether or not a dominant negative action of hGRβ is observed. However, all the studies on hGRβ to date, including our own, have measured reporter gene activity at 48 h post-transfection. These data also raise serious doubts about the suitability of complex transient transfection systems for the study of multiple protein interactions. Recent data have illustrated that while transient transfection of potent transcriptional activators into eukaryotic cells suppressed the transcription of co-transfected target genes by squelching, transcription from integrated chromatin-embedded target genes was unaffected by high levels of transiently introduced activators (Natesan et al., 1997). Future work directed at the interaction of hGRα and hGRβ at endogenous, physiological promoters might be valuable in understanding the differences observed.

Our data indicates, however, that hGRβ is unlikely to behave as a physiologically relevant dominant negative inhibitor of hGRα action on either transrepression or transactivation in all situations. These data do not discount the possibility that hGRβ modulates tissue sensitivity to glucocorticoid by another, as yet undetermined mechanism. For instance, cytokine modulation of hGR gene expression to favour hGRβ rather than hGRα production may represent a mechanism by which sensitivity to glucocorticoids is modulated by the production of less ligand-binding receptors, rather than by an active inhibitory mechanism: such a phenomenon has recently been described in peripheral blood mononuclear cells in patients with glucocorticoid-insensitive asthma (Leung et al., 1997).

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