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Identification of endogenous glucocorticoid repressed genes differentially regulated by a glucocorticoid receptor mutant able to separate between NF-κB and AP-1 repression

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Running title: NF-κB and AP-1 discriminating GR mutant

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Abbreviations:

GR, glucocorticoid receptor; GRE, glucocorticoid response element; nGRE, negative GRE; DBD, DNA-binding domain; LBD, ligand-binding domain; NF-κB, nuclear factor-κB; AP-1, activator protein-1; Luc, luciferase; ALP, alkaline phosphatase; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; MMP-1, matrix metalloproteinase-1 (collagenase-1); ICAM-1, intercellular adhesion molecule-1; IL-6R, interleukin-6 receptor ; qRT-PCR, quantitative real-time PCR

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Abstract

Glucocorticoids are commonly used in the clinic, but long-term treatment is often associated with severe side effects. One way to reduce unwanted effects is to restrict glucocorticoid receptor (GR) signaling through defined pathways. In this study we examine endogenous target genes regulated by a GR mutant that in contrast to the wild type GR is unable to repress stimulated nuclear factor-κB (NF-κB) activity, while repression of activator protein-1 (AP-1) activity is maintained. This GR mutant (GR_{R488Q}) harbors a point mutation in the second zinc finger of the DNA binding domain. Its ability to distinguish between NF-κB and AP-1 repression is defined using reporter genes regulated by both simple and natural promoters. The inability of GR_{R488Q} to repress NF-κB was not related to its inability to activate target genes through a glucocorticoid response element. Furthermore, the discriminating property was observed in three different cell lines, suggesting that this is not a cell specific effect. These results show that different receptor surfaces or mechanisms are involved in repression of NF-κB and AP-1, respectively. Interestingly, the GR_{R488Q} still interacted physically with NF-κB. Gene expression profiling of HEK293 cells, which express the wild type GR and the GR_{R488Q} mutant allowed identification of endogenous genes preferentially repressed by GR interference with NF-κB activity. The genes differentially regulated by GR_{R488Q} mutant versus the wild type GR after 2 h of treatment seem mainly to be involved in control of transcription and cell growth. At 8 h no such distinction could be seen.

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Introduction

Glucocorticoids are widely used in the clinic, but long-term treatment is often associated with severe side effects (Schäcke et al., 2002). Both wanted and unwanted effects of glucocorticoids are mediated via the intracellularly located glucocorticoid receptor (GR), which is present in most cell types. The GR belongs to the superfamily of nuclear receptors, which function as ligand-dependent transcription factors. Like other nuclear receptors, the GR contains three main functional domains, a C-terminal ligand binding domain (LBD), a central DNA binding domain (DBD) and an N-terminal domain. Ligand activation of the GR leads to activation or repression of target gene expression. Activation of gene transcription by the GR typically requires an interaction of the DBDs of a GR homodimer with specific DNA sequences, so called glucocorticoid responsive elements (GREs), usually located in the promoter regions of target genes (De Bosscher et al., 2003). In some cases, activation occurs by GR interaction with other transcription factors without a direct GR DNA interaction (Stöcklin et al., 1996; Subramaniam et al., 2003). The GR inhibits gene expression via at least two mechanisms. Both occur at the transcriptional level. One mechanism involves a direct interaction of the GR with specific DNA sequences, so called negative GREs (nGREs), and displacement of positively acting transcription factors. A second mechanism is mediated via a direct physical interaction between the GR and other transcription factors, a process which does not involve a direct GR DNA binding. This latter mechanism has been shown to be responsible for the relatively well-described GR mediated repression of genes regulated by activator protein (AP)-1 and nuclear factor (NF)-κB. In both cases, GR interferes with the activity of NF-κB and AP-1 while these factors still occupy their respective binding sites in the target genes, a mechanism usually referred to as tethering (De Bosscher et al., 2003). While direct DNA binding of the GR is not involved in tethering, the DBD of the GR still seems to participate in this mechanism (Heck et al., 1994; Jonat et al., 1990; Lidén et al.,

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1997; Schüle et al., 1990; Yang-Yen et al., 1990). The tethering mechanism seems to be physiologically important for the anti-inflammatory responses of glucocorticoids, since mice containing a GR mutant unable to transactivate via GR binding to GREs but still able to repress NF-κB and AP-1, maintain an anti-inflammatory activity *in vivo* (Reichardt et al., 2001).

The transcription factors NF-κB and AP-1 bind to specific DNA sequences in promoter regions of target genes. Both transcription factors have been shown to be crucial for the induction of a number of genes involved in many biological processes e.g. inflammation, differentiation, cell proliferation, apoptosis and oncogenesis (Ghosh and Karin, 2002; Karin et al., 2002; Karin and Chang, 2001; Shaulian and Karin, 2001). The transcription factor AP-1 is a protein dimer composed of members of the Fos and Jun families of proto-oncogenes. Fos and Jun proteins may also dimerize with other transcription factors belonging to the ATF and Maf family of proteins. A variety of stimuli activate AP-1 such as growth factors, cytokines, UV irradiation, and phorbol esters leading to altered gene expression dependent on cell and promoter context (Shaulian and Karin, 2001). NF-κB consists of a dimer of proteins belonging to the Rel family, typically a heterodimer of RelA (p65) and NF-κB1 (p50). In its non activated state, NF-κB resides in the cytoplasm bound to inhibitory proteins, IκBs. Upon exposure of the cells to e.g. cytokines, oxidative stress, phorbol ester or UV irradiation, the IκBs become phosphorylated and degraded allowing the NF-κB complex to translocate to the nucleus where it binds to specific DNA sequences and stimulates gene transcription (Ghosh and Karin, 2002).

Glucocorticoids are among the most potent anti-inflammatory and immunosuppressive class of drugs available. However, long-term treatment is associated with serious side effects

including osteoporosis, diabetes, growth retardation, impaired wound healing, muscle wasting and hypertension (Schäcke et al., 2002). The conceptual view is that the side effects are mediated through the receptor binding to GREs found in genes involved in various metabolic pathways, while the anti-inflammatory actions of glucocorticoids are mediated through protein-protein interactions that do not involve the GRE.This has led to an interest in so called dissociating glucocorticoids, compounds that induce the GR into a conformation that maintains the ability to repress gene transcription but has a poor capacity to transactivate genes. To date, however, such compounds have met with little success *in vivo* (Belvisi et al., 2001). An additional step to achieve more specific effects would be to further dissociate GR cross-talk with NF-κB and AP-1, respectively, since they may be differentially important for various biological processes. By studying GR DBD mutants, we demonstrate that GR mediated repression of NF-κB and AP-1 can be separated, indicating that GR utilizes different mechanisms to repress NF-κB and AP-1 signaling. Finally, we identify endogenous genes that are preferentially repressed by glucocorticoids through GR cross-talk with NF-κB.

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Materials and Methods

Reagents and chemicals. Dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione), cortisol, triamcinolone acetonide (TA, 9α-fluoro-16α-hydroxyprednisolone 16α,17α-acetonide) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were purchased from Sigma (St Louis, MO, USA). The culture media DMEM (high glucose) and F12 (HAM), penicillin/streptomycin, zeocin, hygromycin, L-glutamine and Lipofectin® reagent were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Integra b.v. (Netherlands) and the chemiluminescence reagents used for measurement of alkaline phosphatase and luciferase activity were purchased from Perkin Elmer Life Sciences (Boston, MA, USA) and BioThema (Sweden), respectively. Primers were obtained from DNA Technology A/S (Aarhus, Denmark) and all reagents used for qRT-PCR were purchased from Applied Biosystems (Foster City, CA, USA).

Reporter and expression plasmids. The luciferase reporter plasmids 3x(NF-κB)tk-Luc, ICAM1-Luc (pIC-277-Luc) 2x(GRE)tk-Luc and Cox2-Luc (-327/+59) have previously been described (van de Stolpe et al., 1994; Inoue et al., 1995).). The MMP1-Luc (-517/+63col-Luc) reporter gene was a kind gift from M Göttlicher (Neuherberg, Germany). The 5x(AP1)-ALP reporter vector contains five copies of the consensus TPA responsive element fused to the mouse mammary tumor virus core promoter, including the NF1 binding site (Bruggemeier et al., 1990) but lacking the GREs, and cloned 5' of the cDNA coding for human placental alkaline phosphatase (ALP) (Berger et al., 1988). RSV-Luc or CMV-ALP was used as internal controls to normalize for differences in transfection efficiency. The rat GR expression plasmids used have been described previously (Lidén et al., 1997). To generate stable cell

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lines, the cDNAs encoding the rat wild type GR and GR mutant were subcloned into the pcDNA5/FRT expression vector obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture and generation of stable cell lines. CV-1 cells and HEK293 Flp-In cells (Invitrogen, Carlsbad, CA, USA) were grown at 37ºC in 5% CO_2 in a 1:1 mixture of high glucose DMEM and F12 (HAM) containing 10% FBS, penicillin/streptomycin 10 IU/ml and 100 µg/ml, respectively and 2 mM L-glutamine. Non-transfected HEK293 Flp-In cells were grown in the presence of 100 µg/ml Zeocin and HEK293 Flp-In cells stably expressing wild type GR or GR mutant were selected and grown in the presence of Hygromycin 100 µg/ml. The GR cDNAs (KpnI-DraI fragments) containing the complete coding sequence were subcloned into the KpnI-EcoRV sites of the pcDNA5/FRT expression vector (Invitrogen) to generate stably expressing Flp-In HEK293 cell lines (www.invitrogen.com). Flp-In cells, which contain a single integrated Flp recombination target site, allows stable integration of cDNAs at a specific genomic site and subsequently, similar expression in individual cell clones. Furthermore, the HEK293 Flp-In cell line was chosen, as it does not contain functional endogenously expressed GR. In line with this, similar expression levels of stably transfected GR were obtained in all hygromycin resistant clones and clone mixes tested.

Transfection. Lipofectin® reagent was used in all transfections according to the manufacturer's instructions. The GR expression plasmids and the reporter gene plasmids were used at a concentration of 25 ng/well and 200 ng/well, respectively. The plasmids CMV-ALP and RSV-Luc were used as internal controls at a concentration of 1 ng/well and 10 ng/well, respectively. Briefly, 30,000 cells/well were seeded in 24-well plates 24 h prior to transfection. Twenty hours post transfection, cells were exposed to treatment, 100 nM dexamethasone or triamcinolone acetonide or 1 µM cortisol and/or 5 ng/ml TPA, for 20 h and

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the cell medium was collected and cell extract was prepared for measuring alkaline phosphatase (Barkhem et al., 1998) and luciferase activities.

Western blot analysis. Whole cell extract was prepared from cells cultured to subconfluence in 10 cm plates by lysing the cells in ice-cold Nonidet P-40 (NP-40) buffer (0.5% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA) for 20 min. Cell debris was removed by centrifugation at 14,000 x g for 15 min at 4ºC and an equal volume of 2 x SDS loading buffer was added to the supernatant and the mixture was boiled for 2 min. Protein concentrations were determined with the BioRad protein assay kit according to the instructions from the manufacturer (BioRad, Hercules, CA, USA). Samples were separated by 9% SDS-polyacrylamide gel electrophoresis and electroblotted onto a Hybond C-extra membrane (Amersham Biosciences, UK). The immunoblot was probed with a mouse monoclonal antibody against GR (Okret et al., 1984) followed by a secondary horseradish peroxidase-labeled anti-mouse antibody (Amersham Biosciences, UK). GR immunoreactivity was visualized using the enhanced chemiluminescence kit (Amersham Biosciences, UK) according to the manufacturer's instructions.

Immunoprecipitation assay. Whole cell extracts were prepared from TPA + dexamethasone treated parental HEK293 cells and cell lines stably expressing GR_{wt} and GR_{R488Q}, respectively. In another experiment, cells were treated with vehicle alone, 100 nM dexamethasone alone and 100 nM dexamethasone in the presence of 5 ng/ml TPA for 30 min at 37^0 C. The cells were freeze-thawed twice in ice-cold EPG buffer (1 mM EDTA, 20 mM Na-PO_4 pH 7.4, 10% glycerol, 2 mM mercaptoethanol) containing 400 mM NaCl, homogenized, and cell debris was removed by centrifugation at 14,000 x g for 10 min at 4ºC. The extract was incubated with an anti-GR antibody (Okret et al., 1984) for 4 h at 4ºC and

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subsequently the extract-antibody mixture was incubated with Protein A-Sepharose beads (Amersham Biosciences, UK) for 24 h. After three washes with low-salt buffer (EPG + 50 mM NaCl) the proteins bound to the extract-antibody-Sepharose mixture were eluted with high-salt buffer (EPG + 1 M NaCl) and the supernatant (eluate), after centrifugation, was mixed with 2 x SDS buffer and subjected to SDS-PAGE and analyzed by immunoblotting using an anti-GR antibody (Okret et al., 1984) and an anti-p65 antibody (sc-109 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). An aliquot of the extract-antibody mixture, i.e. prior to the Protein A-Sepharose incubation, was also analyzed by immunoblotting to check for the input of GR and p65, respectively.

Microarray assay and data analysis. To investigate endogenous genes that are modulated by TPA ± dexamethasone treatment in the presence of wild type or mutant receptors the Human Genome Focus Array (Affymetrix) was used. This array represents approximately 8,500 well annotated human transcripts from the NCBI RefSeq database. Affymetrix analysis was conducted according to the Affymetrix manual (www.affymetrix.com). Cells were treated for 2 and 8 h. Three independent experiments were performed for each time point. Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA, USA) followed by RNA quality assessment using the Nano 6000 Chip in the Bioanalyzer from Agilent Inc. Eight μg total RNA was used for target cDNA synthesis according to the Affymetrix manual (www.affymetrix.com). The raw intensity data was normalized and gene expression levels estimated using the robust multichip analysis (Irizarry et al., 2003). An initial four way ANOVA (analysis of variance) was performed and parameters analyzed (treatment, time of treatment, GR type and day of experiment) showed that the only significant combinations of interactions were treatment in relation to GR type and treatment in relation to treatment time. We noted that the day of experiment was not an

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effecting parameter. Consequently, for further statistical analysis a two way ANOVA was performed, testing only GR type, treatment and relation between the two, for 2 h and 8 h, separately. Discriminating genes were selected on the basis of the following four criteria, 1) mean repression of at least 30% for GR_{wt}, 2) p value of the one-sided t-test for repression by $GR_{wt} < 0.01$, 3) repression by the GR_{R488Q} mutant less than half of what is observed for the GR_{wt}, 4) p value for the change in regulation by treatment between the wild type and mutant GRs from the ANOVA was less than 0.01.

Quantitative Real-Time PCR (qRT-PCR) analysis. For validation of array results, cDNA was prepared from the three independent experiments, using one microgram of total RNA, random hexamer primers and Superscript II (Invitrogen, Carlsbad, CA, USA). The expression of specific mRNAs was quantified by qRT-PCR, normalized to GAPDH expression, using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and ABI Prism 7700 sequence detection system. The primers used for PCR analysis were: **GAPDH** forward, 5'-GAAGGTGAAGGTCGGAGTCAAC-3', reverse 5'-CAGAGTTAAAAGCAGCCCTGGT-3'; **Activin A** forward, 5'-TTGCCGAGTCAGGAACAGC-3', reverse, 5'-GGGACTTTTAGGAAGAGCCAGAC-3'; **COX-2** forward, 5'-TGAATCATTCACCAGGCAAATT-3', reverse, 5'-TCTGTACTGCGGGTGGAACA-3'; **GADD45B** forward, 5'-GTCGGCCAAGTTGATGAATGT-3', reverse, 5'-GGATTTGCAGGGCGATGT-3'; **JunB** forward 5'-AAATGGAACAGCCCTTCTACCA-3', reverse, 5'-CGTATCCCGTAGCTGTGTATGAGTC-3'; **IL-6R** forward, 5'-CCTTTCAGGGTTGTGGAATCTT-3', reverse, 5'-TGACTGTGATGTTGGCAGGC-3'; **MMP-1** forward, 5'-TTGAAGCTGCTTACGAATTTGC-3', reverse, 5'-GTCCCTGAACAGCCCAGTACTT-3'.

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Results

A point mutation in the second zinc finger of the GR DBD reduces cross-talk with NF-κB but not with AP-1. Previous studies have shown that the GR DBD is involved in mediating repression of both AP-1 and NF-κB signaling (Lidén et al., 1997; Schüle et al., 1990; Yang-Yen et al., 1990). More specifically, the C-terminal zinc finger of the GR DBD has been shown to be important for transrepression of NF-κB activity (Lidén et al., 1997). We have previously shown that a point mutation, arginine to glutamine, at position 488 (GR_{R488Q}, amino acid number refers to the rat GR) in the C-terminal zinc finger impaired glucocorticoid induced transactivation and significantly decreased the GR mediated inhibition of NF-κB activity (Lidén et al., 1997). To test the effect of this mutant on GR mediated repression of AP-1 activity, transient transfections were performed using GR deficient CV-1 cells. Expression vectors for wild type GR (GR_{wt}) or GR_{R488Q} mutant were co-transfected with reporter genes regulated by NF-κB or AP-1 followed by stimulation with the phorbol ester TPA in the absence or presence of dexamethasone. To assay for glucocorticoid effects on NF-κB activity, a reporter gene with three single NF-κB sites up-stream of the luciferase reporter gene was used. To assay for glucocorticoid effects on AP-1 activity, a luciferase reporter gene controlled by the promoter region (-517/+63) from the matrix metalloproteinase 1 gene (MMP-1, collagenase-1) was used. Repression of MMP-1 gene expression by glucocorticoids has previously been shown to be mediated by an interaction between GR and AP-1 at the AP-1 binding site in the MMP-1 promoter (Jonat et al., 1990). The results showed that the GR_{R488Q} mutant failed to suppress the NF-κB reporter gene activity, whereas its capacity to inhibit AP-1 activity was preserved (Fig. 1). This suggested that the GR_{R488Q} mutant could discriminate between AP-1 and NF-κB repression.

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In order to further investigate the NF-κB and AP-1 discriminating properties of the GR_{R488Q} mutant, cell clones stably expressing GR_{wt} and GR_{R488Q} mutant, respectively, were established in HEK293 Flp-In cells that lack functional endogenous GR. Receptor expression was verified by immunoblotting using a GR specific monoclonal antibody. Both isolated individual clones and clone mixes were screened, all showing similar GR expression levels (Fig. 2A and data not shown). As determined by ligand binding, the total number of receptors was approximately 80,000 receptors/cell (data not shown). To reduce the risk of clone specific effects, clone mixes were used for further studies. In order to investigate the effect by stably expressing the GR_{R488Q} mutant on NF-κB and AP-1 activity, transient transfections of AP-1 and NF-κB regulated reporter genes were performed. Initial studies using simple reporter genes harboring multiple AP-1 or NF-κB sites upstream of minimal promoters, showed that in contrast to the wild type receptor the GR_{R488Q} mutant activated by dexamethasone lacked the ability to repress TPA stimulated NF-κB activity (Fig. 2B), while its inhibitory effect on AP-1 signaling was preserved (Fig. 2C). The same discriminating effect was seen when cells were treated with cortisol or triamcinolone acetonide (TA) (Fig. 2D). A dose-response analysis of the dexamethasone concentration that was required to give 50% repression (ED_{50}) showed a value of 0.6-0.9 nM for repression of NF-κB and AP-1 activity by the GR_{wt} and AP-1 repression by the GR_{R488Q} mutant, while repression of NF-κB activity by the GR_{R488Q} mutant did not occur at any dexamethasone concentration (Fig. 2E). The ED_{50} was found to be in line with the K_d = 1 nM for dexamethasone binding to both GR_{wt} and GR_{R488Q} (data not shown). At low NF-κB activity, as in the absence of TPA stimulation, the GR_{R488Q} was able to repress NF-κB activity (Fig. 2B). This may indicate a remaining weak NF-κB repressing activity in the GR_{R488Q} mutant that is sufficient to repress low NF-κB activity in non-stimulated cells.

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We also analyzed the ability of the GR_{R488Q} mutant to transactivate a target gene in HEK293 cells. In contrast to the GR_{wt}, the GR_{R488Q} mutant lacked the ability to transactivate a GRE-regulated reporter gene (Fig. 2F). The fact that the NF-κB and AP-1 discriminating property of the GR_{R488Q} mutant was seen in both CV-1, HEK293 (see above) and U2OS cells (data not shown) demonstrated that this effect was not cell specific.

To determine if the specificity of NF-κB versus AP-1 repression, displayed by the GR_{R488Q} mutant, was maintained on more complex promoters, reporter genes that are under the control of natural promoters were analyzed. For this purpose intercellular adhesion molecule-1 (ICAM-1) and cyclooxygenase-2 (Cox-2) reporter genes consisting of the ICAM-1 or Cox-2 promoter combined with the luciferase reporter gene, which both have previously been shown to mainly be regulated by NF-κB (van de Stolpe et al., 1994, Newton et al., 1997; Schmedtje et al., 1997) and the MMP-1 reporter gene were used. The failure of the GR_{R488Q} mutant to repress NF-κB signaling, as assayed using the more complex ICAM-1 or Cox-2 promoters (Fig. 3A), together with a preserved ability to down-regulate the natural promoter regulated by AP-1, MMP-1 (Fig. 3B), further substantiated the GR_{R488Q} mutant's discriminatory property. These results indicate that the GR mediated repression of AP-1 and NF-κB signaling operates through separate GR surfaces and/or mechanisms.

The inhibitory effect on NF-κB signaling does not involve GR mediated transactivation. The fact that the GR_{R488Q} mutant lacked transactivation activity (Fig. 2E) suggested the possibility that the separation of NF-κB and AP-1 repression was linked to the failure of the GR_{R488Q} to transactivate GRE-regulated target genes. To investigate this, we compared the GR_{R488Q} with another second zinc finger GR DBD mutant (LS7), containing two point mutations in the second zinc finger, P493R and A494S, previously shown to be

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transactivation deficient (Yang-Yen et al., 1990). Transient transfections of expression vectors coding for wild type and mutant GRs together with reporter genes showed that GR_{LS7}, in contrast to GR_{R488Q}, repressed TPA induced NF-κB activity (Fig. 4). Similar to GR_{R488Q}, GR_{LS7} also repressed AP-1 as previously shown (Yang-Yen et al., 1990). These experiments demonstrated that the NF-κB and AP-1 dissociation activity was restricted to the GR_{R488Q} mutant, and not linked to the inability of GR_{R488Q} to transactivate GRE-regulated target genes.

Both GR_{wt} and GR_{R488Q} physically interact with p65 *in vivo*. Based on the reporter gene assay, transactivation of genes via a GRE dependent mechanism does not seem to be involved in GR mediated inhibition of NF-κB. Since a direct protein-protein interaction has been demonstrated in GR_{wt} repression of NF-κB (Caldenhoven et al., 1995), an explanation for the GR_{R488Q} mutant's inability to repress NF-κB could be that the GR_{R488Q} mutant has lost its capacity to physically interact with the NF-κB complex. A possible intracellular association between endogenous p65 (RelA) and GR *in vivo* was examined by co-immunoprecipitation. Extracts from parental HEK293 cells and cells stably expressing the GR_{wt} and GR_{R488Q} mutant, respectively, were used for immunoprecipitation experiments. The precipitates were analyzed for GR and p65 by immunoblotting. As expected, GR was only detected in GR expressing cells, while p65 was present in equal amounts in both parental HEK293 cells and cells expressing the GRs ('Input' Fig. 5A and B). Precipitation using a monoclonal anti-GR antibody followed by immunoblotting for GR and p65, respectively ('Eluate' Fig. 5A and B), showed that the p65 protein was co-immunoprecipitated in both GR_{wt} and GR_{R488Q} mutant cells, but not in parental cells. This demonstrated that the GR physically interacted, directly or indirectly, with p65 in HEK293 cells *in vivo* and that a point mutation in the C-terminal zinc finger, GR_{R488Q}, did not abrogate this interaction. The

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interaction of both dexamethasone activated GR_{wt} and GR_{R488Q} mutant with p65 was visible only after TPA stimulation of the cells (Fig. 5C).

Identification of endogenous genes that are differentially down-regulated by GR_{wt} and GR_{R488Q} mutant using microarray analysis. Collectively, the transfection data strongly supported that the GR_{R488Q} mutant was unable to repress NF-κB activity, while it still repressed AP-1 dependent signaling. To identify endogenous glucocorticoid repressed genes that are preferentially down-regulated by GR cross-talk with NF-κB signaling, we used the Affymetrix expression platform. Cells were treated with TPA ± dexamethasone for 2 h and 8 h, respectively. The experiment was repeated three times at separate days. Subsequently gene expression levels were analyzed using the human genome focus array (Affymetrix) detecting approximately 8,500 transcripts. This analysis demonstrated that several endogenous genes were differently repressed by GR_{R488Q} compared to GR_{wt} in the presence of dexamethasone. Of the 39 genes significantly down-regulated by GR_{wt} by at least 30% in the presence of dexamethasone at 2 h, 29 (74%) genes were not repressed by the GR_{R488Q} mutant (Fig. 6A and Table 1). After 8 h of treatment 109 genes were down-regulated by GR_{wt} with 28 (26%) not repressed by the GR_{R488Q} mutant (Fig. 6B and Table 2). As revealed in Table 1, many of the genes repressed by the GR_{wt} at 2 h of treatment are involved in regulation cell cycle/cell growth and regulation of transcription. A majority of these genes are not as efficiently repressed by the GR_{R488Q} mutant. At 8 h of treatment no correlation to a particular biological process and genes differentially repressed by the GR_{wt} and GR_{R488Q} mutant could be seen (Table 2).

In contrast to what one would have expected from the transfection results using the MMP-1 luc reporter gene (Fig. 3B) and MMP-1 being a well-characterized AP-1 regulated gene (Jonat et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990), it did not turn up in the

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extracted microarray data using the criteria set as one of the down-regulated genes with non-discriminating property. Careful analysis of the microarray data revealed that the reason for this was that the criteria of >30% repression by the GR_{wt} was not fulfilled. However, both the GR_{wt} and GR_{R488Q} mutant significantly ($p<0.01$) down-regulated the MMP-1 gene transcription to a similar degree (29 vs. 26%, no significant difference) following dexamethasone treatment. Down-regulation of the endogenous MMP-1 gene by the GR_{wt} or GR_{R488Q} mutant was confirmed by qRT-PCR analysis (see below). Regulation of the endogenous ICAM-1 gene was not detected in the array due to a very low expression level in the HEK293 cells (data not shown).

qRT-PCR analysis of some glucocorticoid repressed genes identified in the microarray analysis. For verification of the data generated using array analysis, six genes were selected for qRT-PCR, three discriminating genes, Cox-2, JunB and IL-6R and three non-discriminating genes, Activin A, GADD45B and MMP-1 (see above). Expression analysis of the non-discriminating genes Activin A and GADD45B by qRT-PCR showed that dexamethasone treatment repressed TPA stimulated gene transcription in both GR_{wt} and GR_{R488Q} mutant expressing cells (Fig. 7). Both the GR_{wt} and GR_{R488Q} mutant repressed endogenous MMP-1 expression (99 and 96%, respectively) when analyzed by qRT-PCR (Fig. 7). (The small difference in repression turned out to be significant due to a very low standard deviation). In contrast, the GR_{wt} extensively repressed the discriminating genes Cox-2, JunB and IL-6R, while the GR_{R488Q} mutant did not repress transcription of these genes to the same extent (statistical significant difference) (Fig. 7). In view of the fact that similar results were obtained by the two techniques, microarray and qRT-PCR, the microarray analysis seems to reliably distinguish glucocorticoid repressed and non-repressed genes, although the sensitivity may be less as compared to qRT-PCR.

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Discussion

The DBD of the GR is an essential domain for the receptor's ability to inhibit the activity of both NF-κB and AP-1 (Caldenhoven et al., 1995; Jonat et al., 1990; Lidén et al., 1997; Nissen and Yamamoto, 2000; Schüle et al., 1990; Yang-Yen et al., 1990). Based on this knowledge, we have used GR DBD mutant(s) to investigate the possibility to specifically impair GR cross-talk with one of the two transcription factors. Here we provide evidence that a GR DBD mutant that contains a point mutation in the second zinc finger (R488Q) of the DBD has an impaired capacity to repress NF-κB activity following stimulation, while its AP-1 repressive function is intact. This suggests that the inhibitory cross-talk mechanism between the GR and the two transcription factors, NF-κB and AP-1, operates through different mechanisms and/or involves separate GR regions. Furthermore, the use of this discriminating GR mutant allowed the determination of the relative importance of negative GR cross-talk with NF-κB and AP-1 on individual genes *in vivo*, which previously has been difficult to address. The ability of GR_{R488Q} to repress NF-κB activity in non-stimulated cells as seen in Fig. 2-3, may represent some residual NF-κB repressing activity of GR_{R488Q} that is sufficient to repress minor amounts of NF-κB activity in non-stimulated cells but not sufficient to repress higher amounts NF-κB activity in stimulated cells.

It has been proposed that the mechanism of GR mediated inhibition of both NF-κB and AP-1 involves competition for a common factor, e.g. the cAMP response element binding protein (CREB) binding protein (CBP), that is present in limiting amounts in the cell and is involved in the activation of both NF-κB and AP-1 (Kamei et al., 1996; Sheppard et al., 1998). Since the GR_{R488Q} mutant still repressed AP-1 but not NF-κB activity, it is not likely

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that a competition model involving a common cofactor is responsible for GR repression of the activity of both these transcription factors. This is consistent with evidence that the GR inhibits NF-κB and AP-1 independently of the levels of common coactivators such as CBP (De Bosscher et al., 2001; De Bosscher et al., 2000). Furthermore, this is in line with a recent report suggesting a coactivator independent repression of NF-κB by the GR (Wu et al., 2004), while the involvement of coactivators has been demonstrated in GR mediated AP-1 repression (Rogatsky et al., 2001).

To further explore a molecular mechanism explaining the failure of the GR_{R488Q} mutant to repress NF-κB, the GR_{R488Q} mutant's ability to physically interact with the NF-κB was investigated. Given the fact that a direct protein-protein interaction between the GR_{wt} and NF-κB has been described to be involved in the GR mediated repression of NF-κB (Caldenhoven et al., 1995), a potential explanation for the GR_{R488Q} mutant's inability to repress NF-κB could be that the GR_{R488Q} mutant has lost its capacity to physically interact with the NF-κB complex. However, while the point mutation in the C-terminal zinc finger, GR_{R488Q}, leads to an impaired capacity to inhibit NF-κB activity, the interaction with p65, *in vivo* is still intact. Thus, a physical interaction between GR and NF-κB seems not to be sufficient for a functional GR mediated repression of NF-κB dependent signaling. Possibly, the mutant is not able to recruit or interfere with other factors necessary for a functional inhibition of NF-κB activity. For example, tethered to DNA-bound p65 the GR has been suggested to recruit a so far unidentified co-repressor which interferes with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain (Nissen and Yamamoto, 2000).

To our knowledge this is the first report describing the repression of endogenous genes by a dissociating GR mutant able to discriminate between NF-κB and AP-1 cross-talk. In a

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previous report a GR DBD mutant harboring a point mutation in the first zinc finger, S425G (human nomenclature), was described (Heck et al., 1994). This mutant, in contrast to the GR_{R488Q} mutant, has a preserved ability to transactivate a GRE dependent reporter gene (Heck et al., 1994) but similarly to the GR_{R488Q} mutant lacks the ability to repress a simple NF-κB dependent reporter gene, while its ability to repress a simple AP-1 dependent reporter gene is preserved (Tao et al., 2001). These results, however, are in disagreement with the earlier report showing an impaired GR mediated AP-1 repression using the GR_{S425G} mutant (Heck et al., 1994). Furthermore, in our hands the GR_{S425G} mutant is still able to repress NF-κB activity (data not shown). The reasons for the conflicting results obtained by the various laboratories are unclear. However, since the discriminatory property between NF-κB and AP-1 cross-talk using the GR_{R488Q} mutant is maintained in three different cell lines, we demonstrate that this finding is most likely not a cell specific event. In addition, both the transfection experiments and qRT-PCR analysis of known NF-κB and AP-1 target genes, respectively, confirm that the GR_{R488Q} mutant distinguishes NF-κB and AP-1 repression.

We used the stable cell lines expressing the GR_{wt} and GR_{R488Q} mutant, respectively, to investigate the regulation of endogenous genes by these receptors using the microarray technology. As expected, given the fact that the two transcription factors, NF-κB and AP-1, regulate a diverse set of genes, examples of genes that were similarly and differently regulated by the GR_{wt} and GR_{R488Q} mutant, respectively, were detected. Although other discriminating effects between the GR_{wt} and the GR_{R488Q} mutant than the one described in the present study can not be excluded, the gene regulation observed in the array provided evidence supporting the reporter gene experiments demonstrating the impaired ability of the GR_{R488Q} mutant to repress NF-κB while its ability to repress AP-1 was conserved. In fact, genes such as Cox-2, JunB and NR4A2 (Nurr1) were significantly down-regulated by the GR_{wt} whereas the

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influence of the GR_{R488Q} mutant was marginal, which is in line with earlier reports showing a dependence on functional NF-κB sites in the promoter region of these genes (Frazier-Jessen et al., 2002; McEvoy et al., 2002; Newton et al., 1997; Schmedtje et al., 1997). Genes down-regulated to a similar extent by both the GR_{wt} and the GR_{R488Q} mutant were also identified, e.g. MMP-10, GADD45B and Activin A, that have previously been shown to have a functional or a putative AP-1 site in a regulatory region of the gene (Balliet et al., 2001; Benbow and Brinckerhoff, 1997; Tanimoto et al., 1996). The same was seen when analyzing the MMP-1 regulation by qRT-PCR (see "Results" for the explanation for not being scored positive in the microarray). The reason for not detecting regulation of endogenous ICAM-1 in HEK293 cells by the array despite being regulated in the transfection experiment (Fig. 3), may be due to very low endogenous expression possibly explained by a lack of an essential component/modification required to activate the intact gene in its proper chromatin environment.

To further examine the array data, genes down-regulated by GR_{wt} were sorted with regard to their involvement in various biological processes. Many of the down-regulated genes by the GR_{wt} at 2 h belong to genes involved in cell growth/cell cycle control or in regulating transcription of which several are immediate early response genes, e.g. Fos and Jun. Interestingly, most of these respond differentially to the GR_{wt} and GR_{R488Q} mutant. Overall 74% of the genes down-regulated at 2 h by the GR_{wt} scored as differentially regulated when compared to repression by the GR_{R488Q} mutant. At 8 h several more genes were down-regulated by the GR_{wt} as compared to the 2 h time point. However, at 8 h only 26% of the genes were differently regulated by the GR_{wt} and GR_{R488Q} mutant. At this time point no obvious subpopulation of target genes for discriminated or non-discriminated genes was revealed. Some genes repressed by the GR_{wt} at both 2 h and 8 h scored as discriminated at 2 h

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but were no longer discriminated at 8 h by the GR_{R488Q} mutant. This suggests that in the initial response phase these genes are mainly regulated by NF-κB, while at later time points regulation by other transcription factors, e.g. AP-1, may take over.

In summary, our results demonstrate that GR mediated repression of NF-κB and AP-1 can be separated by a point mutation in the second zinc finger of the GR DBD, a region within the DBD previously shown to be important for NF-κB repression (Lidén et al., 1997). The impairment of NF-κB repression by the GR_{R488Q} mutant seems not to involve a defect in physical interaction between the GR_{R488Q} mutant and NF-κB, rather an alternative explanation seems more likely, which remains to be established. Moreover, the ability to repress NF-κB by another transactivating deficient GR mutant, LS7, and the relative short time of treatment (2 h) for one of the microarray analysis, provides evidence in favor of a direct repression mechanism rather than an indirect effect such as up-regulation of a negatively acting factor, e.g. IκBα (Auphan et al., 1995; Scheinman et al., 1995). Although no clear pattern emerged from the gene expression profiling of GR mediated repression of NF-κB regulated genes, the diversity of genes involved in different biological processes highlight the fact that GR modulate a multitude of functions. In addition, as indicated by the employment of the GR_{R488Q} mutant, several of the down-regulated genes seem, to some extent, to be regulated in an NF-κB dependent manner. Accordingly, it would be interesting to investigate the biological consequences of a GR mutant able to discriminate between NF-κB and AP-1 dependent gene transcription in a more physiological context e.g. in an *in vivo* animal model. Furthermore, the possibility to discriminate between AP-1 and NF-κB repression may open up an opportunity to generate GR interacting drugs with more restricted and beneficial GR mediated therapeutic effects.

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Footnotes

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Figure legends:

FIG. 1. **The GR_{R488Q} mutation reduces the receptor's ability to repress NF-κB but not AP-1 activity in transiently transfected CV-1 cells.** CV-1 cells were transiently transfected with GR expression plasmid, reporter gene plasmid regulated by NF-κB (3x(NF-κB)tk-Luc) or AP-1 (MMP1-Luc) and the internal control vector CMV-ALP. 20 h post transfection cells were exposed to vehicle (vertical striped bars), 5 ng/ml TPA (black bars) or 5 ng/ml TPA + 100 nM dexamethasone (grey bars) for 20 h. The luciferase activity was normalized to the activity of the internal control (alkaline phosphatase). TPA stimulation in each experiment was given the nominal value of 100 and control and TPA + dexamethasone results were expressed relative to this nominal value. Data represent mean ± SD. Each experiment was performed in triplicate and repeated three times. The stars indicate a significant inhibitory effect by TPA + dexamethasone relative to TPA alone (*, $p<0.05$; **, $p<0.01$; ns, not significant as analyzed by Student's *t*-test).

FIG. 2. **The GR_{R488Q} mutant fails to repress NF-κB but not AP-1 activity in stably transfected HEK293 cells.** (A) Similar expression levels of wild type GR and R488Q mutant in stably transfected HEK293 cells. GR expression levels in parental (-), wild type GR (GRwt) and GR_{R488Q} mutant (R488Q) clones were determined by Western blotting. (B) GR_{R488Q} mutant fails to repress a simple NF-κB gene reporter. HEK293 clones were transiently transfected and treated as described in Fig. 1 using 3x(NF-κB)tk-Luc reporter plasmid and CMV-ALP plasmid as internal control. Treatment: vehicle (vertical striped bars), dexamethasone (open bars), TPA (black bars) and TPA + dexamethasone (grey bars). TPA stimulation in each experiment was given the nominal value of 100 and the results for the other conditions were expressed relative to this nominal value. (C) GR_{R488Q} mutant's ability to

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repress AP-1 is preserved. Same protocol and conditions as in "B" with the exception that the reporter plasmid 5x(AP1)-ALP and the internal control RSV-Luc were used. (D) demonstrates the ability of triamcinolone acetonide (TA, diagonal striped bars) and cortisol (horizontal striped bars) to repress TPA stimulated 3x(NF-κB)tk-Luc expression via the GR_{wt} or GR_{R488Q} mutant relative to TPA treatment alone (black bars). Data in B-D represent mean ± SD. Each experiment was performed in triplicate and repeated three times. The stars indicate a significant inhibitory effect by TPA + dexamethasone (cortisol or triamcinolone acetonide (TA)) relative to TPA alone or dexamethasone treatment relative vehicle treatment (**, $p<0.01$; ***, $p<0.001$; ns, not significant (Student's *t*-test)). (E) Dose response curve for dexamethasone repression of NF-κB and AP-1 activity by the GR_{wt} and GR_{R488Q} mutant, respectively. GR_{wt} and GR_{R488Q} mutant containing HEK293 cells were transiently transfected with 3x(NF-κB)tk-Luc or 5x(AP1)-ALP reporter genes as in "B" and "C" and treated with increasing concentration of dexamethasone in the presence of 5 ng/ml TPA. Error bars denote SD (n=3). (F) The R488Q mutation impairs the receptor's ability to transactivate. HEK293 clones were transiently transfected as described in Fig. 1 using 2x(GRE)tk-Luc reporter plasmid and CMV-ALP plasmid followed by treatment with vehicle or 100 nM dexamethasone for 20 h. Data presented are average of three independent experiments, each performed in triplicate. Error bars denote SD.

FIG. 3. **The separation of NF-κB and AP-1 repression by the GR_{R488Q} mutant is maintained on reporter genes regulated by natural promoters.** (A) and (B) GR_{R488Q} mutant fails to repress a NF-κB dependent reporter gene, ICAM1-Luc and Cox2-Luc, but maintains its ability to repress an AP-1 dependent gene reporter, MMP1-Luc. HEK293 clones, parental cells (-), wild type GR expressing cells (GRwt) and GR_{R488Q} mutant expressing cells (R488Q), were transiently transfected with reporter genes and treated as

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described in Fig. 1 using ICAM1-Luc or Cox2-Luc reporter gene (A) and MMP1-Luc reporter gene (B) and internal control plasmid, CMV-ALP. Treatment: vehicle (vertical striped bars), dexamethasone (open bars), TPA (black bars) and TPA + dexamethasone (grey bars). TPA stimulation in each experiment was given the nominal value of 100 and the results for the other conditions were expressed relative to this nominal value. Data represent mean ± SD. Each experiment was performed in triplicate and repeated three times. The stars indicate a significant inhibitory effect by TPA + dexamethasone relative to TPA alone (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant (Student's *t*-test)).

FIG. 4. **In contrast to the GR_{R488Q} mutant, the transactivation deficient GR DBD mutant, LS7, represses NF-κB activity.** HEK293 cells were transiently transfected with GR expression plasmid, reporter gene plasmid regulated by NF-κB (3x(NF-κB)tk-Luc) or AP-1 (MMP1-Luc) and the internal control vector CMV-ALP using the same protocol and conditions as described in Fig. 1. Treatment: vehicle (vertical striped bars), TPA (black bars) and TPA + dexamethasone (grey bars). TPA stimulation in each experiment was given the nominal value of 100 and control and TPA + dexamethasone results were expressed relative to this nominal value. Values are mean ± SD. Each experiments was performed in triplicates and repeated two times. The stars indicate a significant inhibitory effect by TPA + dexamethasone relative to TPA alone (**, $p<0.01$; ***, $p<0.001$; ns, not significant (Student's *t*-test)).

FIG. 5. **Physical interaction of GR_{R488Q} with p65 *in vivo*.** Extracts from parental HEK293 cells (-) and cell clones stably expressing the GR_{wt} and GR_{R488Q}, respectively, treated with TPA + dexamethasone, were analyzed by immunoprecipitation with an anti-GR antibody, followed by immunoblotting using an anti-GR (A) or anti-p65 (B) antibody as

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described in Materials and Methods. "Input" shows the amount of GR (A) and p65 (B) present in the extracts prior to immunoprecipitation whereas the "Eluate" shows the amount of GR (A) and p65 (B) bound to the anti-GR antibody following immunoprecipitation. (C) shows that GR_{wt} and GR_{R488Q} interaction with p65 is only detected when cells have been treated with TPA. Upper panel shows the p65 input and the lower panel the p65 eluate following immunoprecipitation with the anti-GR antibody. -, vehicle treated cells; D, dexamethasone and D/T, dexamethasone and TPA treated cells. Cells were treated with 100 nM dexamethasone and 5 ng/ml TPA for 30 min at 37^0 C.

FIG. 6. **Dexamethasone induced repression of endogenous genes stimulated by TPA in the presence of GR_{wt} and GR_{R488Q} mutant.** HEK293 clones were treated and subjected to microarray analysis as described in Materials and Methods. Significantly repressed genes by the GR_{wt} were extracted from the array data, i.e. genes displaying a mean repression >30% ($p<0.01$) following dexamethasone treatment based on three independent experiments. Correlation plots, GR_{R488Q} versus GR_{wt}, of the identified genes, at 2 h and 8 h respectively, are presented where filled circles denote discriminating genes and open circles denote non-discriminating genes. (A) In total 39 genes were significantly repressed at 2 h by the GR_{wt} but only 10 of those genes were also repressed by the GR_{R488Q} mutant to a similar extent or within 50% of the efficacy of GR_{wt}. (B) At 8 h a total of 109 genes were significantly repressed by the GR_{wt} and 81 of those genes were also repressed by the GR_{R488Q} mutant to a similar extent or within 50% of the efficacy of GR_{wt}. Data presented are average of three independent experiments. The line represents the limit for genes repressed by the GR_{R488Q} mutant less than half of what is observed for the GR_{wt}. Note that the open circles below the line correspond to genes that have a *p*-value >0.01 for change in regulation by treatment between GR_{wt} and GR_{R488Q} mutant, thus these genes scored as non-discriminating.

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FIG. 7. **Repression of gene expression in GR_{wt} and GR_{R488Q} mutant cells as determined by qRT-PCR analysis.** HEK293 clones stably expressing the wild type GR (GRwt) and the GR_{R488Q} mutant (R488Q) were treated with TPA and TPA + dexamethasone for 2 h or 8 h as indicated in the figure, followed by isolation of total RNA, cDNA preparation and qRT-PCR. MMP-1, Activin A, GADD45B, Cox-2, JunB and IL-6R expression was determined and normalized to GAPDH expression. Bars represent percent dexamethasone dependent repression of TPA stimulated gene expression. Mean ± SD from three independent experiments are shown. The stars indicate a significant inhibitory effect by TPA + dexamethasone relative to TPA alone (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant (Student's *t*-test)).

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Table 1 Reduced gene transcription following TPA+dexamethasone treatment compared to TPA alone (2 h)

Genebank Accession no.	Probe ID	Gene Name	% repression WT	% repression R488Q		Genebank Accession no.	Probe ID	Gene Name	% repression WT	% repression R488Q	
Apoptosis						U90304	210239_at	IRX5	40	-13	●
NM_003897	201631_s_at	IER3	71	15	●	**NM_002229**	**201473_at**	**JUNB**	**70**	**31**	●
NM_016639	218368_s_at	TNFRSF12A	37	1	●	NM_005384	203574_at	NFIL3	46	23	○
						S77154	216248_s_at	NR4A2	57	0	●
Cell growth/Cell cycle						NM_003107	201417_at	SOX4	31	12	○
AK023795	222162_s_at	ADAMTS1	55	-26	●	BF343007	204653_at	TFAP2A	47	21	●
NM_001554	201289_at	CYR61	52	-15	●	NM_005655	202393_s_at	TIEG	44	-3	●
NM_002010	206404_at	FGF9	58	23	●						
BC004490	209189_at	FOS	40	7	●	**Stress response**					
NM_006732	202768_at	FOSB	87	38	●	NM_001924	203725_at	GADD45A	38	24	○
NM_005542	201625_s_at	INSIG1	37	-3	●	**NM_015675**	**207574_s_at**	**GADD45B**	**48**	**38**	○
BE327172	213281_at	JUN	41	17	●	NM_005904	204790_at	MADH7	62	19	●
M24779	209193_at	PIM1	32	-15	●						
						Other genes					
Inflammation/Immune response						AF127481	209535_s_at	AKAP13	41	-15	●
NM_000963	**204748_at**	**COX-2**	**45**	**15**	●	NM_016201	203002_at	AMOTL2	33	13	○
NM_005261	204472_at	GEM	79	41	○	NM_025195	202241_at	C8FW	65	-6	●
						NM_004907	202081_at	ETR101	45	10	●
Regulation of transcription						NM_005242	206429_at	F2RL1	35	1	●
NM_001186	204194_at	BACH1	46	25	○	NM_001450	202949_s_at	FHL2	42	16	○
NM_003670	201170_s_at	BHLHB2	69	13	●	U41813	214651_s_at	HOXA9	42	17	●
NM_004405	207147_at	DLX2	36	18	○	NM_000527	202068_s_at	LDLR	52	15	●
NM_004430	206115_at	EGR3	60	17	●	NM_014575	204030_s_at	SCHIP1	37	6	●
NM_001427	207060_at	EN2	39	4	●	AL574096	209277_at	TFPI2	64	29	●
NM_004496	204667_at	FOXA1	44	5	○	NM_020127	205807_s_at	TUFT1	31	8	●

○ No discrimination; ● Discrimination. Boldface genes were validated by Real-Time PCR. Negative values means transcriptional activation. Some genes scored as non-discriminating, although repression by the GRR488Q is less than 50% as compared to the repression by the GRwt, due to not fulfilling the statistical requirement ($P<0.01$)

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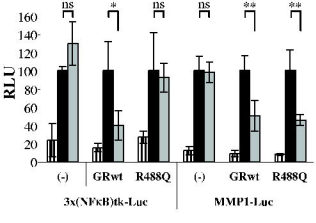
Table 2 Reduced gene transcription following TPA+dexamethasone treatment compared to TPA alone (8 h)

Genebank Accession no.	Probe ID	Gene Name	% repression WT	% repression R488Q	
	Apoptosis				
U83981	37028_at	PPP1R15A	57	48	○
NM_002575	204614_at	SERPINB2	94	32	●
NM_016639	218368_s_at	TNFRSF12A	39	21	○
	Cell growth/Cell cycle				
M13436	**210511_s_at**	**Activin A**	**69**	**80**	○
AK023795	222162_s_at	ADAMTS1	64	-34	●
NM_001657	205239_at	AREG	68	52	○
NM_001718	206176_at	BMP6	32	8	○
AI650819	202213_s_at	CUL4B	30	-1	○
M60278	38037_at	DTR	35	27	○
NM_001423	201324_at	EMP1	70	-85	●
NM_000127	201995_at	EXT1	53	56	○
NM_013394	208240_s_at	FGF1	33	13	○
X59065	205117_at	FGF1	31	22	○
NM_002010	206404_at	FGF9	65	50	○
NM_000875	203627_at	IGF1R	43	43	○
NM_002189	207375_s_at	IL15RA	33	21	○
BE620457	212298_at	NRP1	41	7	○
M24779	209193_at	PIM1	45	32	○
NM_002826	201482_at	QSCN6	31	10	○
NM_019845	219370_at	REPRIMO	35	-24	●
	Inflammation/Immune response				
NM_001627	201952_at	ALCAM	31	13	○
NM_000700	201012_at	ANXA1	40	-59	●
NM_006273	208075_s_at	CCL7	31	16	○
NM_005238	214447_at	ETS1	35	11	○
NM_005261	204472_at	GEM	82	82	○
NM_000882	207160_at	IL12A	31	32	○
NM_000418	203233_at	IL4R	35	29	○
NM_000565	**205945_at**	**IL6R**	**58**	**9**	●
M74447	204769_s_at	TAP2	37	7	●
BE568134	214581_x_at	TNFRSF21	39	35	○
	Regulation of transcription				
NM_001186	204194_at	BACH1	37	46	○
NM_003670	201170_s_at	BHLHB2	72	59	○
AF109161	209357_at	CITED2	60	35	○
NM_001878	202575_at	CRABP2	50	27	○
NM_001427	207060_at	EN2	53	46	○
AF044263	35265_at	FXR2	31	20	○
R61374	44783_s_at	HEY1	36	-17	●
NM_002114	204512_at	HIVEP1	43	36	○
NM_018951	213150_at	HOXA10	48	29	○
NM_019102	213844_at	HOXA5	39	13	●
NM_001546	209291_at	ID4	62	34	○
U90304	210239_at	IRX5	54	37	○
NM_002229	201473_at	JUNB	53	44	○
AF288571	221558_s_at	LEF1	41	27	○
NM_000381	203637_s_at	MID1	66	34	○
S77154	216248_s_at	NR4A2	65	10	●
NM_002971	203408_s_at	SATB1	36	18	○
BF343007	204653_at	TFAP2A	75	71	○
	Stress response				
NM_005904	204790_at	MADH7	58	44	○
NM_003330	201266_at	TXNRD1	39	32	○

Genebank Accession no.	Probe ID	Gene Name	% repression WT	% repression R488Q	
	Other genes				
AF016535	209994_s_at	ABCB1	45	34	○
AF241787	221641_s_at	ACATE2	32	-5	●
NM_001105	203935_at	ACVR1	35	23	○
NM_016201	203002_at	AMOTL2	66	49	○
L14561	215716_s_at	ATP2B1	40	46	○
NM_025195	202241_at	C8FW	54	20	●
M36532	209301_at	CA2	43	33	○
NM_004056	220234_at	CA8	33	21	○
M24915	204490_s_at	CD44	61	17	●
BE903880	212063_at	CD44	52	-18	●
NM_000781	204309_at	CYP11A1	30	-1	●
NM_000574	201925_s_at	DAF	71	57	○
NM_004734	205399_at	DCAMKL1	34	17	○
NM_006465	218964_at	DRIL2	49	44	○
NM_004415	200606_at	DSP	54	12	●
NM_014501	202779_s_at	E2-EPF	31	6	○
BF001670	202668_at	EFNB2	60	6	●
NM_004431	203499_at	EPHA2	49	18	●
NM_001983	203719_at	ERCC1	34	19	○
NM_005242	206429_at	F2RL1	71	51	○
NM_001450	202949_s_at	FHL2	54	43	○
NM_014286	218266_s_at	FREQ	30	30	○
NM_003902	203091_at	FUBP1	32	20	○
BF063271	203397_s_at	GALNT3	36	26	○
NM_000169	214430_at	GLA	35	12	○
NM_005328	206432_at	HAS2	36	-1	●
BG035985	221750_at	HMGCS1	58	51	○
NM_006042	219985_at	HS3ST3B1	31	30	○
U77914	216268_s_at	JAG1	54	12	●
NM_000216	205206_at	KAL1	38	5	●
NM_002245	204679_at	KCNK1	55	44	○
NM_002293	200771_at	LAMC1	36	4	●
NM_012302	206953_s_at	LPHN2	32	12	○
NM_005167	200885_at	MGC19531	31	15	○
NM_022443	204783_at	MLF1	39	21	○
NM_002425	205680_at	MMP10	86	57	○
NM_000270	201695_s_at	NP	32	18	○
U53823	209925_at	OCLN	37	23	○
NM_002844	203038_at	PTPRK	45	4	●
BE789881	217762_s_at	RAB31	60	40	○
AL514445	204337_at	RGS4	59	39	○
BF059159	213194_at	ROBO1	30	24	○
AA906056	203843_at	RPS6KA3	48	31	○
NM_014575	204030_s_at	SCHIP1	60	21	●
NM_002640	206034_at	SERPINB8	42	24	○
NM_003896	203217_s_at	SIAT9	49	31	○
NM_004595	202043_s_at	SMS	31	-16	●
NM_021972	219257_s_at	SPHK1	35	47	○
NM_003122	206239_s_at	SPINK1	44	-5	●
NM_005842	204011_at	SPRY2	33	-3	○
BE966922	209238_at	STX3A	36	23	○
BC002616	210978_s_at	TAGLN2	37	12	○
J03225	209676_at	TFPI	37	26	○
AL574096	209277_at	TFPI2	48	50	○
NM_004817	202085_at	TJP2	69	48	○
NM_003364	203234_at	UP	61	19	●
AA824386	201099_at	USP9X	43	19	●
	Unknown				
NM_005491	205088_at	CXorf6	41	30	○
AI143879	205501_at		59	36	○

○ No discrimination; ● Discrimination. Boldface genes were validated by Real-Time PCR. Negative values means transcriptional activation. Some genes scored as non-discriminating, although repression by the GRR488Q is less than 50% as compared to the repression by the GRwt, due to not fulfilling the statistical requirement ($P<0.01$)


ns
*
ns
ns
**
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160
140
120
100
80
60
40
20
0
RLU
(-)
GRwt
R488Q
(-)
GRwt
R488Q
3x(NFκB)tk-Luc
MMP1-Luc

Fig. 1

Fig. 2

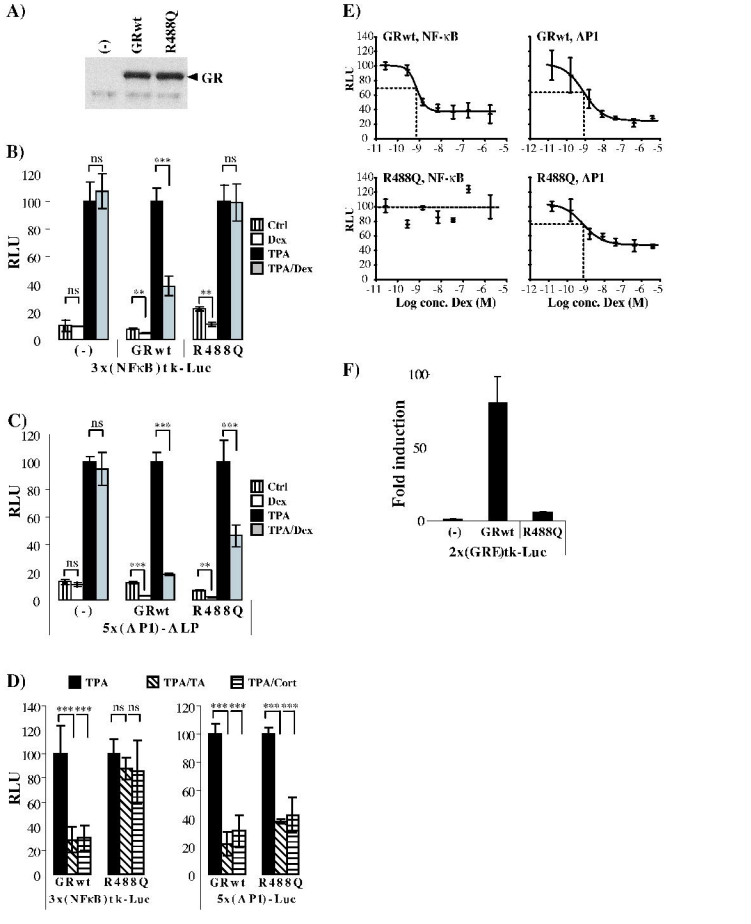
A)
(-)
GRwt
R488Q
GR
B)
RLU
Ctrl
Dex
TPA
TPA/Dex
ns
**
***
(-)
GRwt
R488Q
3x(NFκB)tk-Luc
C)
RLU
Ctrl
Dex
TPA
TPA/Dex
5x(AP1)-ALP
D)
TPA
TPA/TA
TPA/Cort
RLU
GRwt
R488Q
3x(NFκB)tk-Luc
5x(AP1)-Luc
E)
GRwt, NF-κB
GRwt, AP1
R488Q, NF-κB
R488Q, AP1
RLU
Log conc. Dex (M)
F)
Fold induction
(-)
GRwt
R488Q
2x(GRE)tk-Luc

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Fig. 3

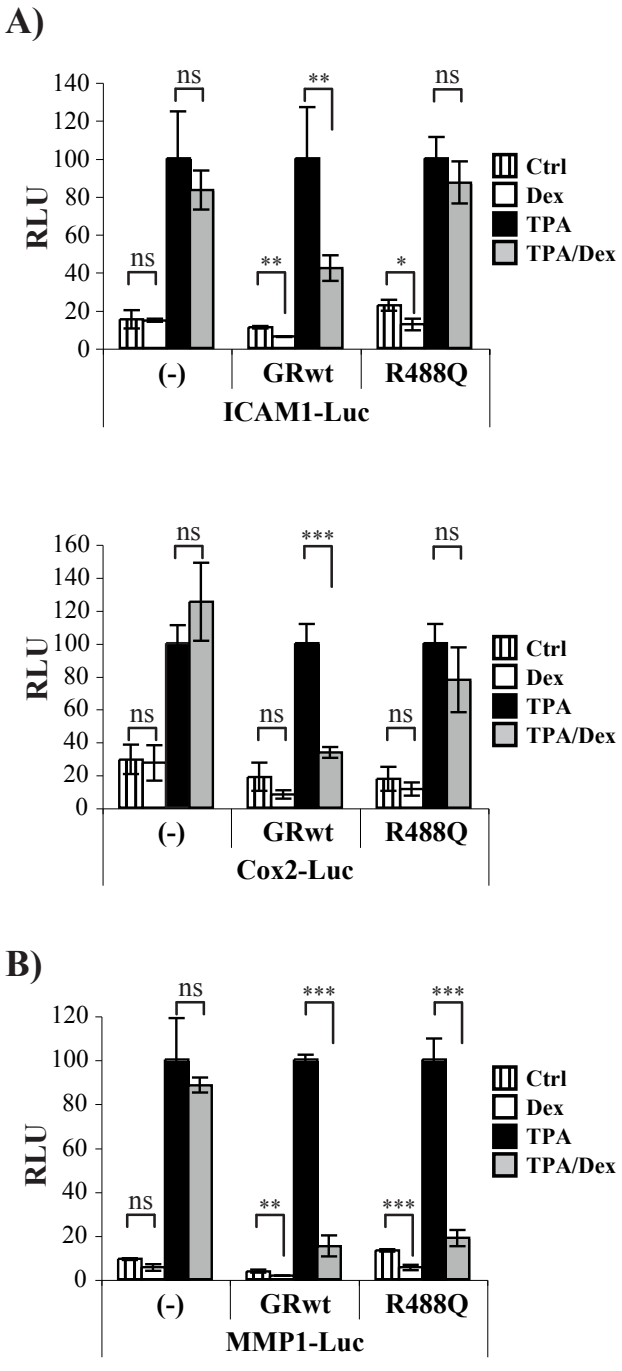
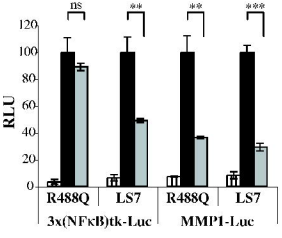


Fig. 4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**
***
120
100
80
60
40
20
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RLU
R488Q
LS7
R488Q
LS7
3x(NFκB)tk-Luc
MMP1-Luc

Fig. 5

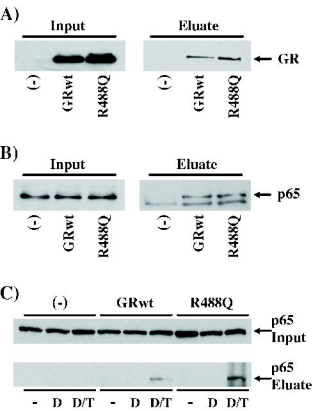
A)
Input
Eluate
GR
(-)
GRwt
R488Q
(-)
GRwt
R488Q
B)
Input
Eluate
p65
(-)
GRwt
R488Q
(-)
GRwt
R488Q
C)
(-)
GRwt
R488Q
p65
Input
p65
Eluate
-
D
D/T
-
D
D/T
-
D
D/T

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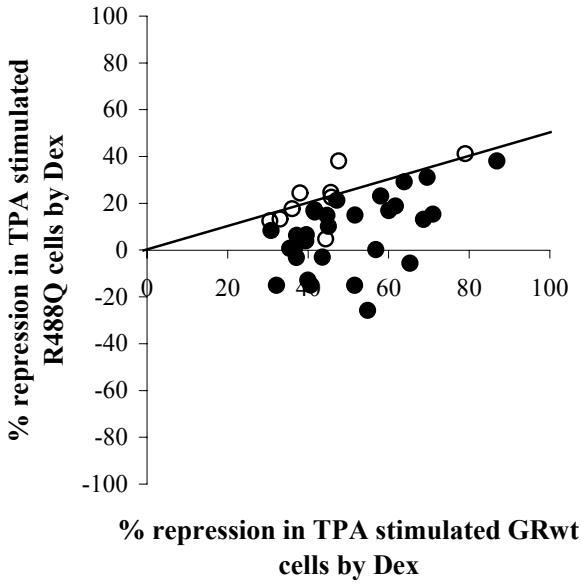
Fig. 6

A)



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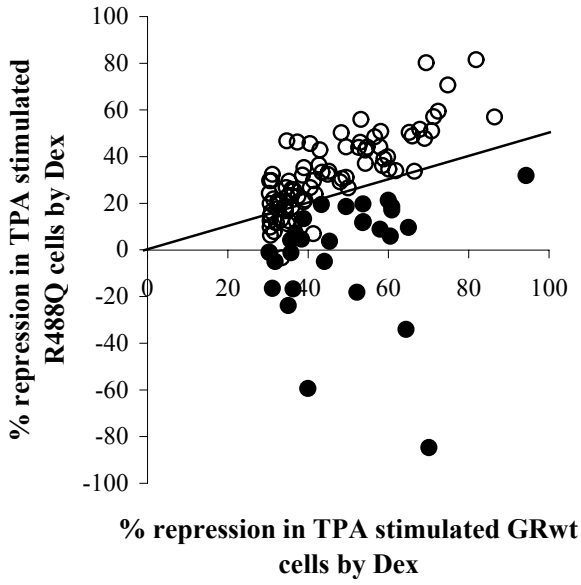
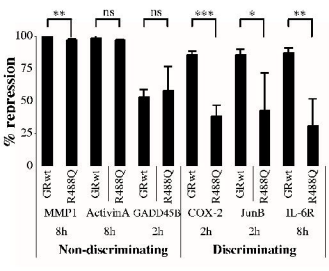


Fig. 7


**
ns
ns
***
*
**
% repression
100
75
50
25
0
GRwt
R488Q
MMP1
ActivinA
GADD45B
COX-2
JunB
IL-6R
8h
8h
2h
2h
2h
8h
Non-discriminating
Discriminating