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Identification of Endogenous Glucocorticoid Repressed Genes Differentially Regulated by a Glucocorticoid Receptor Mutant Able to Separate between Nuclear Factor-κB and Activator Protein-1 Repression

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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, whereas 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. It is interesting that the GR_{R488Q} still interacted physically with NF-κB. Gene expression profiling of human embryonic kidney 293 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.

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 that function as ligand-dependent transcription factors. Like other nuclear receptors, the GR contains three main functional domains: a C-terminal ligand binding domain, 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, and displacement of positively acting transcription factors. A second mechanism is mediated via a

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ABBREVIATIONS: GR, glucocorticoid receptor; DBD, DNA binding domain; GRE, glucocorticoid response element; AP-1, activator protein-1; NF-κB, nuclear factor-κB; Luc, luciferase; TA, triamcinolone acetonide; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; qRT-PCR, quantitative real-time-polymerase chain reaction; ALP, alkaline phosphatase; HEK, human embryonic kidney; IL-6R, interleukin-6 receptor; ANOVA, analysis of variance; MMP-1, matrix metalloproteinase-1 (collagenase-1); ICAM-1, intercellular adhesion molecule-1; Cox-2, cyclooxygenase-2.

direct physical interaction between the GR and other transcription factors, a process that 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-1 (AP-1) and nuclear factor-κB (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). Whereas direct DNA binding of the GR is not involved in tethering, the DBD of the GR still seems to participate in this mechanism (Jonat et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990; Heck et al., 1994; Lidén et al., 1997). The tethering mechanism seems to be physiologically important for the anti-inflammatory responses of glucocorticoids, because 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) (Karin and Chang, 2001; Shaulian and Karin, 2001; Ghosh and Karin, 2002; Karin et al., 2002). 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 nonactivated state, NF-κB resides in the cytoplasm bound to inhibitory proteins, IκBs. Upon exposure of the cells to cytokines, oxidative stress, phorbol ester, or UV irradiation, to cite only a few examples, 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, whereas 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, because 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 uses 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.

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-Aldrich (St. Louis, MO). The culture media Dulbecco's modified Eagle's medium (high glucose) and F12 (Ham's), penicillin/streptomycin, Zeocin, hygromycin, L-glutamine, and lipofectin reagent were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Integra B.V. (Lekystad, The Netherlands), and the chemiluminescence reagents used for measurement of alkaline phosphatase and luciferase activity were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA) and BioThema (Haninge, Sweden), respectively. Primers were obtained from DNA Technology A/S (Aarhus, Denmark), and all reagents used for quantitative real-time-polymerase chain reaction (qRT-PCR) were purchased from Applied Biosystems (Foster City, CA).

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 been described previously (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 (Institute of Toxicology and Genetics, Forschungszentrum Karlsruhe Branch, Eggenstein, 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 (Brüggemeier 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 lines, the cDNAs encoding the rat wild-type GR and GR mutant were subcloned into the pcDNA5/FRT expression vector obtained from Invitrogen.

Cell Culture and Generation of Stable Cell Lines. CV-1 cells and HEK293 Flp-In cells (Invitrogen) were grown at 37°C in 5% CO_2 in a 1:1 mixture of high-glucose Dulbecco's modified Eagle's medium and F12 (Ham's) containing 10% fetal bovine serum, 10 IU/ml/100 μg/ml penicillin/streptomycin, respectively, and 2 mM L-glutamine. Nontransfected 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 100 μg/ml hygromycin. 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. 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 because it does not contain functional endogenously expressed GR. In line with this, similar expression levels of stably transfected GRs 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 and 200 ng/well, respectively. The plasmids CMV-ALP and RSV-Luc were used as internal controls at a concentration of 1 and

10 ng/well, respectively. In brief, 30,000 cells/well were seeded in 24-well plates 24 h before transfection. Twenty hours after 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 the cell medium was collected and the 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 buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 5 mM EDTA) for 20 min. Cell debris was removed by centrifugation at 14,000*g* for 15 min at 4°C, and an equal volume of 2× SDS loading buffer was added to the supernatant and the mixture was boiled for 2 min. Protein concentrations were determined with the Bio-Rad protein assay kit according to the instructions from the manufacturer (Bio-Rad, Hercules, CA). Samples were separated by 9% SDS-polyacrylamide gel electrophoresis and electroblotted onto a Hybond C-extra membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, 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). GR immunoreactivity was visualized using the enhanced chemiluminescence kit (Amersham Biosciences) 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°C. The cells were freeze-thawed twice in ice-cold EPG buffer (1 mM EDTA, 20 mM $NaPO_4$, pH 7.4, 10% glycerol, and 2 mM mercaptoethanol) containing 400 mM NaCl, homogenized, and cell debris was removed by centrifugation at 14,000*g* for 10 min at 4°C. The extract was incubated with an anti-GR antibody (Okret et al., 1984) for 4 h at 4°C and subsequently the extract-antibody mixture was incubated with protein A-Sepharose beads (Amersham Biosciences) 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× SDS buffer and subjected to SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using an anti-GR antibody (Okret et al., 1984) and an anti-p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). An aliquot of the extract-antibody mixture (i.e., before 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, Santa Clara, CA) was used. This array represents approximately 8500 well annotated human transcripts from the National Center for Biotechnology Information 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) followed by RNA quality assessment using the Nano 6000 Chip in the Bioanalyzer from Agilent Inc. Eight micrograms of total RNA was used for target cDNA synthesis according to the Affymetrix manual. The raw intensity data were normalized and gene expression levels were estimated using the robust multichip analysis (Irizarry et al., 2003). An initial four-way analysis of variance (ANOVA) 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 effecting parameter. Therefore, for further statistical analysis, a two-way ANOVA was performed, testing only GR type, treatment, and relation between the two, for 2 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}, and 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.

qRT-PCR Analysis. For validation of array results, cDNA was prepared from the three independent experiments, using 1 μg of total RNA, random hexamer primers, and Superscript II (Invitrogen). The expression of specific mRNAs was quantified by qRT-PCR, normalized to GAPDH expression, using SYBR Green Master Mix (Applied Biosystems) and ABI Prism 7700 sequence detection system. The primers used for PCR analysis are shown in Table 1.

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 (Schüle et al., 1990; Yang-Yen et al., 1990; Lidén et al., 1997). 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 cotransfected 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 upstream 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

TABLE 1
Primers

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′

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.

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 immuno-blotting using a GR-specific monoclonal antibody. Both isolated individual clones and clone mixes were screened, all showing similar GR expression levels (Fig. 2A; 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. 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), whereas its inhibitory effect on AP-1 signaling was preserved (Fig. 2C). The same discriminating effect was seen when cells were treated with cortisol or 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 to 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, whereas 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

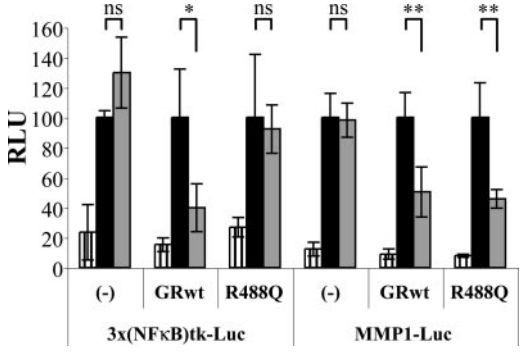

Fig. 1. The GR_{R488Q} mutation reduces the receptor's ability to repress NF-κB but not AP-1 activity in transiently transfected CV-1 cells. CV-1 cells were transiently transfected with GR expression plasmid, reporter gene plasmid regulated by NF-κB [3x(NF-κB)tk-Luc] or AP-1 (MMP1-Luc) and the internal control vector CMV-ALP. Twenty hours after transfection, cells were exposed to vehicle (vertical striped columns), 5 ng/ml TPA (black columns), or 5 ng/ml TPA + 100 nM dexamethasone (gray columns) 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 ± S.D. Each experiment was performed in triplicate and repeated three times. The asterisks 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).

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 nonstimulated cells.

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 whether 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 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 GRE-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 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 shown previously (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. Because 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 coimmunoprecipitation. Ex-

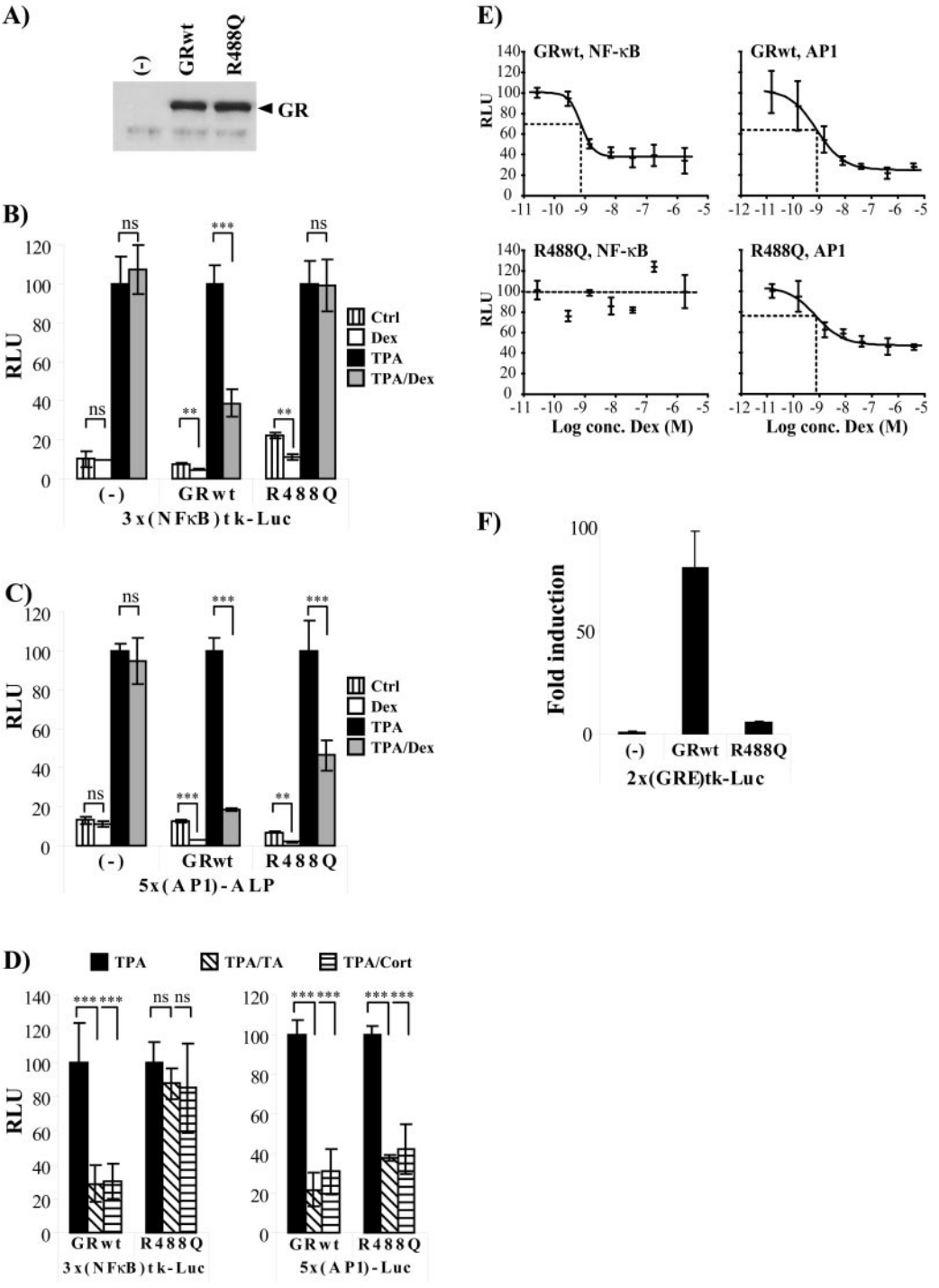

Fig. 2. The GR_{R488Q} mutant fails to repress NF-κB but not AP-1 activity in stably transfected HEK293 cells. A, similar expression levels of wild-type GR and R488Q mutant in stably transfected HEK293 cells. GR expression levels in parental (−), wild-type GR (GRwt), and GR_{R488Q} mutant (R488Q) clones 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 columns), dexamethasone (open columns), TPA (black columns), and TPA + dexamethasone (gray columns). 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 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 TA (diagonal striped columns) and cortisol (horizontal striped columns) to repress TPA-stimulated 3x(NF-κB)tk-Luc expression via the GR_{wt} or GR_{R488Q} mutant relative to TPA treatment alone (black columns). Data in B to D represent mean ± S.D. Each experiment was performed in triplicate and repeated three times. The asterisks indicate a significant inhibitory effect by TPA + dexamethasone (cortisol or TA) relative to TPA alone or dexamethasone treatment relative vehicle treatment (**, $p<0.01$; ***, $p < 0.001$; ns, not significant by 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, 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 S.D.

tracts 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, whereas p65 was present in equal amounts in both parental HEK293 cells and cells expressing the GRs (Fig. 5, A and B, Input). Precipitation using a monoclonal anti-GR antibody followed by immunoblotting for GR and p65, respectively (Fig. 5, A and B, Eluate) showed that the p65 protein was coimmunoprecipitated in both GR_{wt} and GR_{R488Q} mutant cells but not in parental cells. This demonstrated that the GR physically

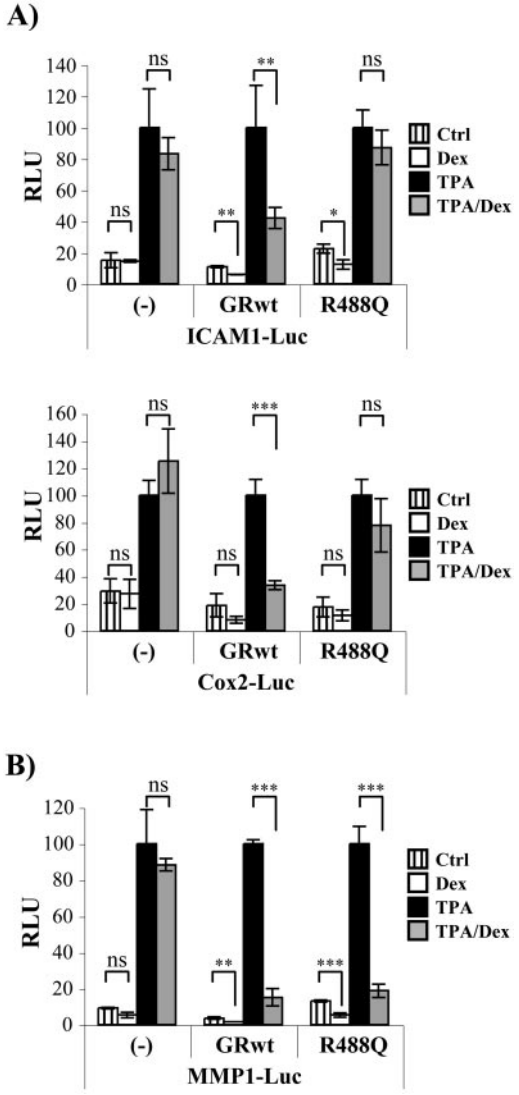

Fig. 3. Separation of NF-κB and AP-1 repression by the GR_{R488Q} mutant is maintained on reporter genes regulated by natural promoters. A and B, GR_{R488Q} mutant fails to repress a NF-κB-dependent reporter gene, ICAM1-Luc and Cox2-Luc, but maintains its ability to repress an AP-1-dependent gene reporter, 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 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 columns), dexamethasone (open columns), TPA (black columns), and TPA + dexamethasone (gray columns). 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 ± S.D. 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 by Student's t test).

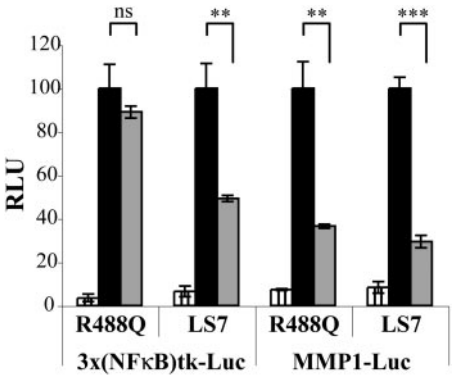

Fig. 4. In contrast to the GR_{R488Q} mutant, the transactivation deficient GR DBD mutant, LS7, represses NF-κB activity. HEK293 cells were transiently transfected with GR expression plasmid, reporter gene plasmid regulated by NF-κB [3x(NF-κB)tk-Luc] or AP-1 (MMP1-Luc), and the internal control vector CMV-ALP using the same protocol and conditions as described in Fig. 1. Treatment: vehicle (vertical striped columns), TPA (black columns), and TPA + dexamethasone (gray columns). 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 ± S.D. Each experiment 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 by Student's t test).

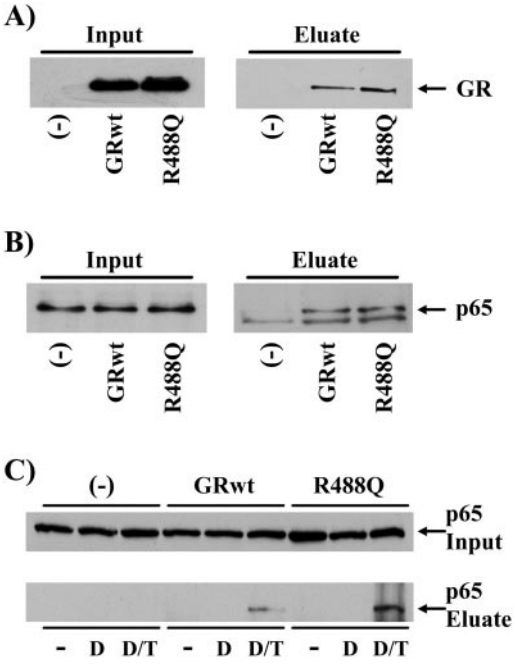

Fig. 5. Physical interaction of GR_{R488Q} with p65 in vivo. Extracts from parental HEK293 cells (−) and cell clones stably expressing the GR_{wt} and GR_{R488Q}, respectively, treated with TPA + dexamethasone were analyzed by immunoprecipitation with an anti-GR antibody, followed by immunoblotting using an anti-GR (A) or anti-p65 (B) antibody as described under *Materials and Methods*. Input shows the amount of GR (A) and p65 (B) present in the extracts before immunoprecipitation, whereas the Eluate shows the amount of GR (A) and p65 (B) bound to the anti-GR antibody after immunoprecipitation. C shows that GR_{wt} and GR_{R488Q} interaction with p65 is only detected when cells have been treated with dexamethasone + TPA. p65 input (top) and p65 eluate (bottom) after immunoprecipitation with the anti-GR antibody. −, vehicle-treated cells; D, dexamethasone; D/T, dexamethasone and TPA-treated cells. Cells were treated with 100 nM dexamethasone and 5 ng/ml TPA for 30 min at 37°C.

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 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. Together, the transfection data strongly supported that the GR_{R488Q} mutant was unable to repress NF-κB activity, whereas 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 and 8 h, respectively. The experiment was repeated three times at separate days. Thereafter, gene expression levels were analyzed using the human genome focus array (Affymetrix) detecting approximately 8500 transcripts. This analysis demonstrated that several endogenous genes were differently repressed by GR_{R488Q} compared with 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; Table 2). 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; Table 3). As revealed in Table 2, 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 3).

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 extracted microarray data using the criteria set as one of the down-regulated genes with nondiscriminating 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 versus 26%, no significant difference) after 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 because of 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 nondiscriminating genes, Activin A, GADD45B, and MMP-1 (see above). Expression analysis of the nondiscriminating 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} mutants 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 because of a very low standard deviation.) In contrast, the GR_{wt} extensively repressed the discriminating

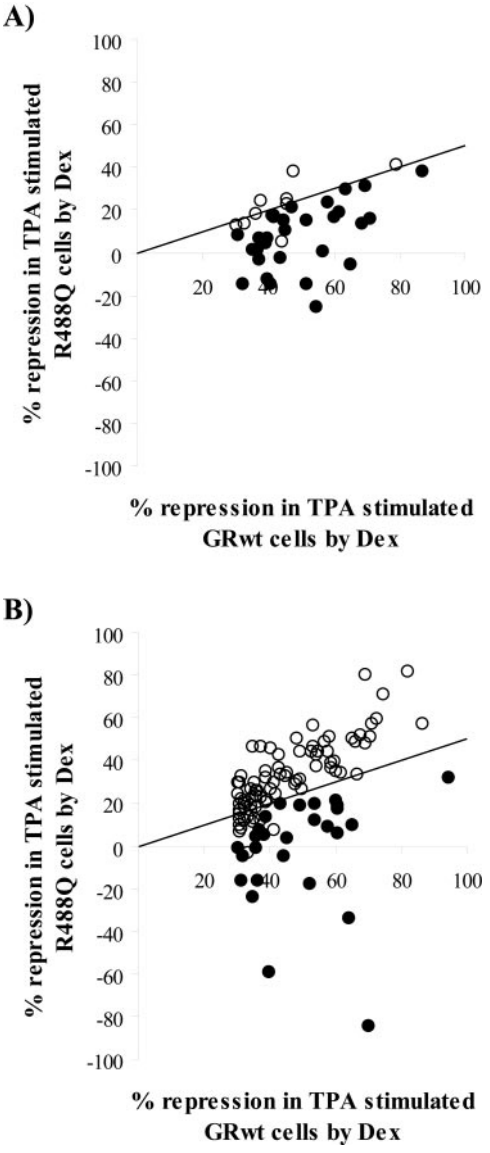

Fig. 6. Dexamethasone induced repression of endogenous genes stimulated by TPA in the presence of GR_{wt} and GR_{R488Q} mutant. HEK293 clones were treated and subjected to microarray analysis as described under *Materials and Methods*. Significantly repressed genes by the GR_{wt} were extracted from the array data [i.e., genes displaying a mean repression >30% ($p < 0.01$)] after dexamethasone treatment based on three independent experiments. Correlation plots, GR_{R488Q} versus GR_{wt}, of the identified genes, at 2 and 8 h, respectively, are presented where filled circles denote discriminating genes and open circles denote nondiscriminating 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, 109 genes in total 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 nondiscriminating.

genes Cox-2, JunB, and IL-6R, whereas the GR_{R488Q} mutant did not repress transcription of these genes to the same extent (statistically 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 nonrepressed genes, although the sensitivity may be less compared with qRT-PCR.

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 (Jonat et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990; Caldenhoven et al., 1995; Lidén et al., 1997; Nissen and Yamamoto, 2000). 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 after stimulation, whereas 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 nonstimulated cells as seen in Figs. 2 and 3 may represent some residual NF-κB-repressing activity of GR_{R488Q} that is sufficient to repress minor amounts of NF-κB activity in nonstimulated cells but not sufficient to repress higher amounts NF-κB activity in stimulated cells.

It has been proposed that the mechanism of GR-mediated

TABLE 2
Reduced gene transcription after TPA + dexamethasone treatment compared with TPA alone (2 h)
Genes shown in bold were validated by real-time PCR. Negative values mean transcriptional activation.

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

○, no discrimination; ●, discrimination; WT, wild type. Some genes were scored as nondiscriminating, although repression by the GRR488Q was less than 50% compared with the repression by the GRwt, because they did not fulfill the statistical requirement ($p < 0.01$).

TABLE 3

Reduced gene transcription after TPA + dexamethasone treatment compared with TPA alone (8 h)

Genes shown in bold were validated by real-time PCR. Negative values mean transcriptional activation.

GenBank Accession no.	Probe ID	Gene Name	% Repression		
			WT	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	○
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	○

TABLE 3 *Continued*

GenBank Accession no.	Probe ID	Gene Name	% Repression		
			WT	R488Q	
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. Some genes were scored as nondiscriminating, although repression by the GRR488Q was less than 50% compared with the repression by the GRwt, because they did not fulfill the statistical requirement ($p < 0.01$).

inhibition of both NF-κB and AP-1 involves competition for a common factor (e.g., the cAMP response element binding protein binding protein 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). Because the GR_{R488Q} mutant still repressed AP-1 but not NF-κB activity, it is not likely 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 cAMP response element binding protein binding protein (De Bosscher et al., 2000; De Bosscher et al., 2001). Furthermore, this is in line with a recent report suggesting a coactivator independent repression of NF-κB by the GR (Wu et al., 2004), whereas 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, although 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. It is possible that 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 corepressor that interferes with serine-2 phosphorylation of the RNA polymerase II carboxyl-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 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, as with the GR_{R488Q} mutant, lacks the ability to repress a simple NF-κB-dependent reporter gene, whereas 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, because 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 cannot 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, whereas its ability

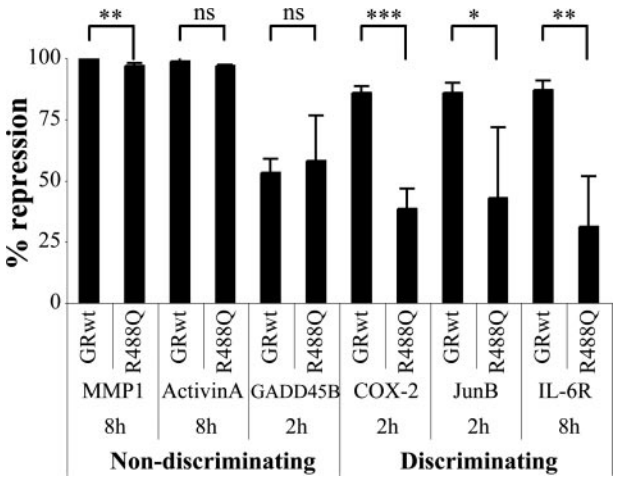

Fig. 7. Repression of gene expression in GR_{wt} and GR_{R488Q} mutant cells as determined by qRT-PCR analysis. HEK293 clones stably expressing the wild-type GR (GRwt) and the GR_{R488Q} mutant (R488Q) were treated with TPA and TPA + dexamethasone for 2 or 8 h as indicated in the figure, followed by 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. Columns represent percentage of dexamethasone-dependent repression of TPA-stimulated gene expression. Mean ± S.D. from three independent experiments are shown. The asterisks indicate a significant inhibitory effect by TPA + dexamethasone relative to TPA alone (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant by Student's t test).

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 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 (Newton et al., 1997; Schmedtje et al., 1997; Frazier-Jessen et al., 2002; McEvoy et al., 2002). 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), which have previously been shown to have a functional or a putative AP-1 site in a regulatory region of the gene (Tanimoto et al., 1996; Benbow and Brinckerhoff, 1997; Balliet et al., 2001). 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 array may not have detected regulation of endogenous ICAM-1 in HEK293 cells, despite their regulation in the transfection experiment (Fig. 3), as a result of 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). It is interesting that 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 compared with repression by the GR_{R488Q} mutant. At 8 h, several more genes were down-regulated by the GR_{wt} compared with 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, no obvious subpopulation of target genes for discriminated or nondiscriminated genes was revealed. Some genes repressed by the GR_{wt} at both 2 and 8 h scored as discriminated at 2 h 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, whereas at later times 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 use of the GR_{R488Q}

mutant, several of the down-regulated genes seem, to some extent, to be regulated in a NF-κB-dependent manner. Therefore, 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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