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Dissociated Glucocorticoids with Anti-Inflammatory Potential Repress Interleukin-6 Gene Expression by a Nuclear Factor-κB-Dependent Mechanism

WIM VANDEN BERGHE, ELISA FRANCESCONI, KAROLIEN DE BOSSCHER, MICHÈLE RESCHE-RIGON and GUY HAEGEMAN

Department of Molecular Biology (W.V.B., K.D.B., G.H.) University of Gent and Flanders Interuniversity Institute for Biotechnology, Gent, Belgium; and Hoechst Marion Roussel (E.F., M.R.-R.), Molecular and Cellular Biology, Bone Disease Group, Romainville Cedex, France

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ABSTRACT

Synthetic glucocorticoids (GCs) remain among the most effective agents for the management of chronic inflammatory diseases. However, major side effects severely limit their therapeutic use. Physiologic and therapeutic activities of GCs are mediated by a nuclear receptor belonging to a superfamily of ligand-inducible transcription factors that, in addition to directly regulating their cognate gene programs, can also mutually interfere with other signaling pathways. We recently identified selective ligands of the glucocorticoid receptor that dissociate transactivation from activator protein 1 transrepression, and most importantly retain in vivo anti-inflammatory activity. To further document the mechanisms of action sustaining the observed in vivo activity, we report here on the interference of dissociated GCs with nuclear factor κB (NF-κB)-driven gene activation. We show that dissociated GCs repress tumor necrosis factor-induced interleukin-6 gene expression by an NF-κB-dependent mechanism, without changing the expression level of inhibitor κB. The DNA-binding activity of induced NF-κB also remained unchanged after stimulation of cells with the various compounds. Evidence for a direct nuclear mechanism of action was obtained by analysis of cell lines constitutively expressing a fusion protein between the DNA-binding domain of the yeast Gal4 protein and the transactivating p65 subunit of NF-κB, which was able to efficiently repress a Gal4-dependent luciferase reporter gene upon addition of the dissociated compounds. We therefore conclude that, in addition to dissociating transactivation from activator protein 1 transrepression, dissociated GCs mediate inhibition of NF-κB signaling by a mechanism that is independent of inhibitor κB induction.

Physiologic and therapeutic activities of glucocorticoids (GCs) are mediated by a nuclear receptor (NR), which is a member of the family of ligand-inducible transcriptional regulators for steroids and thyroid hormones, as well as for retinoids and vitamin D3 (for overview, see Nuclear Receptors Nomenclature Committee, 1999). In addition to directly regulating gene expression via binding as homodimers to cognate response elements in target gene promoters, the glucocorticoid receptor (GR) can also mutually interfere with other signaling pathways such as those utilizing AP-1 or nuclear factor κB (NF-κB). The latter mechanism, referred to as transcriptional crosstalk, is generally not restricted to these two transcription factor families, but can also involve factors such as STAT5, C/EBP, or Oct-2A (Göttlicher et al., 1998; Resche-Rigon and Gronemeyer, 1998).

NF-κB is an inducible transcription factor complex that regulates the expression of various genes involved in inflammatory and immune responses. It is activated upon exposure of cells to proinflammatory cytokines [tumor necrosis factor (TNF), interleukin (IL)-1], oxidants (H_2O_2, ozone, superoxide anions), bacterial compounds (lipopolysaccharide), viral products (dsRNA, HTLV-1 Tax protein), protein kinase C activators (phorbol esters, platelet-activating factor), and UV- or γ-irradiation. In most cells, NF-κB is composed of a heterodimer of RelA (p65) and NF-κB1 (p50), where the RelA protein is responsible for the transactivation potential. In the nonactivated state, NF-κB is sequestered in the cytoplasm by

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ABBREVIATIONS: GC, glucocorticoid; DBD, DNA-binding domain; DEX, dexamethasone; HTC, hepatoma cell; I-κB, inhibitor κB; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; IL, interleukin; TRE, tumor-promoting agent-responsive element; GR, glucocorticoid receptor; NR, nuclear receptor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbant assay; ERK, extracellular signal receptor-activated kinase; MAPK, mitogen-activated protein kinase; AP-1, activator protein 1; GRE, glucocorticoid-responsive element; MMTV, mouse mammary tumor virus; ERK, extracellular signal-regulated kinase.

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interaction with the inhibitor κB (I-κB) protein I-κB-α. During activation, the I-κB-α protein becomes phosphorylated and degraded, allowing NF-κB to translocate to the nucleus, where it binds to specific DNA elements and subsequently activates transcription (Mercurio and Manning, 1999).

In this respect, NF-κB may be a promising target for anti-inflammatory and immunosuppressive therapies (Baeuerle and Baichwal, 1997). Inhibition of NF-κB activity by GC has been well documented, although stimulatory effects have also been observed (for a review see Dumont et al., 1998; McKay and Cidlowski, 1998). This inhibition has been proposed to result from direct interaction between GR and the p65 subunit of NF-κB (Ray and Prefontaine, 1994; Liden et al., 1997; Wissink et al., 1997). Alternatively, induction of I-κB-α synthesis by GC, and the subsequent sequestration of NF-κB, was suggested to be a nonexclusive mechanism (Auphan et al., 1995; Scheinman et al., 1995). However, we and others have recently shown that in many cell types I-κB-α protein levels do not increase, or even decrease after dexamethasone (DEX) treatment, which suggests that the observed effect of GC may be tissue- or cell-specific (De Bosscher et al., 1997; for review, see Dumont et al., 1998 and references included). Furthermore, it should be noted that elevated I-κB-α levels do not necessarily lead to dissociation of NF-κB from its binding site on DNA, nor repress the activity of NF-κB (Heck et al., 1997). Accordingly, GR mutants, in which point mutations in the GR DNA-binding domain (DBD) impaired DBD dimerization and DNA binding, and subsequently abolished GR transactivation, are unable to trigger synthesis of I-κB-α but retain their full ability to inhibit NF-κB activation (Heck et al., 1997; Liden et al., 1997).

Although GCs remain among the most potent immunosuppressive and anti-inflammatory drugs currently available, and are especially effective in the treatment of chronic asthma or rheumatoid arthritis, side effects such as hypothalamic-pituitary-adrenal axis insufficiency, diabetes, altered lipid metabolism, steroid myopathy, osteoporosis, and infectious and neuropsychiatric complications limit the therapeutic use of classical GC agonists (Boumpas, 1993). In an effort to investigate whether the separation of GC-dependent transactivation and transrepression would lead to the development of better tolerated drugs, we have identified a novel class of synthetic GCs that exerted strong AP-1 inhibition, whereas only weakly activating glucocorticoid-responsive element (GRE)-based reporter genes. In addition, these dissociated GCs were shown to inhibit IL-1β secretion and to display anti-inflammatory activity in vivo (Vayssière et al., 1997). In this article, we demonstrate that these compounds are able to inhibit TNF-induced IL-6 secretion in murine fibroblasts and HeLa cells. In addition, these compounds are able to interfere with NF-κB-dependent gene activation. Using a fusion protein between the DBD of the yeast Gal4 and the transactivating p65 subunit of NF-κB stably transfected in murine L929 cells, we further demonstrate that the synthetic GCs inhibit the expression of a Gal4-dependent luciferase reporter gene by a direct nuclear mechanism of action. Finally, we provide evidence that inhibition of NF-κB activity by dissociated GC occurs independently of I-κB-α up-regulation.

Materials and Methods

Cell Culture, Cytokines, and Inhibitors. Murine fibrosarcoma L929sA cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Twenty-four hours before induction, cells were seeded in multiwell dishes such that they were (sub)confluent at the time of the experiment. HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and grown at 37°C and 5% CO_2.

Cytokines and Inducing Compounds. Recombinant murine TNF, produced in *Escherichia coli* and purified to at least 99% homogeneity in our laboratory, had a specific activity of 1.95×10^8 IU/mg protein, as determined in a standardized TNF cytolysis assay on 164 WEHI cl 13 cells, and contained <24 EU endotoxin/mg protein. Reference TNF (code 88/532) was obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). Human TNF was purchased from Preprotech (Rocky Hill, NJ), and used at a final concentration of 10 ng/ml. Staurosporin was purchased from Calbiochem-Novabiochem International (San Diego, CA) and was stored as a 2 mM solution in dimethyl sulfoxide at −20°C. DEX was purchased from Sigma Chemical Company (St. Louis, MO). RU24782, RU24858, RU40066, RU38486, and RU43044 have already been described (Vayssière et al., 1997). Stock solutions were routinely dissolved in dimethyl sulfoxide and stored at room temperature. Dilutions were freshly made in culture medium, and the final concentration of the organic solvent never exceeded 1%. This concentration was shown not to interfere with the different assays used, as tested in control experiments.

Plasmids. Plasmid DNA was prepared using Qiagen (Chatsworth, CA) or PZ-523 columns (5 Prime → 3 Prime, Boulder, CO). The plasmids p1168hu.IL6P-luc+ and p50hu.IL6P-luc+, $p(GAL4)_2$50hu.IL6P-luc+, $p(IL6\kappa B)_3$50hu.IL6P-luc+ (Fig. 2A) have been described previously (Plaisance et al., 1997). The plasmid pIκBα-luc, containing the I-κB-α promoter in front of the luciferase reporter gene was a gift from Dr. A. Israel and was also described before (Le Bail et al., 1993). The vector pPGKβGeobpA, constitutively expressing a neomycin-resistant β-galactosidase fusion protein under control of the 3-phosphoglycerate kinase (PGK) promoter from the mouse housekeeping enzyme PGK, was a gift from Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA). The AP-1-driven reporter gene construct $p(TRE)_3$tkluc, containing three tumor-promoting agent-responsive elements (TRE) in front of a minimal tk promoter coupled to the luciferase reporter gene was a gift from Dr. M. Pons (Institut National de la Sante et de la Recherche Medicale, Montpellier, France). The GR-responsive reporter gene construct pMMTVluc was a gift from Dr. P. Balaguer (Institut National de la Sante et de la Recherche Medicale). The expression plasmids pGal4, pGal4-p65, and pGal4-VP16 expressing the DBD of the yeast Gal4 protein either alone or fused to the complete NF-κB p65 subunit or the viral protein VP16 were obtained from Dr. L. Schmitz (German Cancer Research Center, Heidelberg, Germany) and were also described previously (Vanden Berghe et al., 1998). The plasmid $p(GRE)_2$50hu.IL6P-luc+ was constructed by inserting a (GRE)-containing linker (′5 GATCTCTCT GC***TGTACA***GG A***TGTTCTA***GC GGATCCTGC***T GTACA***GGA***TGTTCTA***GCTAC CTGCAG ′3; in which the GC responsive elements are ***bold underlined***) with protruding *Bgl*II-*Pst*I ends, in the multicloning site of p50hu.IL6P-luc+.

Induction. Cell cultures were transferred to multiwell dishes and were (sub)confluent at the time of induction. For induction, the medium was removed and replaced by DMEM with 5% FCS, 5% NCS, 2 mM L-Glu, 100 I.U./ml penicillin, 0.1 mg/ml streptomycin, and different inducing agents or cytokines as described in the figure legends. After induction, cell culture supernatant was collected for quantification of secreted IL-6 in murine IL-6 enzyme-linked immunosorbant assay (ELISA; R&D Systems, Minneapolis, MN) or human IL-6 ELISA (R&D Systems). Cells were washed with PBS; after careful removal of PBS, cells were lysed according to reporter gene

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assay instructions. Cell extracts were stored at −70°C until further use.

Site-Directed Mutagenesis. Site-directed mutagenesis of the IL-6 promoter was carried out by the gapped heteroduplex method, using a transformer site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA). AP-1 and NF-κB point-mutated variants of the IL-6 promoter have been described previously (Vanden Berghe et al., 1998; Fig. 2A).

Transfection Procedure. Stable transfection of L929sA cells was performed by the calcium phosphate precipitation as described previously (Vanden Berghe et al., 1998), using a 10-fold excess of the plasmid of interest over the selection plasmid pPGKβGeobpA. Transfected cells were selected in 500 μg/ml G418 for 2 weeks, after which the resistant cell clones were pooled for further experiments. In this way, the individual clonal variation in expression was averaged, thus providing a reliable response upon induction. The cotransfected plasmid pPGKβGeobpA, conferring resistance to G418 and expressing constitutive β-galactosidase enzymatic activity, was further used as an internal control for normalization of protein concentration.

For transient transfections, approximately 10^5 exponentially growing L929sA cells were transfected with 1.5 μg of the plasmid of interest, using the dimethylaminoethyl-Dextran procedure, essentially as described (De Bosscher et al., 1997). After 4 h, the transfection mixture was replaced with a 10% dimethyl sulfoxide/Hanks' balanced salt solution for 2 min, after which the solution was immediately diluted with DMEM supplemented with 10% FCS. Finally, fresh medium containing 10% serum was added for another 65 h until experiments were carried out. All transient transfections were performed at least in triplicate.

Reporter Gene Analysis. Luciferase and galactosidase reporter gene assays were performed as described previously (Vanden Berghe et al., 1998).

Electrophoretic Mobility Shift Assay. L929sA cells were seeded in 10-cm-diameter dishes at 500,000 cells/dish at day −1. After appropriate induction, the cells were washed with ice-cold PBS, scraped off, and pelleted in 15 ml of PBS by centrifugation for 5 min at 1100*g*. Lysate preparation and electrophoretic mobility shift assay were performed essentially as described previously (Plaisance et al., 1997). The NF-κB oligonucleotide comprises the sequence: 5′-AGCTATGT***GGGATTTTCCC***ATGAGC-3′, in which the single IL-6 promoter-derived NF-κB motif is bold and underlined.

Western Blot Analysis. Cells were pretreated with 10^{-7} M compound for 150 min, followed by incubation with 2500 IU/ml TNF for several time periods. Cells were harvested and lysed in TOTEX buffer [20 mM HEPES:KOH, pH 7.9/0.35 M NaCl/20% (v/v) glycerol/1% (v/v) Nonidet P-40/1 mM $MgCl_2$/0.5 mM EDTA/0.1 mM EGTA/2 mM Pefabloc/5 mM DTT]. After centrifugation and addition of Laemmli buffer, the samples were loaded onto a reducing SDS/12% polyacrylamide gel and subjected to electrophoresis. Equal amounts of protein, determined according to Bradford, were transferred to a nitrocellulose (Schleicher & Schuell, Inc., Keene, NH), or polyvinylidene difluoride (Bio-Rad, Richmond, CA) membrane. The membrane was soaked overnight in Tris-buffered saline (TBS)/5% nonfat dry milk, after which the solution was replaced by TBS/5% milk/0.05% Tween 20 to which 1:1000 dilution of anti-I-κB-α antibody (provided by Dr. L. Schmitz) was added. After 90-min incubation at room temperature, the membrane was washed six times with TBS/0.05% Tween 20 and incubated for 1 h in TBS/0.05% Tween/5% milk containing 1:3000 dilution of secondary antirabbit antibody coupled to biotin (Amersham Life Science, Buckinghamshire, UK). After extensive washing, filters were further incubated for 45 min with 1:1000 dilution of streptavidin-horseradish peroxidase conjugate (Amersham) in TBS/0.05%/Tween/5% milk. Finally, the membrane was washed 4 times in TBS/0.05% Tween, followed by two additional washing steps in TBS, after which the bound antibodies were detected using enhanced chemiluminescence (DuPont-NEN, Boston, MA).

Results

Dissociated GCs Repress TNF-Induced IL-6 Secretion in Murine and Human Cell Lines. Encouraged by previous results (De Bosscher et al., 1997) demonstrating inhibition of TNF-induced IL-6 gene expression by the classical GC agonist DEX, we examined the effects of a new class of dissociated compounds on endogenous IL-6 secretion in different cell lines. L929sA and HeLa cells were treated for 8 and 6 h, respectively, with TNF in the presence or absence of different compounds that were added 2 h before TNF stimulation. The supernatants were assayed for secreted IL-6 levels by ELISA. Strong repression of TNF-induced IL-6 gene expression was observed with all compounds, both in L929sA and HeLa cells. In L929sA cells, 1 μM each synthetic GC tested exerted strong repression of TNF-induced IL-6 secretion; DEX and RU24782 displayed similar potencies, whereas RU24858 and RU40066 exerted a slightly reduced repression. No or a weak repression only of TNF-induced IL-6 secretion was observed after treatment with the antagonists RU43044 and RU38486 (RU486), respectively (Fig. 1A). In HeLa cells, however, DEX exhibited a stronger inhibition of IL-6 expression than the other synthetic GC (Fig. 1B). In HeLa cells, the IC_{50} of DEX calculated from a dose-response curve ranging from 1 nM to 1 μM was close to 1 nM, whereas that of the dissociated GC was around 50 nM. Similarly, as observed in L929sA cells, the antagonists RU486 and RU43044 were not able to repress IL-6 secretion in HeLa cells (data not shown).

GCs Inhibit TNF-Induced IL-6 Promoter Activity by a Transcriptional Mechanism Acting via the κB Motif. As DEX has been shown previously to exert inhibition of IL-6 expression at the transcriptional level, various IL-6 promoter reporter gene constructs were stably transfected into L929sA cells and tested for their inducibility by TNF and their repression by DEX (Fig. 2, A and B). In stable transfectants of the p1168hu.IL6P-luc+ construct, reporter gene expression could be reproducibly induced with TNF and repressed by DEX and by the dissociated compounds, i.e., RU24782, RU24858, and RU40066 (Fig. 2, B and C), mimicking the endogenous IL-6 gene regulation and confirming that the appropriate regulatory elements for IL-6 gene regulation are present in the 1168-bp promoter fragment. However, DEX exerted almost complete repression of IL-6 production in supernatants of L929 cells (Fig. 1), whereas IL-6 transcription was only partially repressed by DEX in these cells (Fig. 2), which may suggest that the repression by DEX of TNF-induced IL-6 gene expression results from both transcriptional and post-transcriptional events (Chen et al., 1998; Swantek et al., 1997). Because GCs were described to negatively regulate both AP-1- and NF-κB-dependent gene expression (Göttlicher et al., 1998), we further investigated the contribution of both *cis* elements in the repression by DEX of TNF-induced IL-6 gene expression. Specificity was demonstrated by stably transfecting IL-6 promoter variants harboring point mutations in the AP-1 or NF-κB elements (Fig. 2A). Mutation of the AP-1 element largely affected the total promoter activity, whereas the inducibility by TNF (2-fold) and its repression by DEX (75%) remained unaffected as compared with the wild-type promoter. By contrast, the NF-κB point mutation completely abrogated TNF-induced IL-6 promoter function (Fig. 2B). Furthermore, exchanging the IL-6

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TATA box sequence environment for a minimal thymidine kinase promoter in the p1168hu.IL6P reporter gene construct did not abolish DEX repression, and demonstrates that the IL-6 TATA box sequence environment does not largely, if at all, contribute to the GC-mediated repression of IL-6 gene expression (data not shown). Finally, placing three copies of the IL-6 NF-κB-responsive element in front of the unresponsive p50hu.IL6P-luc+ also resulted in a powerful TNF-inducible reporter gene system that could be efficiently repressed by DEX. Taken together, these data indicate that induction of the IL-6 promoter by TNF and its repression by DEX or the dissociated compounds are mainly mediated via the NF-κB-responsive element.

Dissociated GCs Repress TNF-Induced κB-Dependent Reporter Gene Activity. As we established unequivocally the essential role of NF-κB in GC repression of TNF-induced IL-6 gene expression, we further assessed the ability of the various dissociated GCs to repress NF-κB-driven reporter gene expression. Stable L929sA transfectants of p(IL6κB)$_3$.50hu.IL6P-luc+ were stimulated for 10 h with TNF in combination with several doses of the different compounds added 2 h before TNF treatment. The results are expressed relative to the maximal NF-κB transrepression obtained with 1 μM DEX (100%). DEX and RU24782 displayed similar activity, whereas RU24858 and RU40066 were less efficient and repressed the p(IL6κB)$_3$.50hu.IL6P-luc+ reporter activity only 65% as efficiently as the same concentration of DEX (Fig. 3A). Note that the IC_{50} of RU24782 for the κB reporter construct in L929sA was 6 nM, similar to that of DEX, whereas the IC_{50} of both RU24858 and RU40066 was 100 nM, i.e., slightly different than the values obtained in HeLa cells with the endogenous IL-6 promoter.

In contrast, the antagonists RU486 and RU43044 showed only weak repressive activities. In addition, in cotreatment experiments, DEX and the synthetic compounds exerted additive and dose-dependent repression on an NF-κB-dependent promoter reporter gene construct (data not shown).

Dissociated GC-Mediated Repression of NF-κB Activity Is Relieved by the Antagonists RU486 and RU43044. Because the dissociated compounds repressed TNF-induced NF-κB-dependent gene activation in a dose-dependent manner, we tried to relieve the repression with synthetic anti-GC. A pool of stable p(IL6κB)$_3$50hu.IL6P-luc+ transfectants was stimulated with TNF and treated with 100 nM the various dissociated compounds, applied alone or in combination with a 10-fold excess of the classical antagonist RU486 (1 μM) or the class II antagonist RU43044 (1 μM). Repression of TNF-induced luciferase activity could be efficiently relieved by RU486 or RU43044, indicating that the repression of NF-κB activity exerted by the dissociated GC is mediated via the GR (Fig. 3B).

Divergent Potencies of Dissociated GCs in GRE-Dependent Reporter Gene Activation. We established ligand dose-response curves for transactivation of the GRE_5-tk-CAT reporter gene or of the endogenous tyrosine aminotransferase gene, with the endogenous HeLa GR and the hepatoma cell (HTC) GR, and showed that RU24858, RU24782, and RU40066 displayed only a minor transactivation ability (Vayssière et al., 1997). Similarly, the transactivation ability of these compounds

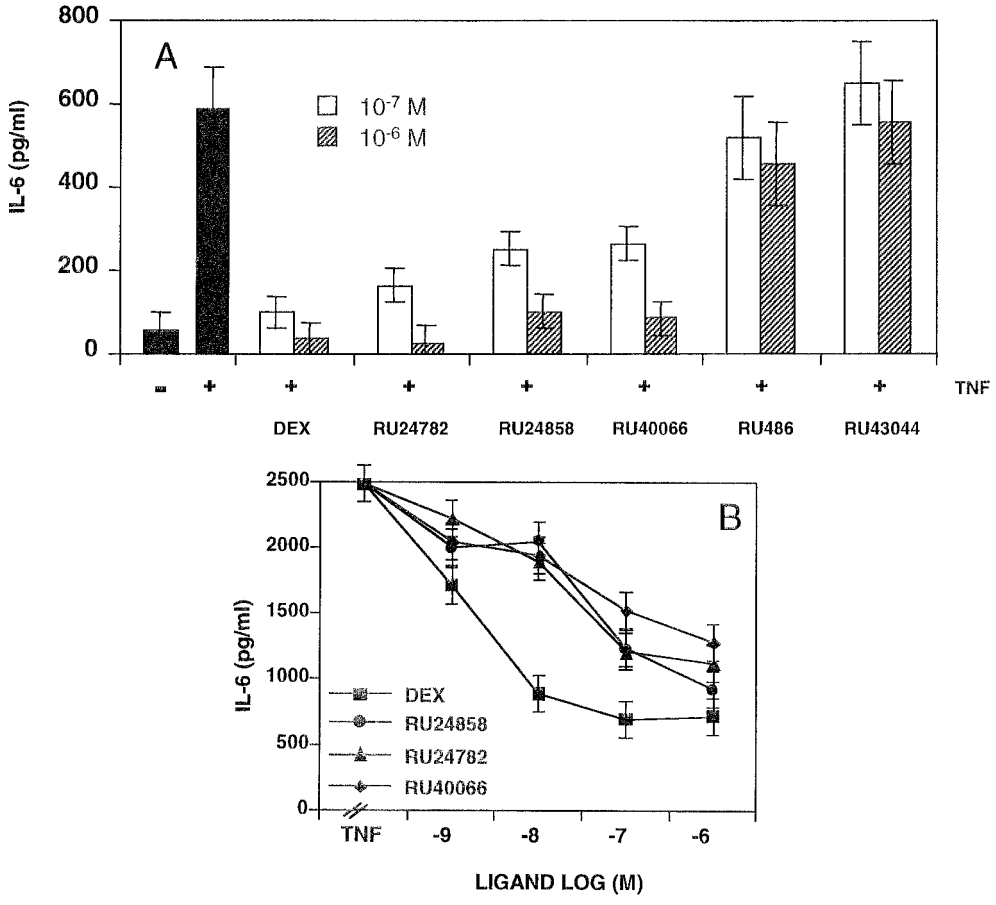

Fig. 1. Dissociated GCs inhibit IL-6 secretion in murine L929sA and human HeLa cells. A, L929sA cells were stimulated for 8 h with 30 ng/ml TNF (2500 IU/ml) and/or treated with, respectively, 10^{-7} or 10^{-6} M compound added 2 h before TNF stimulation. The cell culture supernatants were collected and assayed for IL-6 secretion by ELISA. B, similarly, HeLa cells were stimulated with 10 ng/ml TNF and/or different compounds applied at doses ranging from 1 nM to 1 μM. After 6 h of treatment, the cell culture supernatants were collected and assayed for IL-6 secretion by ELISA. Note that TNF treatment of HeLa cells resulted in a 10-fold stimulation of IL-6 secretion (not shown). The figures are representative of three separate experiments. The results shown are the mean ± S.D. of triplicate inductions.

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was measured in murine L929sA cells stably transfected with a p(GRE)$_2$50hu.IL6P-luc+ reporter (Fig. 2A). In accordance with our previous results, RU24858 and RU40066 did not significantly stimulate the transcription of the stably integrated p(GRE)$_2$50hu.IL6P-luc+ construct in murine fibroblasts. Similarly, both antagonists RU486 and RU43044 did not stimulate the transcription of the p(GRE)$_2$50hu.IL6P- luc+ reporter (Fig. 4A and data not shown). Surprisingly, however, and in contrast with our earlier observations made in HeLa and HTCs, RU24782 displayed a similar activating capacity as DEX on the p(GRE)$_2$50hu.IL6P-luc+ reporter in the L929sA cells. Furthermore, both RU486 and RU43044 were able to reverse the transactivation induced by 100 nM DEX or RU24782 (Fig. 4A and data not shown). Similar results were obtained when a mouse mammary tumor virus (MMTV)-luciferase reporter construct was expressed in the same type of cells (Fig. 4B), demonstrating that the effects observed are not exclusive promoter-dependent phenomena, but rather cell type-specific. In view of the discrepancies observed between transactivation of GRE-based reporters in HeLa and L929 cells, we also investigated the repression capacity of DEX and of the dissociated compounds on activated p(TRE)$_3$tkluc, an AP-1-driven reporter gene construct, in L929 fibroblasts. As shown in Fig. 4, Cand D, DEX and the different compounds exerted comparable repression of the stimulated AP-1-driven reporter, in keeping with the results obtained in transiently transfected Hela cells containing a Coll(−517/+63)CAT reporter.

Dissociated GCs Neither Modify I-κB-α Expression Nor Interfere with NF-κB DNA-Binding Ability. Different mechanisms have been proposed to account for GC inhibition of NF-κB activity. Direct protein-protein interaction between the GR and the p65 subunit of NF-κB has been demonstrated in vitro (see Introduction). More precisely, the N-terminal Rel-homology domain of p65 and the zinc finger structure in the DBD of the GR were shown to be required for these interactions (Liden et al., 1997; Wissink et al., 1997). In addition, we and others have shown previously that the activated GR directly interferes with the transactivation potential of the NF-κB p65 subunit (De Bosscher et al., 1997; McKay and Cidlowski, 1998). Alternatively, it has been sug-

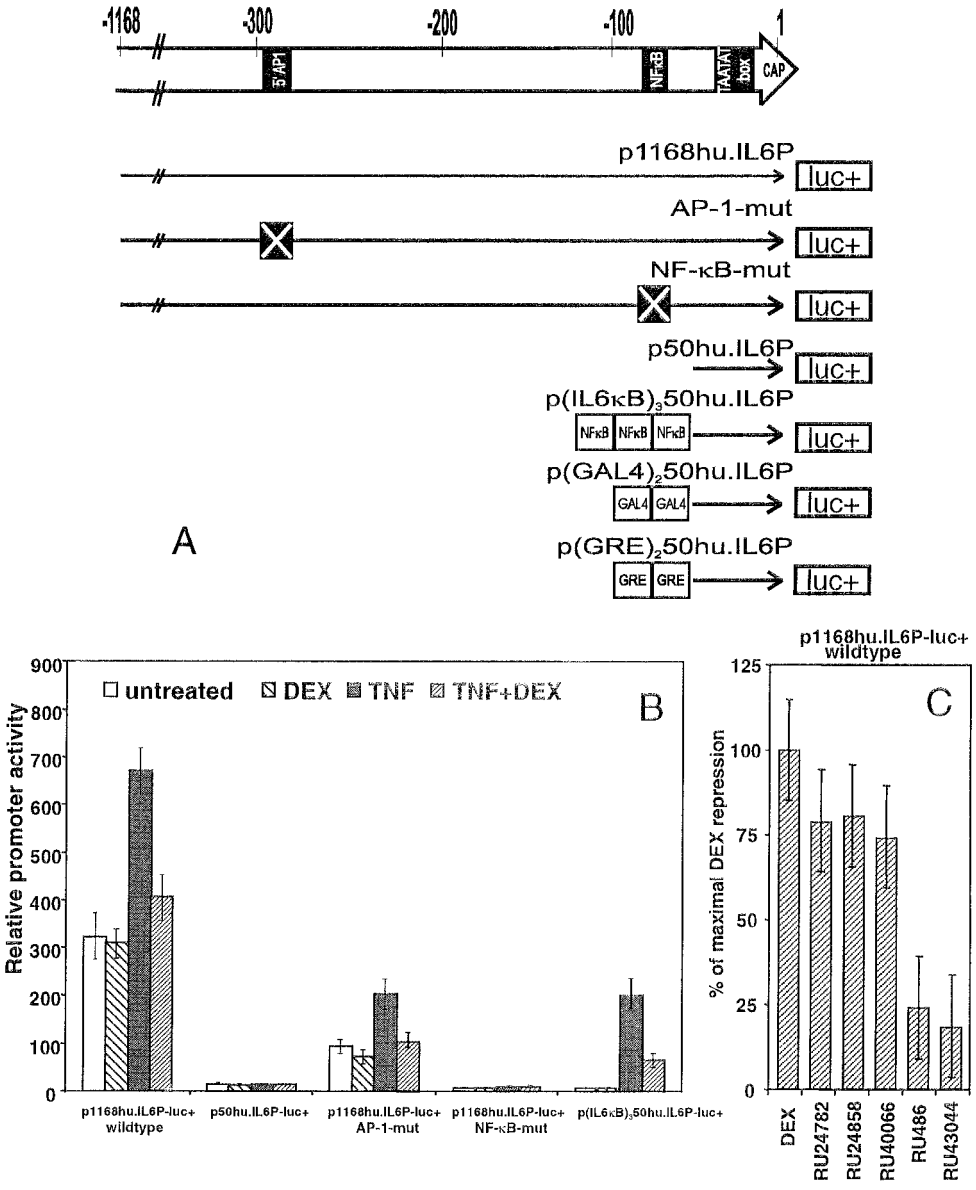

Fig. 2. Inhibition of TNF-induced IL-6 secretion by DEX is mediated by repression of κB-driven promoters. A, representation of the various IL-6 promoter reporter gene constructs. Top, IL-6 promoter. The 1168-bp fragment of the IL-6 promoter is coupled to the luciferase gene, yielding the recombinant reporter gene construction p1168hu. IL6P-luc+. ■, respective binding sites for the indicated transcription factors. Crossed black boxes represent mutated binding sites of the corresponding transcription factors. The reporter gene variants p(IL6κB)$_3$50hu. IL6P-luc+, p(GAL4)$_2$50hu. IL6P-luc+, and p(GRE)$_2$50hu. IL6P-luc+ were constructed by fusing three copies of the NF-κB element of the IL-6 promoter or two copies of the GAL4 or of a consensus GRE-binding site, respectively, to the 50-bp minimal promoter construct p50hu. IL6P-luc+. B, L929sA cells, stably transfected with the different IL-6 promoter-based constructs shown in (A), were left untreated or were treated with DEX alone (10^{-6} M), TNF alone (2500 IU/ml) for 6 h, or with a combination of DEX (added 2 h before TNF stimulation) and TNF. Results are expressed in arbitrary light units and are normalized for protein concentration. Results are the mean of three separate experiments (S.D. is maximum deviation of triplicate results). C, L929sA cells, stably transfected with the p1168hu. IL6P-luc+ reporter gene construction, were stimulated for 7 h with 30 ng/ml TNF (2500 IU/ml) and/or treated with 1 μM compound added 2 h before TNF stimulation. The corresponding lysates were assayed for luciferase activity and were normalized for protein concentration. Results are expressed as a percentage relative to the inhibition obtained with 1 μM DEX set arbitrarily to 100.

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gested that the repressive activity of DEX could be attributed to an increased production of the inhibitory molecule I-κB-α, which in turn would sequester NF-κB in the cytoplasm and prevent the formation of DNA-bound NF-κB complexes in the nucleus (see Introduction). Therefore, the potential influence of the various compounds on I-κB-α expression and on TNF-stimulated NF-κB DNA-binding activity was tested by Western blotting, reporter gene assays, and gel shift analysis, respectively. L929sA cells were exposed to the various GC ligands for 150 min, after which TNF was added. As shown in Fig. 5A, a combined treatment of TNF with different compounds did not substantially raise the I-κB-α expression levels as compared with TNF treatment alone. Thirty minutes after TNF exposure, I-κB-α was entirely degraded and after 150 min it had fully reappeared. Similar results were obtained in HeLa cells, where a combined treatment with TNF and DEX or dissociated compounds did not result in a significant up-regulation of I-κB-α synthesis (data not shown). In addition, the transcriptional activity of the I-κ-B-α promoter was measured in stable transfectants of the pIκBα-luc construct. No stimulation of the IκBα promoter could be observed upon treatment with any of the GCs tested (DEX, RU24782, and RU24858); in contrast, these compounds exerted strong repression of the TNF-induced IκBα promoter activity, and the antagonist RU486 was ineffective (Fig. 5B). Furthermore, gel shift experiments revealed that DEX and the dissociated compounds had no effect on the DNA-binding activity of NF-κB after 30 or 150 min of TNF treatment, as shown in Fig. 6 and quantified with PhosphorImager software (data not shown). In addition, neither DEX nor TNF, as reported previously, nor any of the dissociated compounds altered the levels of the recombination signal sequence-binding protein Jκ. The specificity and qualitative composition of the observed binding complexes has been discussed previously in detail (Plaisance et al., 1997).

Gal4-p65-Mediated Expression of a Reporter Gene Can Be Repressed by Dissociated Compounds. In another attempt to demonstrate that dissociated GC-mediated repression of NF-κB activity occurs independently of I-κB-α induction, we used a mammalian Gal4 "one-hybrid" system. In this assay, we measured the ability of the synthetic GC to interfere with the transactivation potential of a chimeric protein containing the entire transactivating p65 NF-κB subunit linked to the DBD of the yeast Gal4 protein. L929sA cells were stably transfected with pGal4, pGal4-p65$^{1-551}$, pGal4-VP16 expressing the Gal4 DBD alone or fused to the NF-κB subunit p65, or to the viral transactivating protein VP16, respectively (De Bosscher et al., 1997; Vanden Berghe et al., 1998). These proteins are exclusively localized in the cell nucleus, as revealed by immunofluorescence studies (data not shown), and allow monitoring of transcriptional events, independently of I-κB-α effects. The corresponding transactivation capacity of the pGal4 plasmids was measured after transient transfection of the respective cell lines with p(GAL4)$_2$50hu.IL6P-luc+. The constitutive level of transactivation by the nuclear fusion proteins Gal4-p65 and Gal4-VP16, as compared with mock-transfected cells or pools expressing Gal4 alone, is shown in Fig. 7A. In accordance with previous results, basal transactivation levels mediated by pGal4-p65 could be specifically repressed by DEX, whereas no

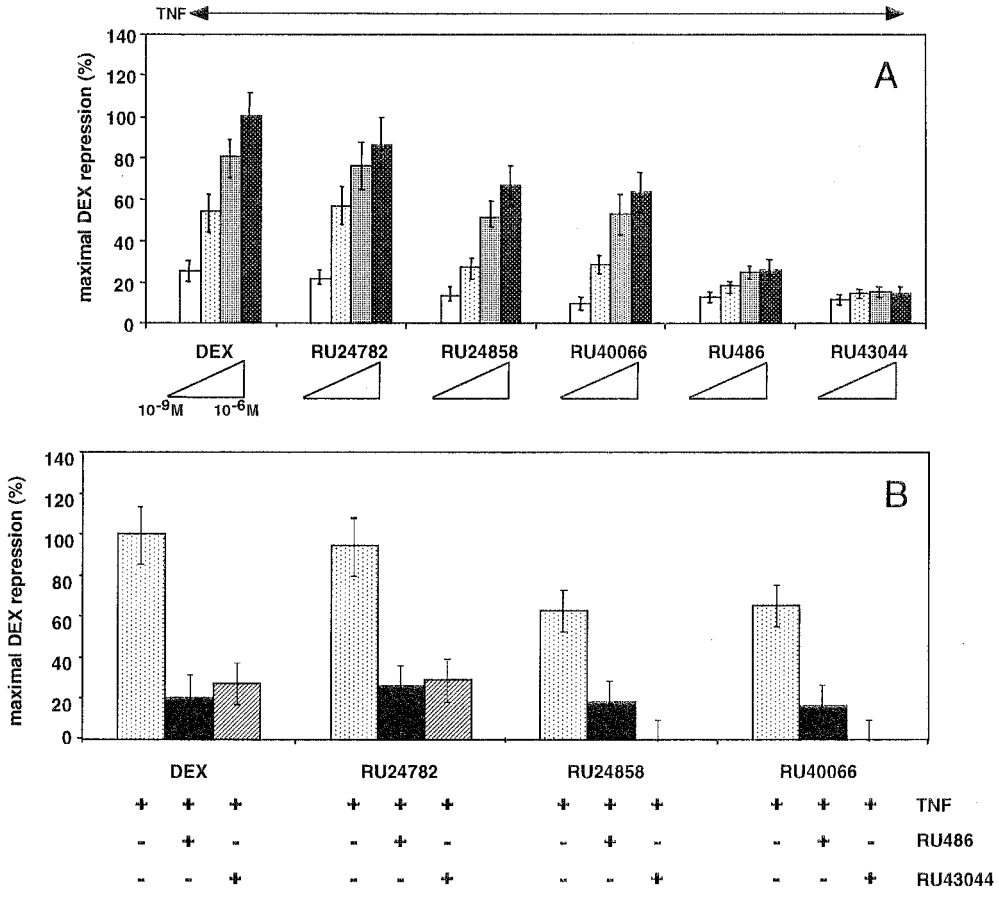

Fig. 3. Dissociated GCs inhibit NF-κB-driven gene expression. A, L929sA cells with the stably integrated construct p(IL6κB)$_3$50hu. IL6P-luc+ were left untreated or were treated for 10 h with TNF alone (2500 IU/ml) or in combination with the indicated compounds (1 nM to 1 μM) added 2 h before TNF stimulation. The corresponding lysates were assayed for luciferase activity and normalized for protein concentration by measuring β-galactosidase expression. The repression obtained with 10^{-6} M DEX was set to 100% and the repressions for the other treatments were expressed relative to the maximum repression level obtained with DEX treatment. Values are mean ± S.D. of at least three independent experiments performed on two independently established stable pools. B, cells were treated with the indicated dissociated compounds (10^{-7} M) and a 10-fold excess of the indicated antagonists (10^{-6} M).

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effect was observed with any of the control set-up cells, i.e., the mock, the pGal4-, or pGal4-VP16-transfected cells (Fig. 7A). Ligand dose-response curves were established after exposure of the cells to DEX, RU24782, RU24858, RU40066, or RU486 (Fig. 7B). RU24782 consistently displayed the highest repressing activity, exceeding even the level of DEX repression. Similar repression levels were achieved for RU24858, RU40066, and DEX. RU486 did not significantly repress the activation of the $p(GAL4)_2$50hu.IL6P-luc+ reporter by pGal4-p65. In addition, the repression of basal pGal4-p65 transactivation exerted by DEX, RU24782, RU24858, or RU40066 (100 nM) could be reversed by adding a 10-fold excess of RU486 (Fig. 7C). The DNA-binding activity of the Gal4 fusion proteins was not affected by the compounds as tested in a control experiment (data not shown), indicating that the repression of p65 activity by the dissociated compounds in the Gal4 one-hybrid system was likely to be due to a down-modulation of p65 transactivation.

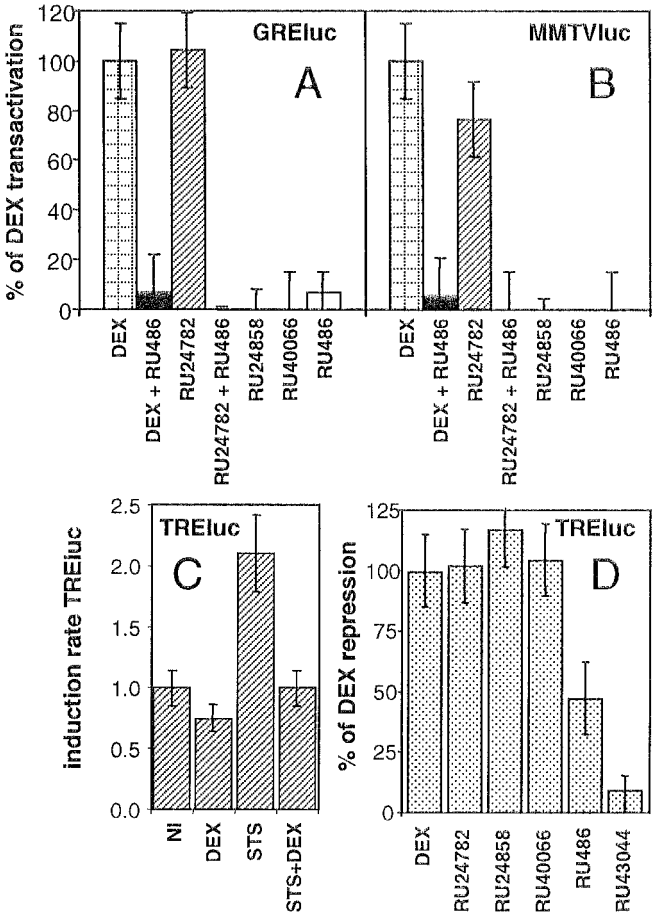

Fig. 4. Transactivation and transrepression ability of dissociated compounds in murine fibroblasts. L929sA cells containing the stably integrated $p(GRE)_2$50hu. IL6P-luc+ (A) or the transient transfected construct pMMTVluc (B) were treated for 7 h with 100 nM the indicated compounds and 10-fold excess of antagonist RU486 (as described in the legend to Fig. 3). The corresponding lysates were assayed for luciferase activity and normalized for protein concentration. The results are expressed as the percentage of activation relative to maximal activation obtained with 100 nM DEX. The figure is representative of three separate experiments (S.D. = maximum S.D. of triplicate induction). (C) and (D), for determination of AP-1 repression, L929sA cells were stably transfected with the $p(TRE)_3$tkluc reporter gene construction and exposed for 6 h to 60 nM staurosporin alone or in combination with 1 μM synthetic GC. Activation of the TRE reporter gene construct and its repression by DEX is shown in Fig. 4C. The repression obtained with the various compounds is also presented in Fig. 4D as a percentage relative to the maximal inhibition obtained with 1 μM DEX, set arbitrarily to 100.

Discussion

Dissociated GCs with Anti-Inflammatory Potential Inhibit IL-6 Synthesis. GCs are highly potent anti-inflam-

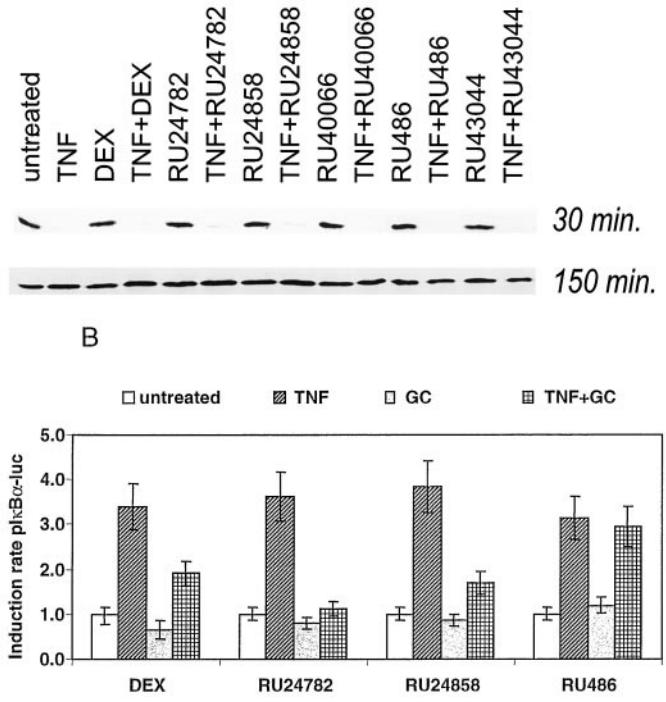

Fig. 5. GC treatment of L929sA cells does not result in up-regulation of the I-κB-α gene expression. A, L929sA cells were treated for 150 min with various GC ligands (10^{-7} M), after which TNF (2500 IU/ml) was added for 30 or 150 min. Equal amounts of total cellular protein extract were loaded onto SDS-polyacrylamide gels for Western blot analysis. B, L929sA cells stably transfected with the pIκBα-luc construct were left untreated or were treated for 6 h with TNF alone (2500 IU/ml) or with a combination of TNF and the indicated compounds (1 μM) added 2 h before TNF stimulation. The corresponding lysates were assayed for luciferase activity and normalized for protein concentration by measuring β-galactosidase expression. Results are expressed as an induction rate and are representative for three separate experiments (S.D. is maximum deviation of triplicate results).

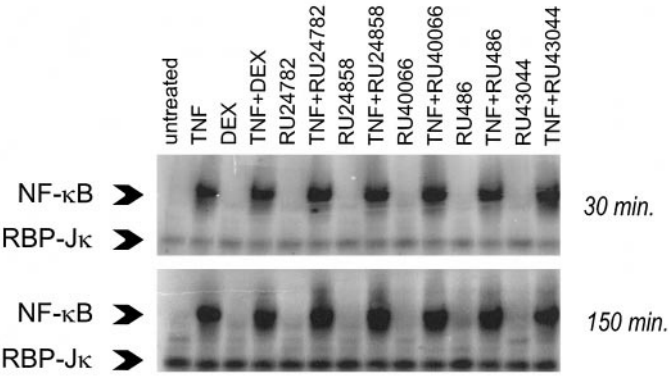

Fig. 6. GCs do not prevent the formation of an NF-κB/DNA complex. L929sA cells were treated as described in the legend to Fig. 5. Equal amounts of total cellular protein extract were incubated with a ^{32}P-radiolabeled oligonucleotide containing the IL-6 NF-κB-responsive element for analysis in electrophoretic mobility shift assay. Arrowheads indicate the activated NF-κB complex, and the constitutively expressed recombination binding protein Jκ (RBP-Jκ).

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matory agents. However, their therapeutic use is seriously limited by severe side effects occurring during long-term treatment. Understanding the molecular mechanism of action underlying each of the physiologic and pharmacologic activities of GCs is of great importance, as it may enable the development of novel GC derivatives with an increased therapeutic value and specificity (Hofmann et al., 1998; Resche-Rigon and Gronemeyer, 1998; Gustafsson, 1998).

We previously described synthetic GCs that exerted strong AP-1 inhibition whereas only weakly activating GRE-based reporter genes and the endogenous tyrosine aminotransferase gene. In addition, these dissociated GCs were shown to inhibit IL-1-β secretion and to display anti-inflammatory activity in vivo (Vayssière et al., 1997). Here we investigated the effect of these compounds on NF-κB-dependent target gene regulation. We have studied the molecular activities of the dissociated GC RU24782, RU24858, and RU40066 and found that they strongly repress TNF-induced IL-6 secretion in murine fibroblasts and human HeLa cells, which are major producers of IL-6. IL-6 contributes to a multitude of physiologic and pathophysiologic processes. Among its various functions, IL-6 plays an active role in immunological responses, bone metabolism, reproduction, inflammation, neoplasia, and aging. Overexpression of IL-6 has been implicated in the pathology of a number of diseases (Papanicolaou et al., 1998). Hence, development of selective IL-6 agonists and antagonists may offer therapeutic benefits for many disorders.

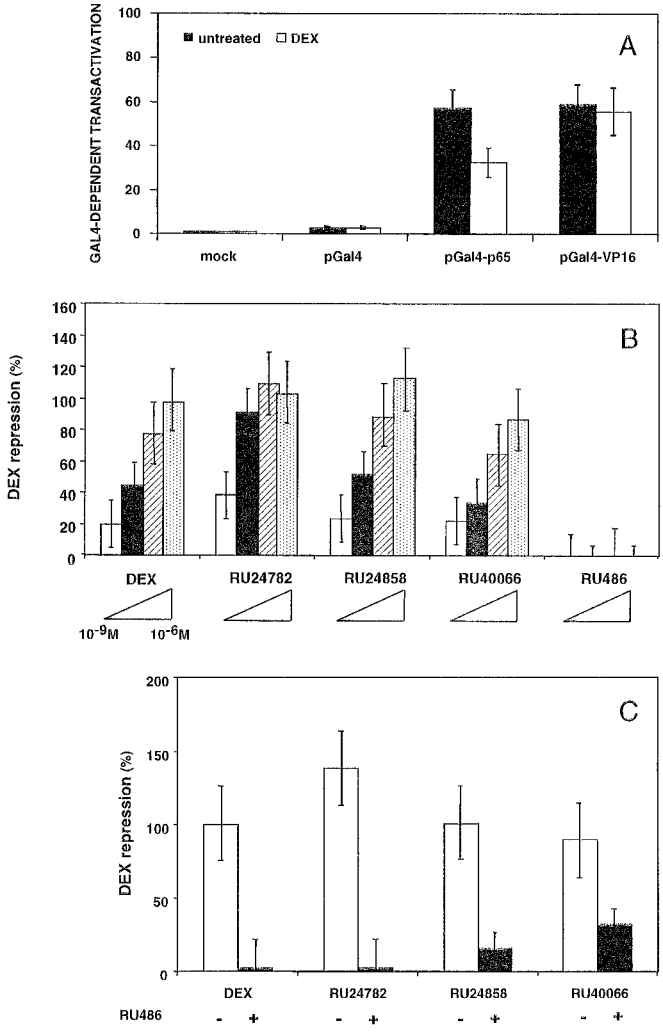

Fig. 7. Gal4-p65-mediated expression of a reporter gene can be repressed by dissociated compounds. A, Gal4 one-hybrid setup. Untransfected cells (mock) or stable pools expressing the nuclear fusion proteins Gal4, Gal4-p65$^{1-551}$, or Gal4-VP16 were transiently transfected with the reporter plasmid p(GAL4)$_2$50hu. IL6P-luc+; 60 h after transfection, cells were left untreated or treated with DEX for 7 h. The transactivation potential of the different Gal4 fusion proteins was determined by measuring the luciferase activity normalized to the galactosidase levels expressed by the internal control plasmid. The results are expressed in arbitrary light units to allow comparison of the transactivation potential of the various Gal4 constructs upon treatment with 10 μM DEX. B and C, stable cells expressing the nuclear fusion protein Gal4-p65$^{1-551}$ were exposed to different compounds as indicated and applied at doses ranging from 1 nM to 1 μM (B) or at a fixed concentration of 100 nM in the presence of a 10 times molar excess of the antagonist RU486 (C). Maximal DEX repression of Gal4-p65 has been arbitrarily set to 100%. Results are expressed relative to the maximal DEX repression and are the mean ± S.D. of at least four independent experiments, each performed in quadruplicate.

Dissociated GCs Retain Both AP-1 and NF-κB Transrepression Ability. Because most of the immunoregulatory genes that are negatively regulated by GCs are positively regulated by AP-1 and/or NF-κB, it has been proposed that the immunosuppressive and anti-inflammatory activities of GC were mediated through interference with these transcription factors (Göttlicher et al., 1998). The characterization of the IL-6 promoter revealed a complex control region that can be triggered by multiple activation pathways (Vanden Berghe et al., 1998). Upon detailed studies of IL-6 promoter reporter gene mutants, we measured the contribution of both the AP-1 and NF-κB motifs in GC repression of TNF-induced IL-6 gene expression and clearly showed that NF-κB is the main transcription factor involved. Further proof for the exclusive role of NF-κB was obtained by inserting multiple κB sites in front of an unresponsive minimal IL-6 promoter. This resulted in a powerful TNF-inducible reporter gene system, which could also be efficiently repressed by DEX. In addition to DEX, the synthetic GC RU24782, RU24858, and RU40066 were also able to efficiently repress the NF-κB-dependent gene activation in a dose-dependent manner from a stably integrated IL-6 or synthetic NF-κB-dependent promoter reporter gene construct. Similar results were obtained using the E-selectine promoter, which contains three κB-responsive elements, instead of the IL-6 promoter (data not shown). Repression could be relieved by the antagonists RU486 or RU43044, indicating that the repression of NF-κB activity exerted by the dissociated GC is mediated via the GR.

DEX has been shown to impair the TNF- or IL-1-induced DNA binding of NF-κB in a glioblastoma cell line, and in HeLa and T cells stably overexpressing the GR (Auphan et al., 1995; Scheinman et al., 1995; Dumont et al., 1998). However, gel shift analysis in murine fibroblasts clearly demonstrates that either classical or dissociated GCs, although distinctly repressing NF-κB activity, do not interfere with NF-κB or RBP-Jκ DNA binding, which points to a different mechanism. Using a mammalian Gal4 one-hybrid system, we further demonstrate that the synthetic GCs also specifically inhibit the transactivation potency of the constitutively expressed fusion protein Gal4-p65, driving a Gal4-dependent luciferase reporter gene by a direct nuclear mechanism of action. Here, too, GC-mediated Gal4-p65 repression with the synthetic ligands could be relieved by cotreatment with the antagonists RU486 and RU43044.

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Recent reports show that NF-κB may exert its transactivating function by interacting with components of the basal transcriptional machinery (Schmitz et al., 1995; Yamit-Hezi and Dikstein, 1998), as well as with the coactivator cAMP responsive element binding (CREB) protein (CBP)/p300 (Zhong et al., 1998). Similarly, NRs interact in a ligand-dependent manner, with multicomponent cofactor complexes (Xu et al., 1999). In analogy with NR-mediated repression of AP-1, competition of NF-κB and GR for limiting amounts of the coactivator CBP/p300 could be responsible for NF-κB repression. However, although CBP overexpression can rescue AP-1-mediated repression of retinoic acid receptor (RAR), GR element (GRE), and androgen receptor (Kamei et al., 1996; Aarnisalo et al., 1998), in our hands it turned out to be ineffective in relieving GR-dependent NF-κB repression (De Bosscher et al., 1998). Interestingly, different regions of the GR DBD are apparently involved in AP-1 and NF-κB repression. Although the N-terminal zinc finger of the GR DBD is critical for AP-1 transrepression (Heck et al., 1994), it does not contribute significantly to the repression of NF-κB activity, as shown by swapping experiments between the corresponding regions of GR and the nonrepressive TR-β (Liden et al., 1997). In addition, the anti-GC ZK98299 was recently reported to be unable to repress NF-κB activity (Liden et al., 1997), whereas it had previously been shown to induce GR transrepression of AP-1 (Heck et al., 1994). From these data and the previously reported results on the differential repression of AP-1 and NF-κB by RAR (Kamei et al., 1996) and MR (Heck et al., 1994), we may infer that different mechanisms are likely to account for GC-mediated repression of AP-1 and NF-κB activities.

Another level of GR regulation concerns mutual interference with other signaling pathways. Recently, GR was shown to antagonize the extracellular signal-regulated kinase (ERK; Rider et al., 1996) and c-Jun N-terminal kinase pathway in AP-1, Elk-1, and activating transcription factor-mediated transcription (Caelles and Gonzalez-Sancho, 1997). Conversely, cyclin-dependent kinases, mitogen-activated protein kinases (MAPKs), protein kinase A, and glycogen synthase kinase-3 were demonstrated to affect directly or indirectly GR (Krstic et al., 1997; Rogatsky et al., 1998a,b; Xu et al., 1999; Zhang and Danielson, 1998). Since we recently reported on the crucial role of p38 and ERK MAPK in TNF-dependent NF-κB transactivation (Vanden Berghe et al., 1998), we have tested whether the p38 and ERK MAPK inhibitors SB20358 and PD098059, respectively, affected GR inhibition of NF-κB. Inhibition of gene expression by GR and the MAPK inhibitors was additive, which suggests that different targets are involved in p38 or ERK MAPK activation and GC repression of NF-κB activity (our unpublished results). Finally, diverse signaling mechanisms may modulate GR recruitment of cofactor complexes (Xu et al., 1999).

I-κB-α Induction by GCs Is Unlikely to Account for NF-κB Repression by the GR. Induction of I-κB-α synthesis by GC and the subsequent sequestration of NF-κB were previously suggested to be a nonexclusive repression mechanism of NF-κB (see introduction). Recent findings, however, challenge this model (Dumont et al., 1998 and references included). Results obtained in mouse fibroblast L929, human fibroblast 293, monkey COS, human T-cell CEM-C7, and mouse endothelial TC10 and BAEC cells, did not show increased I-κB-α protein levels after DEX treatment. In addition, the levels of I-κB-α in the neuronal cortex and hippocampus are lower in DEX-treated rats than in untreated animals. Peripheral cells from the same animals, however, display elevated I-κB-α levels after treatment with DEX, which suggests that up-regulation of I-κB-α in response to steroids is apparently not a universal mechanism and seems to be highly tissue- or cell-specific. Here, we provide evidence that the dissociated GC repress NF-κB without substantially increasing I-κB-α expression levels or interfering in NF-κB or recombination binding protein-Jκ DNA binding. As a matter of fact, neither DEX nor the dissociated GC displayed the ability to stimulate the transcription of an I-κB-α-driven reporter gene stably transfected in L929 cells. Similarly, GR mutants, in which point mutations in the GR DBD impaired DBD dimerization and DNA binding, and subsequently abolished GR transactivation, are unable to trigger synthesis of I-κB-α but retain their full capacity to inhibit NF-κB activation (Liden et al., 1997; Heck et al., 1997). Conversely, GC analogs with transactivating capacity through GRE elements have been described that induced I-κB-α synthesis, but did not inhibit NF-κB activity (Heck et al., 1997). Taken together, these data strengthen the fact that I-κB-α is not required for the anti-inflammatory properties of GC. Although the "I-κB-α" and "protein-interaction" models are not mutually exclusive, it remains to be investigated which model predominates under particular conditions or in specific cell types.

Divergent Potencies of Dissociated GCs in GRE-Dependent Reporter Gene Activation in Human and Murine Cells. In parallel to NF-κB-dependent transrepression, transactivation experiments with GRE-dependent promoter reporter gene variants were also performed in murine fibroblasts (L929sA). In full agreement with results obtained in human HeLa cells and rat HTCs (Vayssière et al., 1997), RU24858 and RU40066 did not substantially stimulate the transcription of a $p(GRE)_2$50hu.IL6P-luc+ or MMTV-luc construct in murine fibroblast cells. Surprisingly, however, and in contrast to our earlier observations in human and rat cells, RU24782 displayed similar activating ability as DEX on the GRE-dependent reporter gene variants in mouse fibroblast cells. Cell type-selective activation of steroid hormone receptors is well known and specially documented for estrogens. Katzenellenbogen and colleagues (1996) previously focused on the pharmacologic mechanisms that underlie this selectivity and introduced the concept of a tripartite model, illustrating that tissue and/or cell type selectivity is determined by three distinct partners, i.e., the ligand, the receptor, and the effector site. They further showed that in a tripartite receptor system, the pharmacologic parameters are not unique characteristics of a ligand, but can be assigned to a ligand only when reference is made to its associated effector molecule.

In accordance with this model, tamoxifen and raloxifen, described previously as estrogen antagonists/partial agonists, were recently renamed SERM (selective estrogen receptor modulators) to integrate the tissue selectivity exhibited by these compounds, i.e., their antagonistic activity in breast and uterus and their full agonistic activity in bone (Gustafsson, 1998).

Does Dissociation of Transactivation and AP-1/NF-κB Repression Lead to Better Tolerated Compounds? Selective agonists/antagonists of the retinoid and

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estrogen receptors have recently been identified, and already demonstrated clinical efficacy and benefit (Gustafsson, 1998, and references therein). Selective GR modulators have been found, too. In addition to their previously reported ability to repress AP-1 and to exhibit in vivo anti-inflammatory activity, we show here that these compounds interfere with NF-κB activity. In parallel, the genetic analysis of GR mutant mice defective in GR dimerization and GR binding to DNA (Reichardt et al., 1998), is in progress to assess the exact contribution of GR-mediated transactivation and transrepression in vivo. A scrutinized analysis of further studies using these GR^{dim} mice will allow to address questions on the molecular mechanism of the GR and indicate how "dissociated ligands", as the ones described here, may exert their effects in a promoter- and tissue-specific manner. In conclusion, dissociated ligands may have a great potential as novel pharmacologic tools in the treatment of a large variety of diseases in which GRs play a critical role, including inflammatory affections and immunological disorders.

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Send reprint requests to: Dr. Michèle Resche-Rigon, Hoechst Marion Roussel, Molecular and Cellular Biology, Bone Disease Group, 102, route de Noisy, 93235 Romainville Cedex, France. E-mail: michele.resche-rigon@hmrag.com

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