Negative Cross-Talk between RelA and the Glucocorticoid Receptor: A Possible Mechanism for the Antiinflammatory Action of Glucocorticoids

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Glucocorticoids are efficient antiinflammatory agents, and their effects include transcriptional repression of several cytokines and adhesion molecules. Whereas glucocorticoids down-regulate the expression of genes relevant during inflammation, nuclear factor (NF)-κB/Rel proteins function as important positive regulators of these genes. The expression of intercellular adhesion molecule-1 (ICAM-1), which plays an essential role in recruitment and migration of leukocytes to sites of inflammation, is also down-regulated by glucocorticoids. We found that a functional NF-κB site in the ICAM-1 promoter, which can be activated by either 12-O-tetradecanoylphorbol-13-acetate or tumor necrosis factor-α (TNFα), is also the target for glucocorticoids. In this report we present evidence that the ligand-activated glucocorticoid receptor (GR) is able to repress RelA-mediated activation of the ICAM-1 NF-κB site. Conversely, transcriptional activation by GR via a glucocorticoid response element is specifically repressed by RelA, but not by other NF-κB/Rel family members. Mutational analysis of GR demonstrates that the DNA binding domain and the ligand binding domain are required for the functional repression of NF-κB activation. Despite the importance of the DNA binding domain, we found that the transcriptional repression of NF-κB, mediated by GR, is not caused by binding of GR to the ICAM-1 NF-κB element, but by a physical interaction between the GR and RelA protein. The repressive effect of GR on NF-κB-mediated activation was not shared by other steroid/thyroid receptors. Only the progesterone receptor, which belongs to the same subfamily as GR and which possesses high homology with GR, was able to repress NF-κB-mediated transcription. These studies highlight a possible molecular mechanism that can explain the antiinflammatory effects of glucocorticoid treatment during inflammation. (Molecular Endocrinology 9: 401–412, 1995)

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INTRODUCTION

Glucocorticoid hormones mediate their effects by binding to the intracellular glucocorticoid receptor (GR) (1), which, like other members of the steroid/thyroid hormone receptor superfamily, functions as a ligand-activated nuclear transcriptional regulator (2). Steroid hormone receptors contain specific functional domains for hormone binding, DNA binding, dimerization, and transactivation (3, 4). After ligand binding the GR protein selectively regulates transcription by binding to specific DNA sequences, termed glucocorticoid responsive elements (GREs) (5) and activates transcription of downstream coding sequences. GR is also capable of repressing gene expression either directly via a negative GRE (nGRE) (6–8) or indirectly via protein-protein interaction (9–12).

Glucocorticoids (GCs) are known for their antiinflammatory effects (13–15) and are therefore used in the treatment of many human diseases, including asthma and other inflammatory ailments (16–18). Dexamethasone (Dex), a synthetic glucocorticoid, is a potent inhibitor of gene transcription of many proinflammatory cytokines including tumor necrosis factor-α (TNFα) (19), granulocyte/macrophage colony-stimulating factor (GM-CSF) (20), interleukin-2 (IL-2) (21, 22), IL-3 (23), IL-5 (24, 25), IL-6 (26), and IL-8 (27). Furthermore the IL-2 receptor is down-regulated by GCs (28). These hormones also block the migration of leukocytes to sites of inflammation. Since adhesion molecules play a key role in this trafficking process (29), steroids are thought to have a direct inhibitory effect on the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) (30), and E-selectin (31). Despite the recognition of the importance of GCs for the inhibition of expression of cytokines and adhesion molecules, not much is known about the molecular mechanisms underlying these inhibitory effects (32).

In a recent report we characterized the human ICAM-1 promoter and described its inducibility by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and the cytokine TNFα, and the repressive effect by Dex on this induction. We found that both effects are mediated through a nuclear factor (NF)-κB-like site present in the ICAM-1 promoter (33).

NF-κB, like the GR, acts as a messenger transmitting the gene induction signal from the cytoplasm to the nucleus (34, 35). NF-κB is present in an inactive form in the cytoplasm where it is associated with an inhibitory molecule IκB (36–38). A great variety of very distinct agents including viral proteins (39, 40), mitogens (41), and several cytokines (42, 43) cause an as yet poorly understood alteration in IκB, allowing NF-κB to be released from the complex and rapid degradation of IκB (44). NF-κB subsequently translocates to the nucleus where it contacts its decameric DNA-binding motif as a homo- or heterodimer composed of 50 kilodaltons (kDa) (NFKB1) and 65 kDa (RelA) DNA-binding subunits and activates transcription (45–47). The RelA protein is primarily responsible for the transactivation function, mediated by a C-terminal transactivation domain that is absent from the NFKB1 protein (48, 49). The NF-κB proteins have been found to have structural similarity to the protein product of *c-rel* protooncogene (50, 51) and the *Drosophila* protein dorsal (52, 53). These transcription factors are all members of the NF-κB/Rel family which shows a high degree of homology in their 300-amino acid N-terminal region, termed the NF-κB/Rel/Dorsal (NRD) homology domain. This region mediates DNA binding, dimerization, nuclear localization, and interactions with IκB (54).

NF-κB/Rel proteins activate transcription of a variety of genes encoding immunologically relevant proteins. Genes like TNFα (19), IL-2 (21), IL-2 receptor (55), IL-6 (56), IL-8 (57), ELAM-1 (58), granulocyte colony stimulating factor (59), and GM-CSF (59) all contain NF-κB sites in their promoter. The observation that all these genes are down-regulated by GCs suggests that NF-κB may be the key target for GCs. Using the ICAM-1 promoter NF-κB element to investigate how GCs exert their negative effects, we describe a direct physical interaction between activated GR and the RelA transcription factor. This interaction results in the repression of transactivation potential of both transcription factors. Further experiments revealed that the DNA-binding and, to a lesser extent, the hormone binding domain of the GR are required for the repression to occur. Despite the importance of the GR-DNA binding domain (DBD), we found that DNA binding of GR to the ICAM-1 NFκB site is not a prerequisite for repression of NF-κB-mediated activation. Taken together, we conclude that the physical interaction between GR and RelA provides a basis for the molecular mechanism of the inhibitory action of GCs on inflammatory processes.

RESULTS

NF-κB Activation Is Repressed by Dex, and Can Be Rescued by RU486

Previously we have shown that phorbol ester TPA- and TNFα-induced ICAM-1 protein and mRNA expression was inhibited by Dex in bronchial epithelial cells and in the monocytic cell line U937 (30). We also characterized the human ICAM-1 promoter and identified several *cis*-acting enhancer elements (33, 60). An NF-κB site present in the ICAM-1 promoter was found to be responsible for both the inducibility by TPA and TNFα, as well as for the repression by Dex (33). These results led us to explore the possibility that GR might interfere with the functioning of proteins involved in NF-κB transactivation. Transfection of GR-negative 293 cells with the 3xNF-κB(IC)tkLuc reporter construct, containing 3 NF-κB sites from the ICAM-1 promoter in front of the thymidine kinase (tk) promoter, followed by TNFα treatment of the cells, resulted in a 50-fold induction of luciferase activity (Fig. 1A, lane 2). Cotransfection of GR in the presence of Dex (1 μM) leads to potent inhibition (90%) of this induction (lane 4), and this repression was dose-dependent (lanes 4–8). The repressive effect by Dex was not observed on basal luciferase transcription (data not shown). RU486, a synthetic antiglucocorticoid that acts as a competitor against binding to the GR (61, 62), counteracted the repressive effect of Dex on NF-κB activation by TNFα in a dose-dependent manner, whereas RU486 alone had almost no effect on TNFα-mediated NF-κB activation (Fig. 1B). These data established that both GR and hormone are required for the glucocorticoid-mediated repression of NF-κB activation by TNFα.

Mutual Transcriptional Interference between RelA and the GR

To further explore the transcriptional interaction between NF-κB and GR, we activated the 3xNF-κB(IC)t-

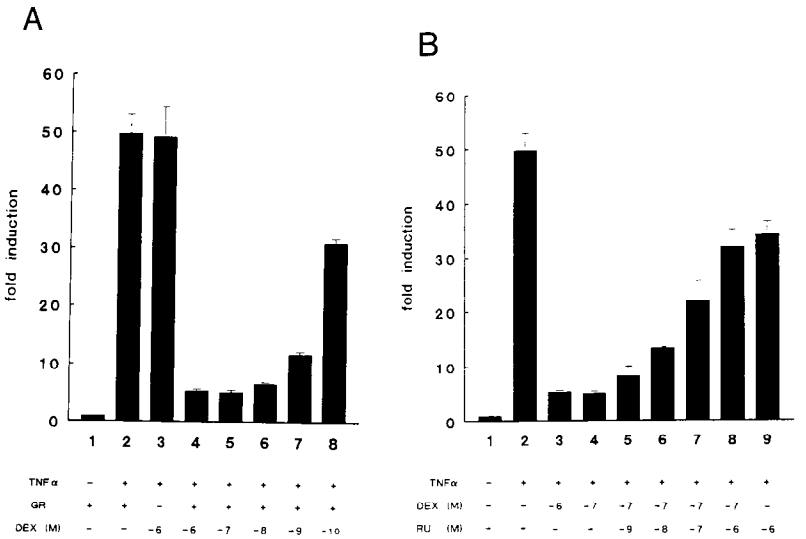

Fig. 1. TNFα-Mediated NF-κB Activation Is Repressed by Dex; This Repression Can Be Rescued by RU486

Human embryonal kidney 293 cells were transiently transfected with 2 μg 3xNF-κB(IC)tkLuc reporter construct, containing 3 NF-κB sites from the ICAM-1 promoter linked to the thymidine kinase promoter, together with 1 μg pCMV4-hGR expression vector. A, 24 h after transfection cells were stimulated for 16 h with TNFα (250 U/ml) either alone or together with variable concentrations of Dex (10^{-6} to 10^{-10} M). B, Cells were stimulated for 16 h with TNFα (250 U/ml) either without hormone or with variable concentrations of Dex (10^{-6} and 10^{-7} M), RU486 (10^{-6} to 10^{-9} M), or combinations of both. The values show the fold inductions of Luc activity in stimulated *vs.* untreated (luciferase units = 2695). These values are averages of five independent experiments performed *in duplo*, and *error bars* indicate the SEM. Details are described in *Materials and Methods*.

kLuc transfectants by overexpressing the RelA protein. RelA, which is primarily responsible for NF-κB transactivation (48), can form homodimers that stimulate transcription from certain enhancer motifs that are not recognized by either NFKB1 homodimers or NFKB1/RelA heterodimers (47, 57). We found that the ICAM-1 κB-like site, 5′-TGGAAATTCC-3′, which does not match the consensus κB-site, is activated by RelA homodimers (our unpublished data). We therefore transfected COS cells with a pCMV4-RelA expression vector either alone or in combination with a pCMV4-expression vector encoding the human (h) GR. COS cells were used in this experiment since they virtually lack endogenous NF-κB and GR proteins. Cotransfection of 100 ng RelA expression vector caused extensive activation of the 3xNF-κB(IC) reporter in COS cells (Fig. 2, lane 1). Cotransfection of RelA together with hGR in the presence of Dex resulted in repression of RelA-mediated transcription, in a dose-dependent manner (lane 2–4). This repression by GR was abolished by further addition of an excess of RelA protein (lane 5), which suggested that RelA and GR are not competing for a limiting common factor(s). Western blotting indicates that this repression was not caused by decreased expression of RelA through inhibition of the cytomegalovirus (CMV) promoter driving the RelA expression vector in the presence of GR (data not shown).

Since GR repressed RelA activity, we were interested to know whether the reverse process was also taking place. To test this possibility we cotransfected a $(GRE)_2$tkluc construct, containing two palindromic GR-binding sites coupled to the *tk* promoter, with expression vectors encoding the GR and different members of the NF-κB/Rel family. As shown in Fig. 3A, cotransfection of this reporter construct with a small amount of hGR expression vector (100 ng) and addition of Dex (10^{-6} M) resulted in a 50-fold increase in luciferase activity (lane 2). However, when GR was cotransfected together with RelA in the presence of Dex, the GR-induced luciferase transcription was reduced by RelA in a dose-dependent manner (lanes 3–6). This RelA-mediated repression was abrogated after addition of excess GR (lane 7). Because RelA shares the N-terminal 300-base pair Rel-homology domain with other members of the NF-κB/Rel family, we were interested to determine whether NFKB1 and c-Rel could also inhibit GRE activation by GR. As can be seen in Fig. 3B, cotransfection of expression vectors encoding the NFKB1 or the c-Rel protein together with GR showed that these two members had no effect on GRE transactivation. The data presented in Figs. 2 and 3 demonstrate that GR and RelA reciprocally repress their transcriptional activation potential.

Both the DBD and Ligand-Binding Domain (LBD) of hGR Are Required for the NF-κB Repression

To determine which region of the GR was required to inhibit transcriptional activation by NF-κB, several GR deletion mutants were analyzed in cotransfection studies in 293 cells. We observed that the three amino-terminal deletion mutants Δ9–205, Δ9–385, and Δ262–404 were at least as effective in repressing

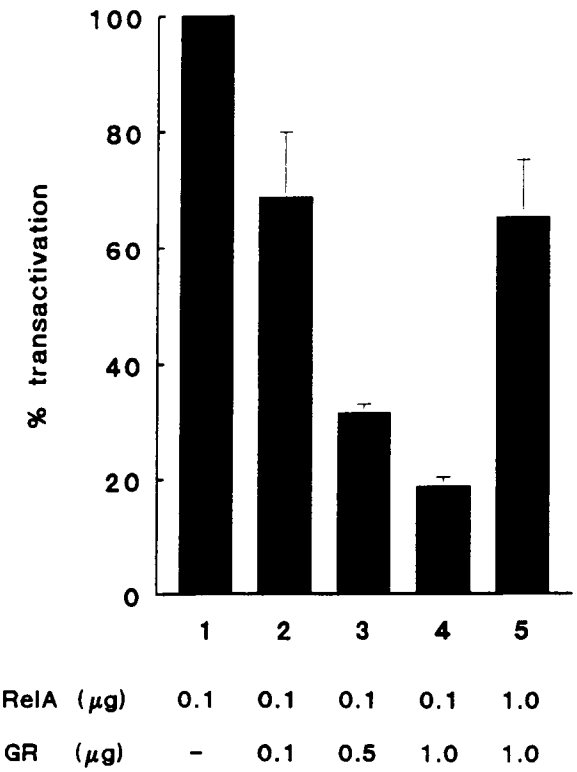

Fig. 2. RelA-Induced Transactivation of the ICAM-1 NF-κB Site Is Repressed by GR

Monkey COS cells were transiently transfected with 2 μg 3xNF-κB(IC)tkLuc construct together with 0.1 μg (lanes 1–4) or 1.0 μg pCMV4-RelA expression vector (lane 5). Various amounts of pCMV4-hGR, expressing the full length hGR, were cotransfected. The total amount of DNA was kept constant using an insertless pCMV4 expression vector. After transfection the cells were grown for 24 h in the presence of Dex (10^{-6} M), and Luc activity was measured. Values show the per cent transactivation compared with cells transfected with the insertless pCMV4 vector (luciferase activity = 750) and are averages of three independent experiments performed *in duplo*. *Error bars* indicate the SEM.

3xNF-kB(IC)tkLuc activation by TNFα as mediated by the wild type receptor (Fig. 4, lanes 2–4). However, after cotransfection of deletion mutant Δ428–490, which lacks the DBD, a complete loss of repression of NF-κB activation was observed (lane 5). Transfection experiments with a construct containing only the GR-DBD showed no repression of NF-κB activation (data not shown), indicating that probably other parts of the receptor are also involved. We also tested the carboxy-terminal truncation mutants 515* and 550* which both lack the LBD and are transcriptionally active in the absence of hormone. Transfection of these mutants resulted in a hormone-independent NF-κB repression (lanes 6 and 7). However, the repression was lower than observed with the wild type GR, suggesting that a part of the LBD is important for the repressive effect of GR. The importance of both the DBD and LBD for the repression was confirmed by the use of chimeric GR receptors in which these functional domains had been replaced with the corresponding domains of the retinoic acid receptor-α (RARα) receptor. Substitution of the GR-DBD for that of RARα resulted in a chimeric receptor (GRG) that failed to repress activation by NF-κB (lane 8). Interestingly, a mutant (RGR) in which the GR amino- and carboxy termini had been exchanged with the corresponding domains of RARα, was also unable to repress NF-κB-mediated activation. Together, these results indicate that both the DBD and the LBD of GR are probably involved in the negative cross-talk between GR and RelA.

GR and RelA, but Not NFKB1, Physically Associate with One Another, Independently of DNA Binding

The observed mutual interference of transcription could occur either by competition for a common or overlapping DNA binding site(s) or by physical interaction between GR and RelA. To test the possibility that DNA binding of GR to the NF-κB site is the mechanism for repression, we performed a transfection experiment with the 3xNF-κB(IC)tkLuc vector and a GR mutant GTG3A (63) in which three amino acids in the P-box of the DBD were mutated (Fig. 5A). This GR mutant recognizes a thyroid hormone response element and is unable to bind to a GRE. As can be seen in Fig. 5B (lane 2), cotransfection of this chimeric receptor results in a clear repression of NF-κB activation by TNFα, which is comparable to the result obtained with wild type GR receptor. In addition, no repression was observed with the GR mutant GTG, in which the entire GR-DBD was exchanged with that of thyroid receptor (TR) (64) (Fig. 5B, lane 3). The result with this chimeric receptor argues against the possibility that the repressive effect mediated by the GR mutant GTG3A was caused by binding of its DBD to the NF-κB site. We therefore conclude that DNA binding of GR to the ICAM-1 NF-κB site is not necessary for the observed repression. This observation also excludes the possibility that a factor induced by GCs via a GRE plays a significant role in the repression phenomenon. Next, we were interested to determine whether this mutual repression is achieved by a direct interaction between GR and NF-κB proteins. Therefore, we performed immunoprecipitation experiments with *in vitro* translated proteins. For this purpose we used the pRBal-117 vector (65) for *in vitro* translation of rat GR and the pRcCMV-RelA or NFKB1 vector for *in vitro* translation of RelA and NFKB1, respectively. In this experiment RelA or NFKB1 were ^{35}S-labeled and mixed with unlabeled rat GR (rGR), and after cross-linking with dithio*bis*[succinimidyl propionate] (DSP) an immunoprecipitation was performed with a monoclonal antibody against GR. Figure 6 shows that using translation mix with RelA and GR resulted in a specific immunoprecipitated band of 65 kDa (lane 3), which migrates at the same height as the ^{35}S-labeled RelA input (lane 1); this band could not be detected in translation mix without GR (lane 4). In contrast, NFKB1

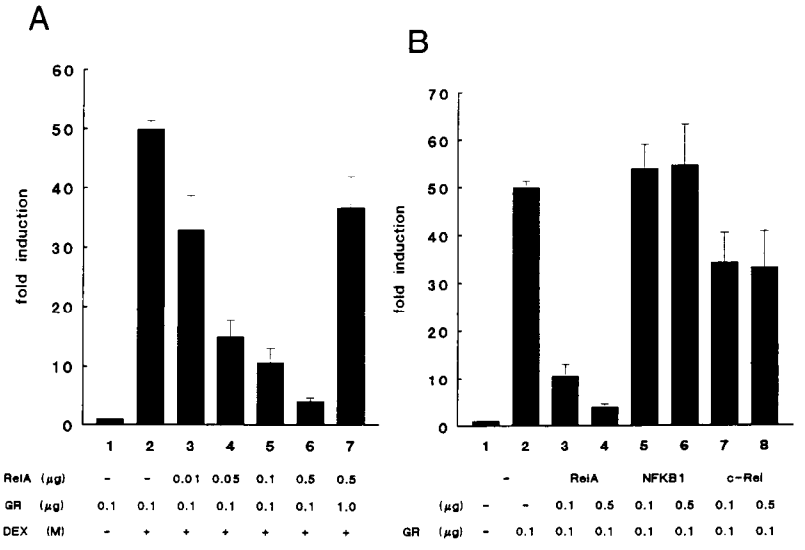

Fig. 3. RelA Inhibits Activation of GRE-tk-Luc Transcription by GCs

Monkey COS cells were transiently transfected with 2 μg $(GRE)_2$tkluc reporter construct. A, Cells were cotransfected with 0.1 μg (lanes 1–6) or 1.0 μg pCMV4-hGR expression vector (lane 7) together with various amounts of pCMV4-RelA expression vector. B, Cells were cotransfected with a constant amount of pCMV4-hGR (0.1 μg) together with different amounts of pCMV4-RelA (lanes 2 and 3), pCMV4-NFKB1 (lanes 4 and 5), and pCMV4-cRel (lanes 6 and 7). The total amount of DNA was kept constant using an insertless pCMV4 expression vector. The cells were either without hormone or treated with Dex (10^{-6} M), and 24 h later Luc activity was measured. The values show fold induction of Luc activity of Dex-stimulated cells *vs.* untreated cells (luciferase activity = 1200) and are averages of three independent experiments performed *in duplo*. *Error bars* indicate the SEM.

did not show any interaction with GR under these conditions (lanes 5 and 6).

Effects of Other Steroid or Retinoid Receptors on NF-κB Transactivation by TNFα

The above experiments demonstrate that GR interferes with the function of NF-κB. Next, we addressed the question whether this inhibitory effect was also seen with other members of the steroid/TR superfamily. We therefore cotransfected 293 cells with the 3xNF-κB(IC)tkLuc vector together with expression vectors encoding for the hGR and rGR, human progesterone receptor (hPR), human estrogen receptor (hER), and the human RARα (hRARα). As shown in Fig. 7, cotransfection of either the hGR or the rGR expression vector in the presence of Dex (10^{-6} M) led to a 10-fold repression of the TNFα-induced NF-κB activity. Addition of an expression vector encoding the hPR_B which is characterized by strong homology within the DBD and LBD of GR, resulted in a ligand-dependent (Org2058, 10^{-7} M) reduction of 50% of TNFα-induced NF-κB activation; this repression could not be further enhanced by an excess of 5 μg PR_B expression vector (data not shown). However, cotransfection of ER in the presence of 17β-estradiol (10^{-9} M) did not reduce NF-κB activation by TNFα. In contrast, addition of an expression vector encoding RARα, in the presence of RA (10^{-6} M), showed an increased NF-κB activation in combination with TNFα. These results demonstrate that the transcriptional repression of TNFα-mediated NF-κB activation is restricted to the members of the steroid/TR superfamily that are known to possess strong homology with the DBD and LBD of GR.

DISCUSSION

It has been known for some time that GCs have antiinflammatory properties and are therefore used for the treatment of inflammatory disorders, *e.g.* asthma (17). They inhibit the release of several proinflammatory mediators, thereby affecting the distribution, growth, differentiation, and function of neutrophils, monocytes, lymphocytes, and eosinophils (66, 67). These antiinflammatory effects of GC on responsive cells are likely to be due to the activation of GRs which subsequently activate or repress transcription of certain target genes. Of particular interest is the repression of gene transcription, since the mechanisms underlying these repressive effects are less well understood. We have previously found that GCs down-regulate basal- and TNFα-induced ICAM-1 expression at the transcriptional level (30). Transcriptional repression by GCs can be achieved by direct interaction with a site on the DNA designated as "nGRE" (6, 8). However, no such nGRE was found in the ICAM-1 promoter. Interestingly, an NF-κB-like site in the ICAM-1 promoter, which is responsible for the activation by TNFα, is also the target for GC-mediated repression (33). In the present study we tried to elucidate the molecular mechanism underlying this transcriptional interference. For the interference between

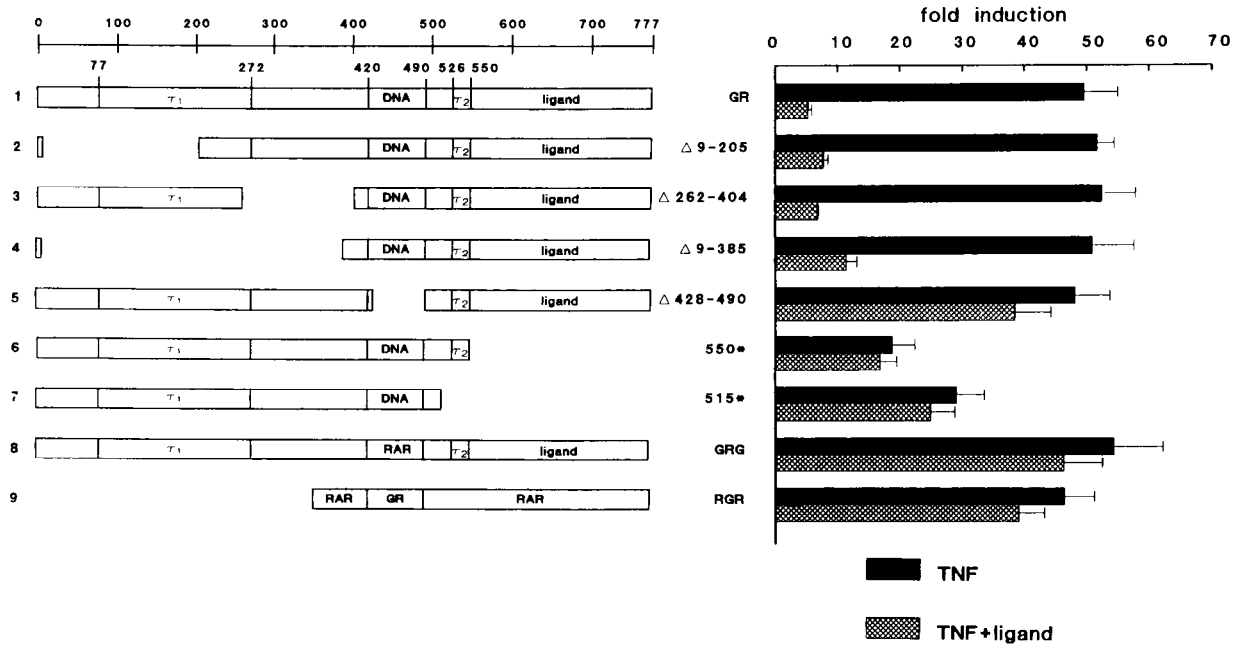

Fig. 4. Deletion Analysis of hGR

Several GR mutants were assayed for their ability to repress activity of the 3xNF-κB(IC)tkLuc reporter. The wild type receptor consists of two activation domains (τ1 and τ2), the DBD (DNA), and a LBD (ligand). On the *horizontal scale* amino acid numbers are indicated. The GR deletion mutants are described elsewhere (82–84); 293 cells were cotransfected with 2 μg 3xNF-κB(IC)tkLuc reporter construct and 1 μg of the indicated GR deletion mutant. After transfection, cells were stimulated for 16 h with TNFα (250 U/ml) alone or in combination with Dex (10^{-6} M). Cells were harvested and Luc activity was measured. Details are described in the legend of Fig. 1.

two transcription factors several mechanisms can be envisaged: 1) Competition for a common or overlapping binding site, 2) physical interaction between two proteins either directly or mediated by a third protein, 3) competition for a common factor required for the activity of either transcription factor, 4) alteration or induced expression of a regulatory protein (*e.g.* IκB) by GCs, thereby inhibiting NF-κB activation.

In this report we demonstrate the functional and physical association between RelA and GR, which are members of two distinct transcription factor families, NF-κB/Rel and the steroid/TR superfamily, respectively. The functional association was characterized by the inhibition by hormone-activated GR of TNFα- and RelA-induced expression of a promoter containing three ICAM-1 NF-κB sites. In the converse experiment, RelA repressed transcriptional activation mediated by GR.

Our results indicate that it is not very likely that this cross-inhibition between GR and RelA is caused by competition for limiting cofactors that mediate their action in the nucleus. This is based on the observation that addition of an excess of either RelA or GR expression vector rescued the repressive effect of GR or RelA, respectively. Furthermore, this mutual repression was limited to GR and RelA, since other steroid/thyroid family members, such as ER, RARα, and TR, were not able to repress NF-κB activity, and NFKB1 and c-Rel did not repress GR activity. This is in contrast to recently published data in which ER was shown to inhibit IL-1 and RelA-mediated IL-6 promoter activation (68). Although we have no explanation for this discrepancy; cell type-, inducer-, and sequence-specific differences could be the reason. We found that only PR, which has high sequence homology within the DBD and LBD with GR, was able to repress TNFα-induced NF-κB activation. This suggests that a specific sequence in these domains is essential for the negative cross-talk between GR and RelA. The importance of the DBD is further supported by the observation that a GR protein that lacks its DBD is unable to repress transcriptional activation by NF-κB. The GR deletion mutants without the LBD are still able to repress TNFα-mediated NF-κB activation, although less effectively than wild type GR. In addition, by the use of chimeric constructs in which the DBD or the LBD were replaced with similar domains of RARα, we concluded that both domains are important for the repression mediated by GR. Interestingly, a mutant receptor with a three-amino acid mutation in the P-box, which is unable to bind to a GRE, was still able to repress NF-κB activity. This means that despite the importance of the DBD, DNA binding of GR to the ICAM-1 NF-κB site is not necessary for the observed NF-κB repression. These findings further argue against the induction by Dex of a secondary inhibitor protein of NF-κB function (*e.g* IκB). This result paralleled the observation that the repressive effect of Dex on induced ICAM-1 expression in cells remained present

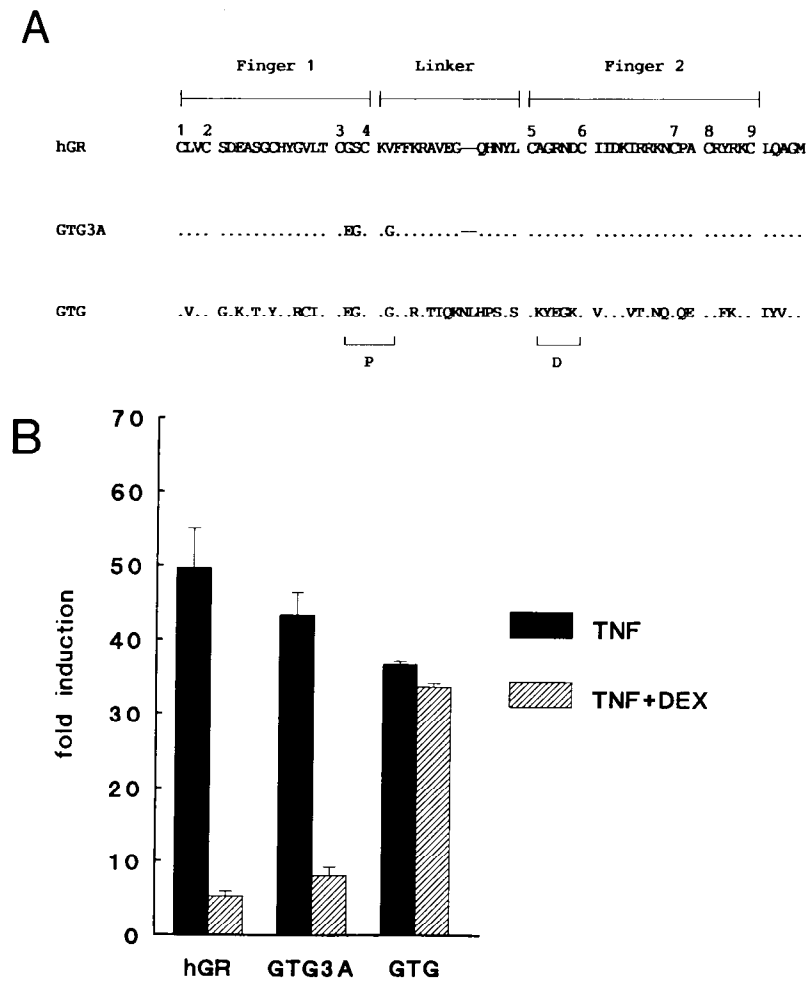

Fig. 5. Effects of GR-DBD Mutants on TNFα-Mediated NF-κB Activation

A, Schematic presentation of the DBDs of the wild type hGR, GTG3A, which contains the hGR-DBD with a three-amino acid mutation, and GTG which is a chimeric GR containing the hTR-DBD. *Upper numbers* (1–9) correspond to nine conserved cysteines. *Dots* show identical amino acids comparing hGR with hTR. The proximal (P) and distal (D) elements are indicated. B, 293 cells were transiently transfected with the 3xNF-κB(IC)tkLuc reporter construct, and 1.0 μg of the indicated mutated GR expression vector. After transfection, cells were stimulated for 16 h with TNFα (250 U/ml) alone or in combination with Dex (10^{-6} M). Cells were harvested and Luc activity was measured. Details are described in the legend of Fig. 1.

after pretreatment with the protein synthesis inhibitor cycloheximide (30).

The observed mutual transcriptional inhibition between RelA and GR is likely to be mediated by a physical interaction between the two proteins. Our cross-linking experiments demonstrated a clear interaction between RelA and GR of *in vitro* translated proteins in the absence of DNA. This finding is perfectly in line with data described recently (69). The specificity of this physical interaction is demonstrated by the finding that NFKB1, which does not interfere with GR-mediated activation, is not able to associate with GR. Recently, increasing evidence has been obtained that protein-protein interactions are involved in the interplay between different transcription factors. Physical and functional interactions, usually by dimerization, of factors within a given family are well documented. However, recent reports show that these interactions also occur between different families. One example is the interaction between the GR and the leucine zipper proteins Fos and Jun (9, 10). Another example is the interaction between the Rel homology domain of NF-κB and the basic leucine zipper (bZIP) region of C/EBP and the bZIP transactivator of Epstein-Barr virus (BZLF1) (70–72). Moreover, interaction has been described between the NF-κB proteins (RelA and NFKB1), and Fos and Jun (73). The domains that are responsible for homodimerization of the respective proteins also appear to be important in the interaction with distinct proteins. We have found that the DBD of GR is necessary for the interference with the NF-κB-mediated response. It may be argued that the most likely region of NF-κB to be involved in this interaction is the conserved Rel-homology domain. However, our results contradict the importance of this domain, since NFKB1 and c-Rel, which also contain this Rel homol-

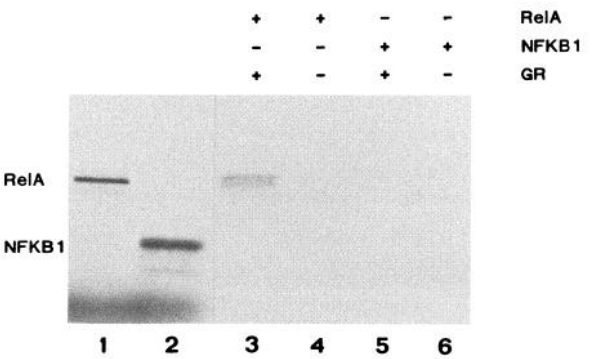

Fig. 6. Coimmunoprecipitation of RelA and the GR

In vitro translated [^{35}S]methionine-labeled RelA or NFKB1 was mixed and incubated with rabbit reticulocyte lysate programmed and unprogrammed with unlabeled GR for 30 min at room temperature. Lanes 3–6 show immunoprecipitated proteins with a monoclonal anti-GR antibody, after cross-linking with 5 mM DSP. Lanes 1 and 2 show the input of labeled RelA and NFKB1, respectively. Proteins were separated on a 8% (wt/vol) SDS-polyacrylamide gel electrophoresis gel followed by autoradiography.

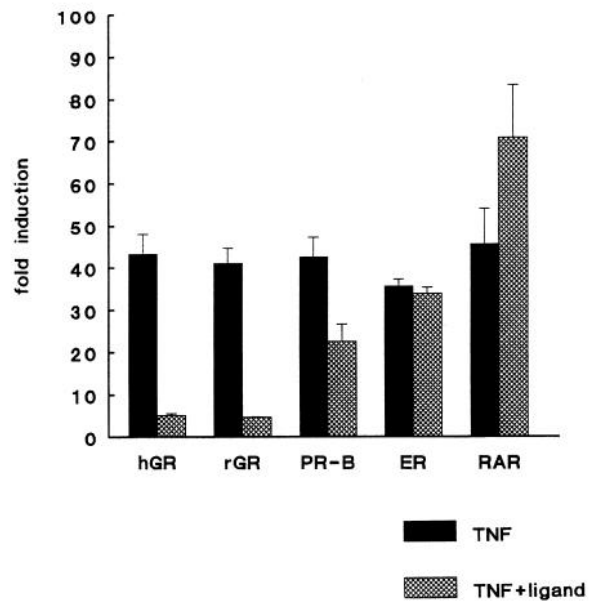

Fig. 7. Effects of Other Steroid or Retinoid Receptors on TNFα-Mediated NF-κB Activation

293 cells were transfected with 2 μg 3xNF-κB(IC)tkLuc reporter construct and 1 μg of the indicated expression vector. After transfection cells were stimulated for 16 h with TNFα (250 U/ml) alone or in combination with either Dex (10^{-6} M), Org2058 (10^{-7} M), E_2 (10^{-9} M), and RA (10^{-6} M). Cells were harvested and Luc activity was measured. Details are described in the legend of Fig. 1.

ogy domain, were not able to repress GR activity. A different region, the (mini) leucine zipper domain in the carboxy terminal of RelA, could be essential for the interaction with GR. This would be in line with the importance of leucine zipper regions of, for instance, c-jun interaction with other proteins. Experiments are in progress to delineate the precise region in RelA that is involved in its interaction with GR. The outcome of our study demonstrates that the interaction between GR and RelA is probably a general mechanism by which GCs down-regulate the expression of several genes during inflammation. This conclusion is further supported by a recent report that this physical interaction between GR and RelA also plays a role in the down-regulation of IL-1-induced IL-6 expression (69). In addition to the observed interaction, we provide evidence that the DBD and LBD are necessary for the repressive effect by GR on TNFα-mediated NF-κB activation. While deletion mapping showed that the DBD of the GR is important, specific mutations in the GR DBD exclude the possibility that GR competes with RelA for the ICAM-1 NF-κB site. Additional support comes from a number of reports that show that inflammatory mediators such as IL-2 (22), IL-2 receptor (28), IL-3 (23), IL-5 (24, 25), IL-8 (74), GM-CSF (20), TNFα (19), the inducible form of nitric oxide synthetase (75), and phospholipase A2 (76) are down-regulated by GCs. Since most of these genes contain NF-κB sites in their respective promoters, it can be assumed that physical interaction between GR and RelA, leading to inhibition of NF-κB activation, could be a general mechanism, whereby GCs mediate their antiinflammatory action. The general significance of this hypothesis is currently being tested.

MATERIALS AND METHODS

Special Reagents and Antibodies

Recombinant human TNFα (hTNFα) was obtained from Boehringer Mannheim (Indianapolis, IN). Dexamethasone, 17β-estradiol (E_2), retinoic acid (RA), and T_3 were obtained from Sigma Chemical Co (St Louis, MO). The progestin Org2058 was provided by Organon International (Oss, The Netherlands). RU486 was obtained from Roussel-UCLAF (Paris, France). Dithio*bis*[succinimidyl propionate] (DSP) was from Pierce Chemical Co (Rockford, IL). *In vitro* translation was done using the rabbit reticulocyte lysate system from Promega (Madison, WI). The monoclonal antibody 250 against the rat GR (rGR) has been described previously (77).

Cell Culture

Human 293 embryonal kidney cells and monkey COS1 cells were grown at 37 C in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium, buffered with bicarbonate and supplemented with 7.5% fetal calf serum.

Expression Vectors and Reporter Plasmids

The luciferase reporter plasmids 3xNF-κB(IC)tkLuc and $(GRE)_2$tkluc have been described elsewhere (33, 78). The pCMV4 expression plasmid contains a fragment of the CMV immediate early promoter/enhancer (79). Plasmid pCMV4-cRel, which contains human c-Rel cDNA (a gift from S. Doerre), has been described elsewhere (80). Plasmid pCMV4-p50 was created by inserting the cDNA (amino acid 1 to 399) from RcCMC-p50 into the *Hin*dIII/*Xba*I sites of pCMV4. pCMV-p65 contains the full length cDNA of RelA which was

derived from RcCMV-p65 (RelA) and cloned into the *Hin*dII/*Xba*I sites of pCMV4 (RcCMV-p50 and RcCMV-p65 were a gift from P. Baeuerle). Plasmids pCMV4-hGR (2) and pCMV4-rGR (81) contain the full length cDNAs encoding hGR and the rGR, respectively, inserted into the pCMV4 expression vector. The hGR deletion mutants Δ9–205, Δ9–385, Δ262–404, Δ428–490, 515*, 550*, GRG, and RGR have been described (82–84). pMT-hGR (85) encodes the hGR and is under control of the SV40 enhancer and metallothionein promoter. Expression vector pRSV-GTG (64) encodes a chimeric receptor in which the hGR N- and C-terminal domains have been fused to the DBD from the human TR. pRSV-GTG3A (63) contains the hGR cDNA in which three amino acids in the P-box in the DBD have been mutated corresponding to the human TR. The SV40 early promoter containing mammalian expression vector pSG5 containing the cDNA of either the hPR (86), hER (87), and hRARα (88) have been described. The pSV-lacZ construct has been previously described (89). The pRBal-117 (65) was used for *in vitro* translation of rGR from the SP6 promoter, and the RcCMV-p50 and RcCMV-RelA were used for *in vitro* translation of p50 and RelA, respectively, from the T7 promoter.

Transient Transfections

For transfection experiments, 293 cells were seeded at 3×10^5 cells per well and COS cells at 2×10^5 cells per well in six-well plates (Costar Corp., Cambridge, MA). Twenty four hours later, the cells were transfected with 10 μg supercoiled plasmid DNA by the calcium phosphate coprecipitation technique. The mixture contained 2 μg reporter plasmid and 3 μg pSV-LacZ plasmid (internal control for transfection efficiency). Cotransfection of additional expression vectors is indicated in the figure legends. The total amount of DNA was always adjusted to 10 μg with SK^-. After 16–20 h exposure to the calcium phosphate precipitate, medium was refreshed and cells were left untreated or incubated for 16 h with human TNFα (250 U/ml) alone or together with Dex (10^{-6} to 10^{-10} M), RU486 (10^{-6} to 10^{-9} M), Org2058 (10^{-7} M), E_2 (10^{-9} M), T_3 (10^{-6} M), or RA (10^{-6} M). Transfected cells were subsequently harvested for luciferase assay (90) and lacZ determination (91).

Association of Proteins *in Vitro*

Proteins were translated *in vitro* using the rabbit reticulocyte lysate system from Promega. Twenty five microliters of rGR translation mix were incubated with 45 μl ^{35}S-methionine labeled RelA or NFKB1 translation mix in lysate incubation buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg aprotinin/ml, and 1 μM Dex) at room temperature for 30 min. As a control a parallel lysate incubation was used, in which unprogrammed rabbit reticulocyte lysate was mixed together with labeled RelA or NFKB1. To cross-link proteins that interacted with GR, 5 mM DSP was added to the reactions and incubated 5 min at room temperature after which reactions were terminated by the addition of 0.1 M ethanolamine. Immunoprecipitation (IP) of rGR and cross-linked proteins was performed with a monoclonal anti-rGR antibody, 250 (also designated as No. 7, Ref. 77) and covalently linked to cyanobromide-activated Sepharose. The IP reactions were performed in IP buffer, containing 10 mM Tris-HCl (pH7.5), 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.1 mM ethanolamine, 1 mM phenylmethylsulfonyl fluoride, 10 μg aprotinin/ml, and 1 μM Dex, at 4 C rotating for 16 h. The immune complexes were washed extensively with 0.05% Tween 20-containing PBS (10 mM Na_2HPO_4, 3 mM KCl, 400 mM NaCl) at pH 7.4 and boiled for 5 min in sodium dodecyl sulfate (SDS) loading buffer and loaded to 8% SDS-polyacrylamide gel electrophoresis gels. After electrophoresis the gel was dried and subjected to autoradiography for 8 h.

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Erratum

In the article "A combination of distal and proximal regions is required for efficient prolactin regulation of transfected rabbit αs_1-casein chloramphenicol acetyltransferase constructs" by Sandra Pierre, Geneviève Jolivet, Eve Devinoy, and Louis-Marie Houdebine (*Molecular Endocrinology* 8:1720–1730, 1994), two inadvertent errors appeared.

1. The reference for the cell culture method in *Materials and Methods* has been omitted. It should have been cited at the end of the first sentence of the first paragraph in this section. The reference is as follows: Lesueur L, Edery M, Paly J, Clark J, Kelly PA, Djiane J 1990 Prolactin stimulates milk protein promoter in CHO cells cotransfected with prolactin receptor cDNA. Mol Cell Endocrinol 71:R7–R12.

2. The authors listed in Reference 18 should be: Edery M, Jolicoeur C, Levi-Meyruels C, Dusanter-Fourt I, Petridou B, Boutin JM, Lesueur L, Kelly PA, Djiane J.