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Characterization of Mechanisms Involved in Transrepression of NF-κB by Activated Glucocorticoid Receptors

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Glucocorticoids are potent immunosuppressants which work in part by inhibiting cytokine gene transcription. We show here that NF-κB, an important regulator of numerous cytokine genes, is functionally inhibited by the synthetic glucocorticoid dexamethasone (DEX). In transfection experiments, DEX treatment in the presence of cotransfected glucocorticoid receptor (GR) inhibits NF-κB p65-mediated gene expression and p65 inhibits GR activation of a glucocorticoid response element. Evidence is presented for a direct interaction between GR and the NF-κB subunits p65 and p50. In addition, we demonstrate that the ability of p65, p50, and c-rel subunits to bind DNA is inhibited by DEX and GR. In HeLa cells, DEX activation of endogenous GR is sufficient to block tumor necrosis factor alpha or interleukin 1 activation of NF-κB at the levels of both DNA binding and transcriptional activation. DEX treatment of HeLa cells also results in a significant loss of nuclear p65 and a slight increase in cytoplasmic p65. These data reveal a second mechanism by which NF-κB activity may be regulated by DEX. We also report that RU486 treatment of wild-type GR and DEX treatment of a transactivation mutant of GR each can significantly inhibit p65 activity. In addition, we found that the zinc finger domain of GR is necessary for the inhibition of p65. This domain is also required for GR repression of AP-1. Surprisingly, while both AP-1 and NF-κB can be inhibited by activated GR, synergistic NF-κB/AP-1 activity is largely unaffected. These data suggest that NF-κB, AP-1, and GR interact in a complex regulatory network to modulate gene expression and that cross-coupling of NF-κB and GR plays an important role in glucocorticoid-mediated repression of cytokine transcription.

Glucocorticoids have long been used as effective immunosuppressive agents in the treatment of conditions involving T-cell- or cytokine-mediated tissue damage. These steroids have been shown to block inflammation, suppress immune system activation, and act as growth-inhibitory agents both in vivo and in vitro (23). Surprisingly, despite the lengthy history of the use of glucocorticoids as therapeutic agents, the mechanism by which they perform these functions is largely unknown.

Studies of the effect of glucocorticoid administration on the immune system have resulted in a number of important observations. Glucocorticoids induce a rapid redistribution of lymphocytes from the circulation to other lymphoid compartments (23). In addition, glucocorticoids potently suppress lymphocyte accessory function, the clonal expansion of T lymphocytes, and the secretion of cytokines (23, 73). Interestingly, cytotoxic T-lymphocyte clones provided with exogenous interleukin 2 (IL-2) are able to proliferate in response to mitogenic stimulation in the presence of glucocorticoids (28). These data suggest that the block of cytokine secretion plays an important role in glucocorticoid-mediated immunosuppression. Indeed, glucocorticoid administration represses the de novo transcription of a number of cytokine genes, including those for IL-1, IL-2, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF-α), and gamma interferon (22, 23, 77).

Glucocorticoids function by binding to specific cytoplasmic receptors, allowing the complex to translocate into the nucleus. Glucocorticoid receptors (GR) are members of the steroid hormone receptor superfamily, all of which contain a homologous DNA-binding domain and divergent C-terminal ligand-binding domains (9, 26). Activated GR have been shown to bind to specific DNA elements, glucocorticoid response elements (GREs) and negative GREs, resulting in transcriptional activation and repression, respectively (25, 62, 81). Many cytokine genes have been cloned, and their promoters have been analyzed. Interestingly, nGRE consensus sequences have not been identified in these promoters, suggesting that GR inhibit cytokine gene transcription through a different mechanism.

Recently, a number of workers have described a mechanism of GR-mediated transcriptional repression involving the physical interaction of GR and AP-1 (24, 35, 46, 68, 82). This interaction, termed cross-coupling, results in glucocorticoid-mediated repression through AP-1-responsive elements. NF-κB, an activator of a broad class of immune system genes, has also been shown to cross-couple with other transcription factor families, including AP-1 (74), SP1 (56), and C/EBPβ (75). Interaction of NF-κB and AP-1 results in the synergistic activation of both κB and AP-1 response element function, while interaction of NF-κB and C/EBPβ results in the enhancement of C/EBPβ and the repression of κB response element function. As NF-κB sites have been mapped for a large number of cytokine promoters and shown to be necessary for stimulation of cytokine gene transcription (3, 29, 36, 44, 45, 66, 72, 83), this transcription factor, in addition to AP-1, is an excellent candidate as a target for glucocorticoid-mediated repression of cytokine expression.

NF-κB, originally identified and named for its role in the regulation of immunoglobulin kappa chain gene expression in B cells (43, 70), is a heterodimer of a 50-kDa subunit (p50; NFKB1) and a 65-kDa subunit (p65; RelA) (6, 39). These

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subunits, along with the more recently cloned p52 (NFKB2) and RelB proteins, share homology with the products of the c-*rel* oncogene and *Drosophila dorsal* maternal effect gene (15, 27, 41, 47, 53, 60, 61, 64). The p50 and p52 subunits are derived from 105- and 100-kDa precursors, respectively, by proteolytic cleavage. It has been shown both in vitro and in vivo that NF-κB/Rel proteins can dimerize to produce a number of different NF-κB-like factors (32, 41, 53, 60, 80). Presynthesized NF-κB is stored in the cytoplasm bound to the inhibitor, IκB (4, 5). In addition, NF-κB subunits can be found sequestered in the cytoplasm by the dimeric association with NF-κB precursor molecules (48, 59, 63). IκB and NF-κB precursors share domains encoding the so-called ankyrin repeats which have been shown to play an important role in both blocking NF-κB binding to DNA and masking the nuclear localization signal (12, 14, 34). A wide variety of stimuli including mitogens, cytokines, lipopolysaccharide, reactive oxygen intermediates, and viral infection rapidly induce the dissociation of IκB, allowing NF-κB to translocate to the nucleus (7, 8, 12, 43, 44, 52, 67, 69). These inducers lead to the phosphorylation and rapid degradation of IκB (11, 17, 76). Thus, NF-κB is part of a richly complex regulatory network which plays a critical role in the rapid induction of cytokines and other immune system genes.

In this study, we demonstrate with transfected cells and with endogenous GR and NF-κB that activated GR are capable of blocking the ability of NF-κB to activate gene expression, as recently described (49, 58). We show that GR can physically interact with NF-κB subunits and also block their ability to bind DNA. In addition, we present new data showing that dexamethasone (DEX) treatment of HeLa cells causes a significant reduction in nuclear p65 protein levels. Thus, glucocorticoids are also able to inhibit NF-κB activity by a novel mechanism involving a block of cytokine-induced nuclear translocation. In addition, we show that the zinc finger region of GR is required for the inhibition of NF-κB but that GR-mediated transactivation cannot entirely account for the repression of NF-κB. Given the role of NF-κB in the processes of T-cell activation and inflammation, we propose that GR interaction with the NF-κB system represents an important mechanism for the immunosuppressive properties of glucocorticoids.

MATERIALS AND METHODS

Cell culture. COS-7 cells were maintained in Iscove's Dulbecco's minimal essential medium (DMEM), as modified by Iscove with 5 mM glutamine, 7.5% fetal calf serum (FCS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml and passaged every 3 days. F9 cells were maintained in the same medium and passaged every 2 days. Plates for F9 cultures were pretreated with 0.1% gelatin (Sigma) in phosphate-buffered saline (PBS) to increase cell adhesion. Adherent HeLa cells were maintained in Eagle's MEM supplemented with 10% FCS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml and passaged every 3 days. For certain transfection experiments, prior to DEX (Steraloids, Inc.) treatment, the medium was replaced with fresh medium containing charcoal-stripped FCS (HyClone).

Plasmid construction. Expression vectors encoding fos, jun, and the NF-κB subunits p50, p65, and c-rel consist of human cDNAs cloned into the pCMV4T plasmid as described elsewhere (2, 74, 75). pCYGR was constructed (by Y. Itoh-Lindstrom and J.A.C.) by isolating a 3.0-kb *Kpn*I-*Xho*I cDNA fragment of the pRSVhGR plasmid (54) encoding the human GR and cloning it into the pCMV5 plasmid. pCMV4T and pCMV5 have identical promoters. Δ77/262GR was constructed by deleting a *Bgl*II fragment from pYCGR. Δ428/490GR was a generous gift from Ron Evans, Salk Institute. The NF-κB reporters, 3xMHC CAT and 3xMHCLUC, consist of three copies of the major histocompatibility complex (MHC) class I *H-2K*^{b} NF-κB site cloned upstream of a minimal *fos* promoter chloramphenicol acetyltransferase (CAT) expression vector (63) and the luciferase reporter construct pGL2-Basic (Promega), respectively. The 3xMHCLUC construct was a generous gift of T. Mitchell, University of Wisconsin. The AP-1 reporter, AP-1CAT, consists of five copies of the collagenase AP-1 site cloned upstream of a TK promoter CAT expression vector (74). The GR reporter, GRECAT, was cloned as described previously (1).

Transfections, CAT assays, luciferase assays, and protein assays. COS cells were transfected by the DEAE-dextran method as described elsewhere (38). DEX was added to a final concentration of 10^{-7} M immediately after the chloroquine-containing medium was replaced by fresh medium. The plates were harvested 24 to 48 h later. For CAT assays, the cells were resuspended in 0.1 ml of 0.25 M Tris (pH 7.4), lysed by four cycles of freeze-thaw, and incubated for 10 min at 70°C to inactivate endogenous inhibitors of CAT. For luciferase assays, plates were incubated with reporter lysis buffer (Promega) and cells were processed as recommended by the manufacturer. F9 and HeLa cultures were transfected by the calcium phosphate method as described previously (31). The DNA-$Ca_3(PO_4)_2$ solution remained in contact with the cells for 16 h, after which the plates were washed with PBS and fresh medium was added. For F9 experiments, DEX was added at this time to a final concentration of 10^{-7} M, and the cells were harvested 18 to 22 h afterwards. HeLa cells were allowed to recover for 4 h after removal of the crystals and then treated with DEX or RU486 (gift of D. Gallet, Roussel Uclaf) as described above. TNF-α (2 ng/ml) or IL-1 (10 ng/ml) was then added 20 h later, and the HeLa cultures were harvested an additional 24 h later. Cell extracts were stored at −20°C until assayed. CAT activities were determined either by a scintillation counting assay (51) or by thin-layer chromatography (30). Luciferase activity was measured by combining 50 to 100 μl of cell extract with a reaction buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), and 15 mM $MgSO_4$. Light output was measured with an Autolumat LB953 luminometer (Berthold). Protein was assayed by method of Bradford (16) by using Bio-Rad dye reagent according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA) and immunoblotting. COS or HeLa cells were washed and scraped in PBS and transferred to microcentrifuge tubes. Cell pellets were resuspended in 100 to 200 μl of lysis buffer (10 mM Tris [pH 8.0], 60 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.1% Nonidet P-40 [NP-40], 1 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated on ice for 5 min. The cell membranes were then pelleted by centrifugation (pulsing for 5 s at 4°C), and the cytoplasmic extracts were removed to fresh tubes. Glycerol was later added to make a 20% solution, and the cytoplasmic extracts were quick-frozen on dry ice and stored at −80°C until used. The pelleted nuclei were immediately washed in 1 ml of lysis buffer without NP-40, spun as described above, and resuspended in 50 μl of buffer C {20 mM HEPES [pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 2 mM DTT, 20% glycerol, 1 mM PMSF}. NaCl (5 M) was then added to bring the salt concentration to 0.4 M, and the nuclei were extracted on ice for 10 min with occasional vortexing. The nuclei were then pelleted, and supernatants were collected as nuclear extracts, quick-frozen on dry ice, and stored at −80°C until used.

EMSA binding reactions were performed by first preincubating 5 to 10 μg of cell extract with 0.5 to 2 μg of poly(dI-dC) in binding buffer (10 mM Tris [pH 7.7], 50 mM NaCl, 20% glycerol, 1 mM DTT, 0.5 mM EDTA) for 10 min at room temperature. Approximately 10,000 cpm (0.2 ng) of ^{32}P-labeled DNA probe encompassing the *H-2K*^{b} NF-κB binding site was then added and allowed to bind for approximately 30 min as described elsewhere (63). The reaction mixture was then loaded onto native 5% acrylamide TBE (89 mM Tris, 89 mM borate, 2 mM EDTA) gels.

Polyclonal antipeptide antibodies raised against human NF-κB p65 and human IκB were obtained from Rockland, Inc. (Boyertown, Pa.). Ab 1141 is a polyclonal antibody raised against a peptide consisting of amino acids 1 to 14 encoded by the human p105 gene and was a generous gift of N. Rice (59). Cell extracts were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and transferred to nitrocellulose by electroblotting in 25 mM Tris base–0.2 M glycine–20% methanol at 100 V for 1 h at 4°C. The nitrocellulose was probed by using 1:1,000 dilutions of our antibodies and visualized by using an ECL kit (Amersham) according to the manufacturer's instructions.

GST fusion protein interaction assay. In vitro-translated ^{35}S-GR protein was incubated with either DEX (10^{-7} M final concentration) or vehicle for 2 h on ice and then for 20 min at 25°C. The coding regions of NF-κB p50 and p65 were fused in frame to the glutathione *S*-transferase (GST) gene by using the pGEX system (Pharmacia) and grown in BL21 bacteria. A GST/NFY-A fusion construct transformed into DH5α bacteria was a generous gift from J. Ting, University of North Carolina. These constructs were grown and harvested according to the instructions of the manufacturer of the pGEX system (Pharmacia). GST fusion proteins were purified by binding to glutathione-Sepharose 8A beads (Pharmacia) as described elsewhere (74). After equilibration in LBST (20 mM HEPES [pH 7.9], 100 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% NP-40, 1.5% Triton X-100), 20 μl of the beads was incubated with 5 to 7 μl of activated or mock-activated ^{35}S-GR and washed as described elsewhere (74). The bound proteins were eluted by boiling for 5 min in SDS-PAGE loading buffer and analyzed by SDS-PAGE.

Metabolic labeling and coimmunoprecipitation. COS cells were transfected as described above and labeled 48 h later. Cultures were washed in PBS and incubated for 1 h in serum-free medium. The medium was replaced, and 200 μCi of [^{35}S]Express Label (NEN) per ml was added. Cultures were labeled for 2 h, and whole-cell extracts were prepared as described elsewhere (63). Extract was mixed by rotation with 1 μl of the GR-specific antibody, EP57, in the absence or presence of blocking peptide, for 1 h at 4°C; combined with protein A-Sepharose (PAS) (Sigma); and mixed for 1 h. The PAS pellets were aspirated, washed, and

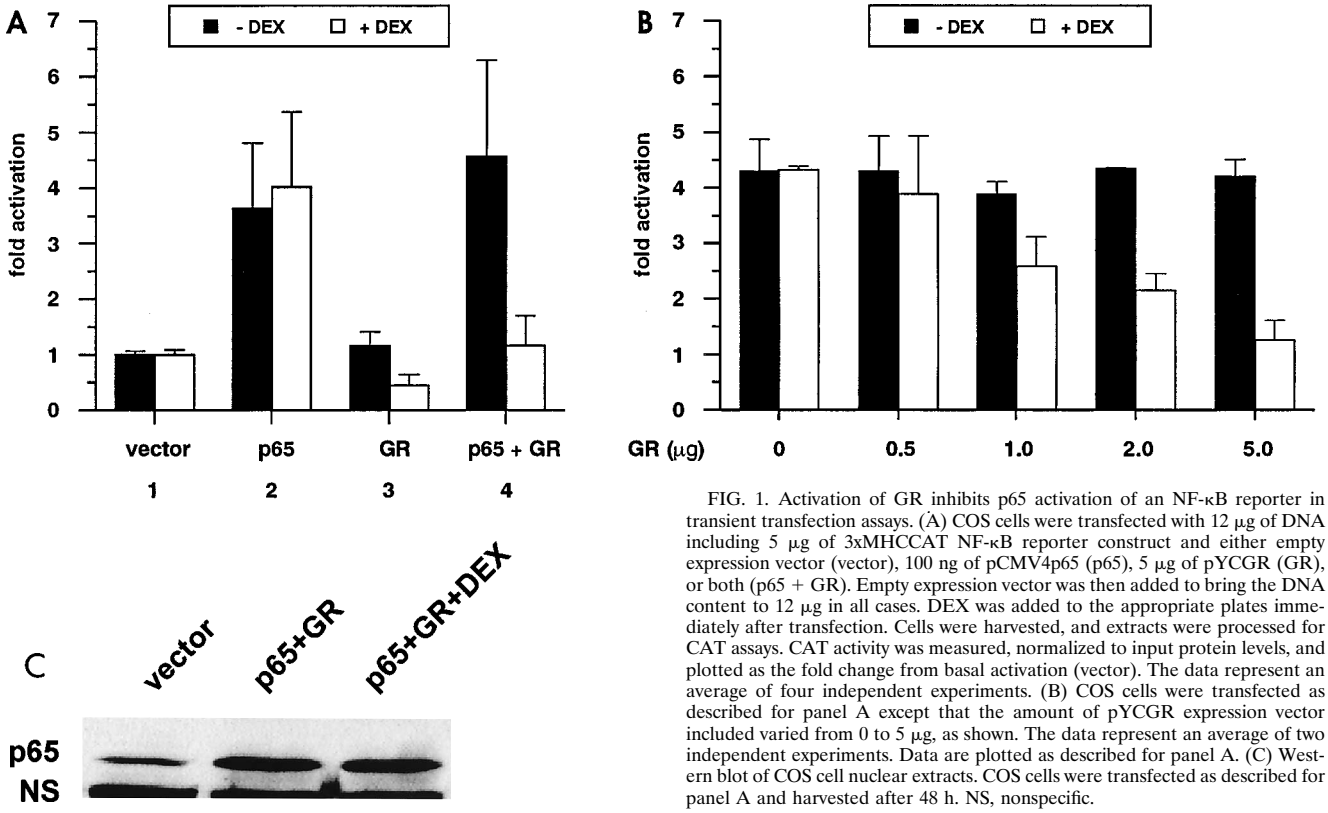


FIG. 1. Activation of GR inhibits p65 activation of an NF-κB reporter in transient transfection assays. (A) COS cells were transfected with 12 μg of DNA including 5 μg of 3xMHCCAT NF-κB reporter construct and either empty expression vector (vector), 100 ng of pCMV4p65 (p65), 5 μg of pYCGR (GR), or both (p65 + GR). Empty expression vector was then added to bring the DNA content to 12 μg in all cases. DEX was added to the appropriate plates immediately after transfection. Cells were harvested, and extracts were processed for CAT assays. CAT activity was measured, normalized to input protein levels, and plotted as the fold change from basal activation (vector). The data represent an average of four independent experiments. (B) COS cells were transfected as described for panel A except that the amount of pYCGR expression vector included varied from 0 to 5 μg, as shown. The data represent an average of two independent experiments. Data are plotted as described for panel A. (C) Western blot of COS cell nuclear extracts. COS cells were transfected as described for panel A and harvested after 48 h. NS, nonspecific.

boiled in radioimmunoprecipitation (RIP) buffer containing 1% SDS as previously described (63). Supernatants were collected, diluted to 0.1% SDS with RIP buffer, and immunoprecipitated overnight with 1 μl of NF-κB subunit antibody and PAS in the absence or presence of peptide as described elsewhere (63). The PAS pellets were washed as described above and boiled in SDS loading buffer, and immunoprecipitates were analyzed by SDS-PAGE and visualized by PhosphoImager.

Immunocytochemistry. COS cells were cultured for 24 h in polystyrene two-chamber slides (Nunc) and transfected as described above but with cell numbers and reagent amounts proportional to the smaller area. Transfected cells were washed twice with PBS, solubilized for 1 min in PBS with 0.1% Triton X-100, and fixed for 45 min in PBS with 3.7% formaldehyde. After three washes in PBS, the slides were blocked with 10% normal goat serum (Cappel) in PBS plus 2% bovine serum albumin (BSA). After an additional three washes, the slides were incubated for 1 h with primary antibody diluted in PBS plus 2% BSA. Antibody EP57 was raised against a peptide from the human GR and affinity purified as described elsewhere (20). It was used for our experiments at a dilution of 1:500. A polyclonal antipeptide antibody raised against human NF-κB p65 was purchased from Santa Cruz Biotechnology and was diluted 1:50 for these experiments. After being washed, the slides were incubated with fluorescein isothiocyanate-conjugated secondary antibody (Chemicon) in the dark for 45 min. After a final wash, a drop of Vectashield (Vector) was added to each chamber area and a coverslip was applied. The slides were stored at 4°C in the dark until visualized by fluorescence microscopy.

RESULTS

Functional repression of NF-κB p65-mediated transcriptional activation by GR. We began this study by determining whether GR had any effect on NF-κB p65-mediated gene expression in transient transfection assays. For these experiments, we transfected expression plasmids encoding p65, GR, or both into COS cells along with the NF-κB reporter, 3xMHC CAT. The p65 subunit contributes several strong activation domains to NF-κB (65). After 48 h, we tested the ability of p65 to transactivate the reporter in the absence or presence of DEX. When COS cells were transfected with empty expression vector, a small but easily detectable amount of CAT activity was measured (Fig. 1A, condition 1). Cotransfection of p65 conferred a three- to fivefold activation over this basal transcription level (Fig. 1A, condition 2). Incubation with DEX had little effect on this level of activation. GR, when transfected alone, resulted in a level of activity comparable to that with the vector alone. However, when these cells were treated with DEX, this activity was reduced (Fig. 1A, condition 3). Experiments described below show that this reduction is not due to a general inhibition of basal promoter activity but is due instead to the inhibition of endogenous NF-κB. When p65 was cotransfected with GR, we observed an increase in CAT activity similar to that seen with p65 alone; however, in this case the increase was profoundly inhibited by DEX (Fig. 1A, condition 4). Similar results were obtained with a luciferase reporter (3xMHCLUC) and by harvesting 16 to 24 h after transfection.

One trivial explanation for this result is that activated GR inhibits the transcriptional activity of the cytomegalovirus (CMV) promoter found in the expression plasmids. After cotransfection of CMVCAT and GR, however, DEX was found to have no effect on CAT activity (data not shown). Activated GR's repression of CAT activity in the absence of cotransfected p65 (Fig. 1A, condition 3) is most likely due to the presence of endogenous NF-κB activity. Indeed, nuclear extracts of vector-transfected COS cells contain detectable p65 (Fig. 1C). In addition, with F9 embryonal carcinoma cells, which contain little detectable basal NF-κB activity, DEX treatment has no effect on basal transcription of the NF-κB reporter in GR-transfected cells (data not shown). This DEX-dependent repression could also be titrated by decreasing the amount of GR cotransfected with p65, suggesting that the ratio of GR to p65 determines the level of repression (Fig. 1B).

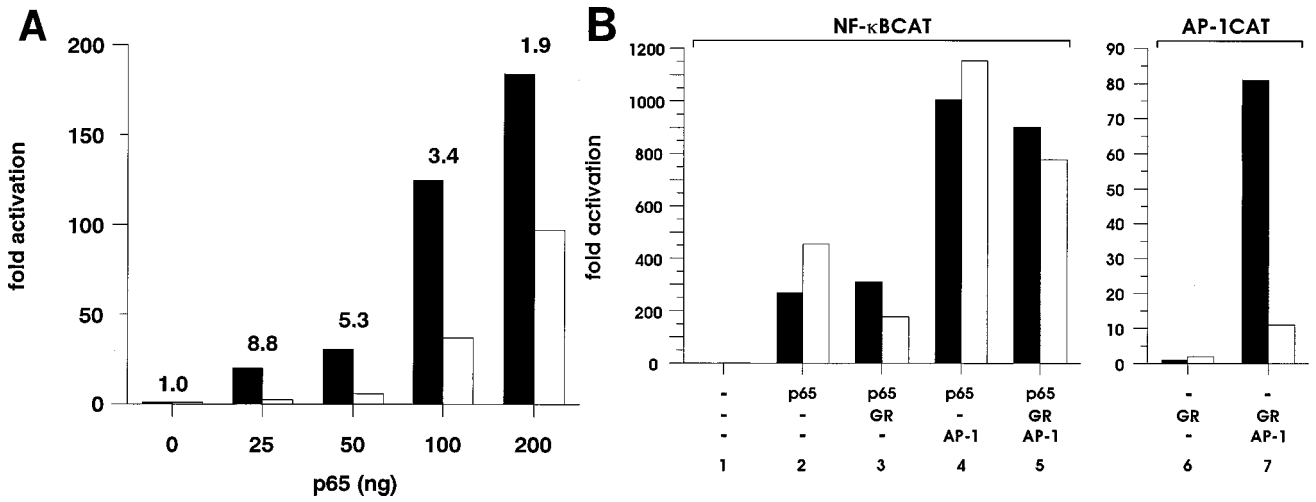


FIG. 2. AP-1/NF-κB synergy is not inhibited by GR in transient transfection assays. F9 cells were transfected with 12 μg of DNA. DEX was added to appropriate plates immediately after transfection. Cells were harvested 16 h after transfection. Data were normalized and plotted as described in the legend to Fig. 1. (A) Each transfection mixture contained 5 μg of 3xMHCCAT NF-κB reporter construct and 5 μg of pYCGR. A variable amount of pCMV4p65 DNA, from 0 to 200 ng, was transfected as shown. Empty expression vector was added to bring all samples to 12 μg of DNA. Numbers shown above the bars for each condition represent the ratio of the transcriptional activation of untreated to DEX-treated samples. (B) F9 cells were transfected with combinations of fos and jun (AP-1), p65, or GR expression plasmids (as indicated below the bars) along with either 5 μg of 3xMHCCAT reporter (conditions 1 to 5) or 5xAP-1CAT reporter (conditions 6 and 7). The amounts of expression plasmids transfected were as follows: p65, 200 ng; GR, 5 μg; fos and jun (AP-1), 500 ng each. Empty expression vector was added to all samples to bring the DNA content to 12 μg. Data are representative of two independent experiments. □, DEX treatment; ■, no DEX treatment.

We then considered the possibility that the activation of GR might somehow affect transfected nuclear p65 protein levels. In order to test this, we performed cotransfection assays similar to those described above and prepared nuclear extracts for Western blot (immunoblot) analysis. We found little detectable difference in p65 immunoreactivity between untreated and DEX-treated cultures (Fig. 1C). In addition, this control was addressed qualitatively by immunocytochemistry (discussed below). Thus, hormone-activated GR is able to specifically inhibit the ability of the NF-κB p65 subunit to activate transcription.

AP-1/NF-κB synergy is not inhibited by activated GR. It has been observed that under some conditions, GR can block AP-1-mediated transcription, and it has also been observed that AP-1 can synergize with NF-κB to enhance transcription (24, 68, 74, 82). As COS cells contain detectable levels of AP-1 activity, we considered the possibility that the observed level of transfected NF-κB activity might be due in part to the presence of AP-1 and to its physical interaction with NF-κB. GR-mediated repression of NF-κB activity might then be caused by an interaction between GR and AP-1, thus blocking AP-1/NF-κB synergy. In order to test this possibility, we utilized F9 cells in which basal NF-κB and AP-1 activities are much reduced. We first tested the F9 cells for basal NF-κB and AP-1 transcriptional activity and found that transfection of a reporter construct plus an empty expression vector resulted in virtually undetectable levels of CAT activity (data not shown). We then determined whether p65-mediated transactivation is blocked by activated GR in F9 cells. Cultures were cotransfected with 3xMHCCAT along with constant amounts of GR expression plasmid and increasing amounts of p65 expression plasmid. As shown in Fig. 2A, activated GR is able to repress p65-mediated activation of the reporter in a manner dependent on the ratio of p65 to GR. As the amount of transfected p65 is increased, the level of p65 activation of 3xMHCCAT is increased; however, as shown in Fig. 2A above the bars for each condition, the ability of GR to repress activation is decreased. Thus, it is clear that AP-1 activity is not required for GR to inhibit p65 activation. In addition, as previously shown with COS cells, the ratio of GR to p65 plays an important role in determining the extent of this inhibition.

In order to determine if activated GR affects the synergistic function of NF-κB and AP-1, we initially determined the conditions for GR-mediated repression of AP-1 and AP-1/NF-κB synergy in our system. As shown in Fig. 2B, a ratio of transcription factors was chosen such that GR blocks p65 activation by 40 to 50% (Fig. 2A, 200 ng of p65 expression plasmid, and B, condition 3), GR blocks AP-1 activation by almost 90% (Fig. 2B, condition 7), and p65 synergizes with AP-1 to increase 3xMHCCAT activity by approximately fourfold (Fig. 2B, compare conditions 2 and 4). DEX treatment of GR-transfected cultures shows no change in basal activity for either 5xAP-1CAT (Fig. 2B, condition 6) or 3xMHCCAT (data not shown). We then compared the effect of activated GR on p65-mediated transcription with the effect of activated GR on p65/AP-1-mediated transcription. Surprisingly, the NF-κB/AP-1 synergistic activity is resistant to glucocorticoid-mediated repression (Fig. 2B, compare conditions 4 and 5), despite the fact that GR represses the two transcription factors separately. Thus, AP-1 seems to protect p65 from the repression mediated by activated GR and further shows that GR does not indiscriminately inhibit transcription factor activity.

Physical association of GR and NF-κB subunits. The previous data persuaded us to determine if GR directly associates with NF-κB subunits. One possible effect of a physical interaction between the two transcription factors would be the inhibition of GR-mediated transactivation. In order to test this, we cotransfected COS cells with a GRECAT reporter in the presence of a constant amount of GR and an increasing amount of p65. GR, when activated, induces a 17-fold increase in reporter gene transcription over the basal activity level (Fig. 3). As an increasing amount of p65 is cotransfected with GR, this activity is decreased to a mere twofold activation in the presence of 2.5 μg of p65 expression plasmid. Thus, when cotransfected, p65 and GR repress each other's ability to transactivate in a manner dependent on the ratio of the two pro-

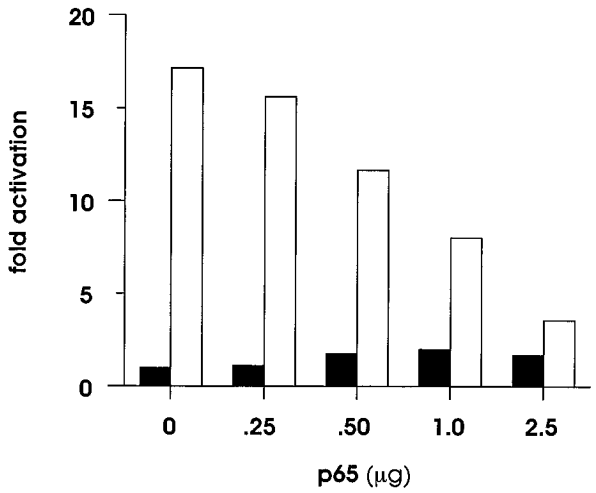


FIG. 3. p65 inhibits GR activation of a GR reporter in transient transfections. COS cells were transfected with 12 μg of DNA. Each transfection mixture contained 5 μg of GRECAT reporter plasmid and 5 μg of pYCGR expression plasmid. Increasing amounts of pCMV4p65 expression plasmid from 0 to 2.5 μg were included as shown. DEX was added immediately after transfection. Data were normalized and plotted as described in the legend to Fig. 1 and are representative of two independent experiments. □, DEX treatment; ■, no DEX treatment.

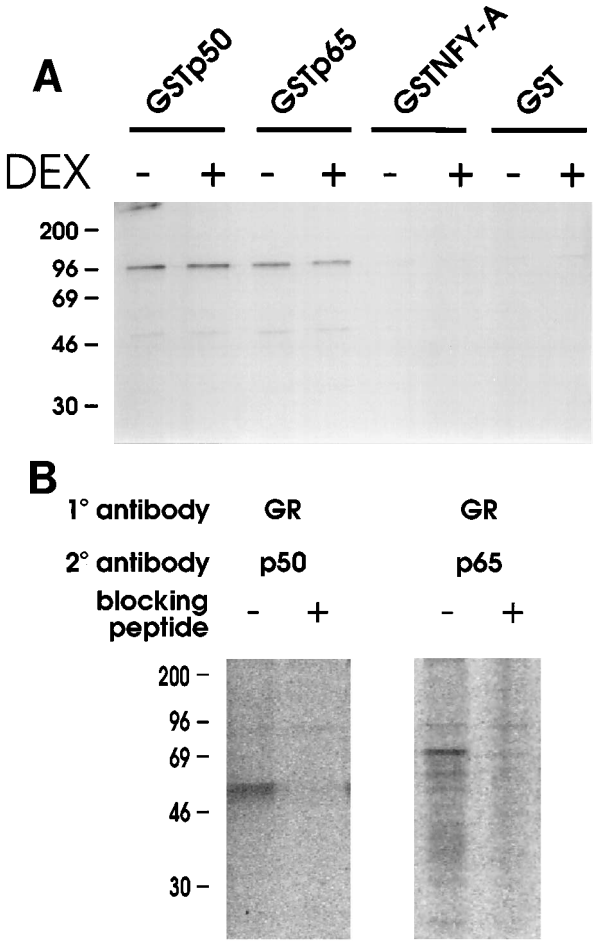


FIG. 4. NF-κB subunits can physically interact with in vitro-translated GR. (A) In vitro-translated ^{35}S-GR was incubated with DEX (+) or vehicle (−) as described in Materials and Methods. GST fusion proteins were grown, extracted from bacteria, and bound to glutathione-Sepharose beads as described in Materials and Methods. Beads bound to GST fusion constructs containing p50, p65, NFY-A, or GST alone were then incubated with activated or mock-activated ^{35}S-GR as described in Materials and Methods. The beads were washed, boiled in SDS-PAGE loading buffer, and size separated by SDS-PAGE. ^{35}S-GR was visualized with a PhosphoImager. (B) COS cells were cotransfected with 1.25 μg of pYCGR (GR) in combination with 1.25 μg of p50 (left panel) or with 1.25 μg of p65 (right panel). Cultures were labeled with [^{35}S]methionine, whole-cell extracts were prepared, and GR-containing complexes were immunoprecipitated as described in Materials and Methods. Washed PAS pellets were boiled in SDS, and diluted supernatants were reimmunoprecipitated with antibody specific for p50 (left panel) or p65 (right panel) in the absence (−) or presence (+) of blocking peptide. Numbers on the left indicate molecular mass (in kilodaltons).

teins. This symmetry lends further credence to the hypothesis that p65 and GR physically interact.

In order to test whether NF-κB subunits are capable of physically interacting with GR, we generated bacterial GST fusion proteins containing either NF-κB p50, NF-κB p65, or NFY-A (a DNA-binding protein which interacts with MHC class II regulatory elements) and bound them to glutathione-Sepharose beads. In vitro-translated ^{35}S-GR was prepared and then activated by incubation with DEX. After the beads had been washed extensively, binding reactions with either activated GR or mock-activated GR were set up. The beads were again washed extensively and boiled in loading buffer, and remaining ^{35}S-GR was analyzed by SDS-PAGE. As shown in Fig. 4A, ^{35}S-GR bound specifically to GSTp50 and GSTp65 but not to GSTpNFY-A or GST alone. Interestingly, mock-activated ^{35}S-GR bound NF-κB subunits as well as DEX-activated ^{35}S-GR did. The implications of this finding are discussed below.

We then extended this observation by attempting to coimmunoprecipitate transfected NF-κB subunits associated with GR using anti-GR antisera. COS cells were cotransfected with GR and p50 or with GR and p65. Cultures were then labeled with [^{35}S]methionine, and whole-cell extracts were prepared. These extracts were then incubated with GR-specific antisera, and GR-containing complexes were precipitated with protein A-Sepharose, washed extensively as described in Materials and Methods, and analyzed by SDS-PAGE. While labeled GR was specifically immunoprecipitated, high background levels precluded visualization of cotransfected NF-κB subunits (data not shown). In order to decrease background, these precipitates were then boiled in RIP buffer containing 1% SDS, diluted to 0.1% SDS, and reprecipitated with antiserum specific for p65 or p50, respectively. The results are shown in Fig. 4B. With COS cells cotransfected with p50 and GR, p50 is clearly present in GR-containing immunoprecipitates. The specificity of this interaction can be determined by inclusion of the p50 peptide (Fig. 4B, left panel). Similarly, with COS cells cotransfected with p65 and GR, p65 is clearly present (Fig. 4B, right panel). In addition, both signals can be blocked by incubation with GR peptide in the first immunoprecipitation step (data not shown). Interestingly, DEX treatment is apparently not necessary for this interaction, consistent with our GST data (see Discussion). Thus, GR is capable of physically interacting with NF-κB subunits both in vitro and in vivo.

GR block NF-κB DNA binding in the presence of DEX. To further characterize the mechanism of glucocorticoid-mediated repression of p65 activity, we analyzed the effect of activated GR on NF-κB subunit DNA binding. COS cells were cotransfected with the GR expression plasmid plus an expression plasmid encoding either p65, p50, or c-rel and were cultured in the presence or absence of DEX. After 3 days, the cells were harvested and extracts were prepared for Western

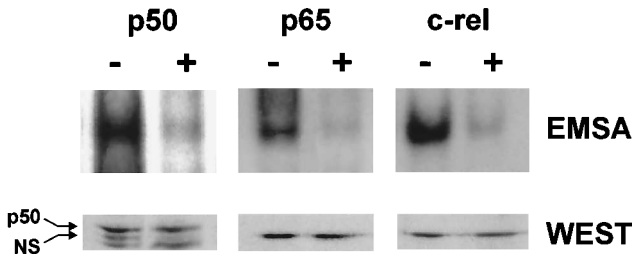


FIG. 5. Activation of GR blocks NF-κB subunit DNA binding. COS cells were transfected with 5 μg of pYCGR plus either 200 ng of pCMV4p50 (left panel), 200 ng of pCMV4p65 (middle panel), or 200 ng of pCMV4rel (right panel). Cultures were either treated with DEX immediately after transfection (+) or left untreated (−), as shown at the top of the figure. Nuclear extracts were prepared for EMSA and Western blot analysis. Each upper panel shows a representative EMSA using the MHC class I *H-2K*^{b} NF-κB binding site as a DNA probe (EMSA). Gels were analyzed with a PhosphoImager. Below each EMSA panel, a Western blot probed with the appropriate NF-κB subunit-specific antisera is shown (WEST). In the p50 Western blot, the top band corresponds to transfected p50. The middle band is nonspecific (NS), and the lower band corresponds to endogenous p50.

blot analysis and EMSA. Figure 5 shows a series of representative experiments. Quantitative Western blots were performed by titrating the untreated extract against the DEX-treated extract in order to ensure that equal amounts of NF-κB subunit protein were present in DEX-treated and untreated EMSA binding reaction mixtures (Fig. 5, WEST). With EMSA analysis, we found that DEX treatment caused a marked reduction in the ability of the NF-κB/Rel subunits to bind DNA despite the presence of equal amounts of NF-κB subunit protein in the EMSA DNA-binding reaction mixtures (Fig. 5, EMSA). Interestingly, in COS cells after 72 h, cotransfection of GR caused a significant reduction of transfected p65 but not transfected p50 or c-rel subunits as measured by Western blots (data not shown). We will return to this observation in a subsequent section.

Analysis of the subcellular localization of GR and p65 following cotransfection. In order to directly visualize the distribution of proteins in the absence or the presence of DEX, we utilized GR and p65 cotransfection followed by immunocytochemistry. Shown in Fig. 6a through c are cotransfected cells stained with antibodies against p65. Cells stained with antibodies against GR are shown in Fig. 6d through f. In untreated transfected cells, p65 staining is both nuclear and cytoplasmic (Fig. 6a, brightly stained cells). The presence of p65 in the cytoplasm is likely due to the fact that p65 transfection leads to an increase in COS IκB protein, which stabilizes cytoplasmic p65. Interestingly, as seen before (12), the distribution of p65 in the nucleus is punctate. One can also see specific staining of endogenous NF-κB in nontransfected cells, primarily in the cytoplasmic and perinuclear regions (Fig. 6a, weakly stained cells). DEX treatment has little effect on the localization of transfected p65 (Fig. 6b). In untreated transfected cells, GR staining is primarily cytoplasmic (Fig. 6d, brightly stained cells). In the presence of DEX, however, GR is entirely nuclear (Fig. 6e). Fig. 6c and f show the staining by peptide-blocked antibodies, thus illustrating the level of background staining for these experiments. In addition, we found that the distribution of cotransfected p65 and GR was identical to that of p65 or GR transfected alone (data not shown). Thus, under conditions in which GR transactivation is inhibited almost 10-fold by the cotransfection of p65 (Fig. 3, rightmost bars), the GR/NF-κB

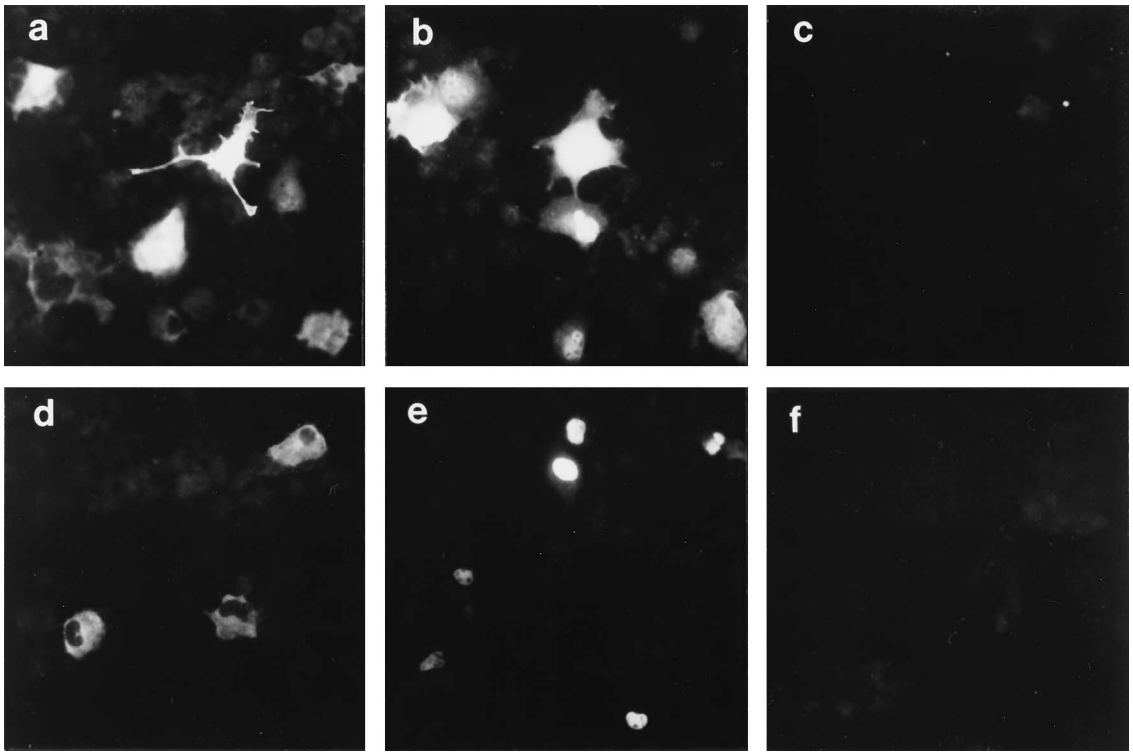
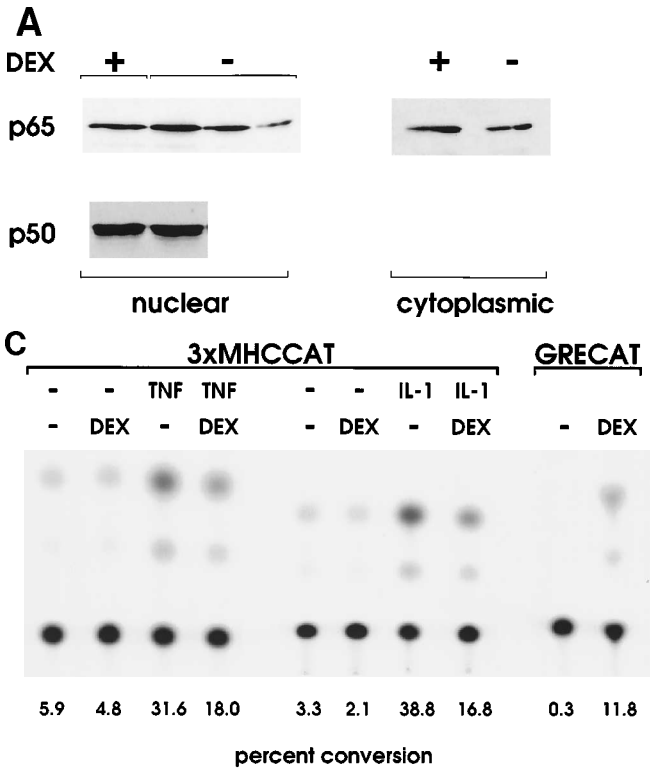


FIG. 6. Immunocytochemical analysis of transfected p65 and GR. COS cells were transfected with equal amounts of p65 and GR expression plasmid, cultured for 24 h in the absence or presence of DEX, and processed for immunocytochemistry as described in Materials and Methods. (a) Untreated cells stained for p65. (b) DEX-treated cells stained for p65. (c) Peptide-blocked p65 staining. (d) Untreated cells stained for GR. (e) DEX-treated cells stained for GR. (f) Peptide-blocked GR staining.



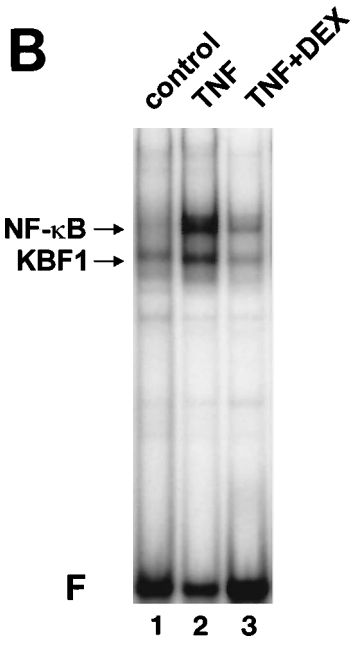


FIG. 7. DEX inhibits the activation of endogenous NF-κB in response to TNF-α. HeLa cells were treated with DEX and TNF-α as described in Materials and Methods. Nuclear and cytoplasmic extracts were then prepared for Western blot and EMSA or CAT assay. (A) Western blot analysis of nuclear and cytoplasmic extracts for p65 and p50 subunit immunoreactivity. For nuclear p65, 42.4 μg of TNF-plus-DEX-treated cell extract (+) was compared with dilutions of TNF-treated cell extract (−) corresponding to 20.8, 10.4, and 4.16 μg, respectively. For cytoplasmic p65, 2.0 μg of TNF-plus-DEX-treated cell extract (+) and TNF-treated extracts (−) were compared. For nuclear p50, 50.0 μg of TNF-plus-DEX-treated cell extract and TNF-treated extracts were compared. (B) EMSA analysis of HeLa nuclear extracts. Extracts from TNF-α-treated cells containing equal amounts of p65 protein as measured by Western blot were compared. Lane 1, control, 11.4 μg; lane 2, TNF-α treated, 11.4 μg; lane 3, DEX-plus-TNF-α treated, 14.5 μg. F, free probe. (C) HeLa cells were transfected with 10 μg of 3xMHCCAT reporter construct (left and middle) or 10 μg of GRECAT (right). At 4 h posttransfection, appropriate plates were treated with DEX or left untreated (−) as shown at the top of the figure. At 24 h posttransfection, appropriate plates were treated with TNF-α (left panel) or IL-1 (middle panel) or left untreated (−). The cells were harvested 48 h posttransfection. Results of representative CAT assays are shown. The percent conversion of [^{14}C]chloramphenicol was measured by using a PhosphoImager and is shown at the bottom of the figure. Data are representative of three independent experiments.

subunit complex is not sequestered as detected by immunocytochemistry (Fig. 6b and e).

Glucocorticoids block activation of endogenous NF-κB activity. In order to determine whether endogenous NF-κB could be repressed by glucocorticoids through the activation of endogenous GR, HeLa cells were pretreated with DEX for 16 h and then treated with TNF-α for 1 h to activate endogenous NF-κB. Nuclear extracts were prepared and analyzed by Western blot and EMSA. We first observed that DEX treatment resulted in a reproducible and significant reduction in TNF-inducible nuclear p65 subunits but not p50 subunits (as described above for transfected COS cells after 72 h). We undertook to more accurately measure the difference in p65 protein levels both to understand the extent of loss of nuclear p65 in DEX-treated cells and to be able to perform EMSA analysis on nuclear extracts containing comparable amounts of p65 protein. Nuclear extracts from DEX- and TNF-α-treated cultures were run next to decreasing amounts of extracts from TNF-α-treated cultures and analyzed by Western blotting. A representative experiment is shown in Fig. 7A. The autoradiograms were scanned, and the quantitated bands from the TNF-α-treated cultures were used to construct a standard curve against which the extract from DEX-treated cultures could be compared. In the presence of DEX, nuclear p65 was measured as 0.62 (±0.19) of that found in the absence of DEX (average of three experiments). We also measured the amount of p50 in nuclear extracts and found it to be unchanged (Fig. 7A, bottom panel). In order to determine if the loss of nuclear p65 was paralleled by a gain of cytoplasmic p65, we also analyzed matched cytoplasmic extracts by Western blotting and found that DEX treatment resulted in a slight but reproducible increase in cytoplasmic p65 (Fig. 7A). For cytoplasmic extracts, the presence of DEX resulted in an average increase of 1.14 ± 0.19 for p65 in comparison with the amount in untreated cultures. In addition, we observed a correlation between the degree of loss of nuclear p65 and the degree of gain of cytoplasmic p65 in these experiments. Figure 7B shows an EMSA analysis of a representative experiment in which nuclear extracts containing similar amounts of p65 protein were compared (lanes 2 and 3). TNF treatment induced the translocation of NF-κB to the nucleus (Fig. 7B, lane 2). Pretreatment with DEX resulted in a profound loss of TNF-α-induced NF-κB as well as p50 homodimer (KBF1) gel shift activity (Fig. 7B, lane 3). Similar data were obtained by treating cells with IL-1 (data not shown). Small amounts of constitutive nuclear NF-κB can be visualized by EMSA with long film exposures. DEX treatment also blocks the ability of this constitutive NF-κB to bind the DNA probe (data not shown). Thus, we have observed that in HeLa cells, GR inhibits NF-κB both by blocking nuclear NF-κB binding to DNA and by generally decreasing NF-κB p65 nuclear protein levels, possibly by sequestering p65 in the cytoplasm.

In order to determine if DEX treatment results in a functional loss of NF-κB activity, HeLa cells were transfected with the NF-κB reporter, 3xMHCCAT, and treated with DEX 4 to

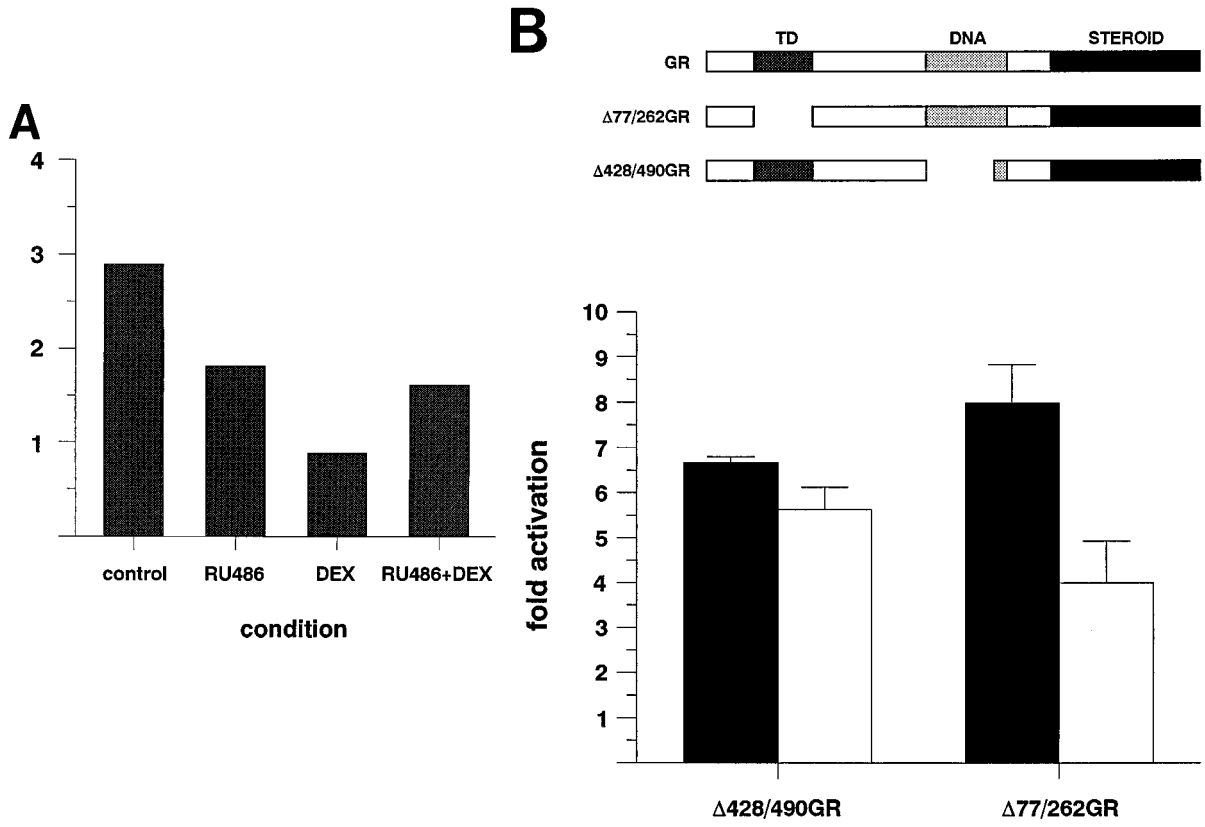


FIG. 8. Effects of RU486 and GR transactivation mutants on p65 inhibition. COS cells were transfected with 12 μg of DNA including 5 μg of 3xMHCLUC NF-κB reporter construct along with p65 and either wild-type GR or mutant GR as described in the legend to Fig. 1. (A) COS cells transfected with p65 plus GR were either left untreated (control), or treated with 10^{-6} M RU486 (RU486), 10^{-7} M DEX (DEX), or both (RU486+DEX) for 24 h, after which the cells were harvested for luciferase assays. Numbers on the left indicate fold activation. (B) At the top is a diagram of wild-type GR and GR transactivation mutants. Shaded rectangles represent domains of GR. TD, transactivation domain Tau 1; DNA, DNA-binding domain containing two zinc finger motifs; STEROID, steroid-binding domain. At the bottom, results are shown for an experiment in which COS cells were transfected with p65 and either Δ77/262GR or Δ428/490GR and were cultured in the absence (■) or presence (□) of 10^{-7} M DEX for 24 h.

6 h posttransfection. After 20 h, cultures were treated with either TNF-α or IL-1. Cells were harvested 24 h later. Representative experiments are shown in Fig. 7C. TNF-α and IL-1 both produced a 5- to 10-fold induction in CAT activity in these experiments. Pretreatment with DEX resulted in a significant reduction in CAT activity. Thus, endogenous HeLa GR is capable of repressing endogenous NF-κB activity. As a control to show that DEX did not block gene expression nonspecifically, HeLa cells were also transfected with the GR reporter, GRECAT. DEX treatment resulted in a 40-fold activation of GRECAT over basal transcription levels. HeLa cells contain numerous forms of TNF-α-inducible NF-κB-like complexes (10). It is possible that while the transfected NF-κB subunits are rendered unable to bind DNA (Fig. 5), certain endogenous NF-κB subunit dimers might be resistant to GR-mediated repression. This and other possible mechanisms which might contribute to the remaining NF-κB-like activity present after DEX treatment are discussed below. In any event, it is clear that endogenous NF-κB activity, induced by a physiological signal, can be significantly repressed by glucocorticoids.

Effect of the antisteroid RU486 on the inhibition of p65 activity. In order to begin to dissect the features of GR which are necessary for the inhibition of NF-κB, we studied the effect of the antisteroid RU486 in our COS cell transfections. In the presence of RU486, GR is able to dissociate from HSP90 and translocate to the nucleus; however, its ability to transactivate is markedly reduced (13). GR and p65 were cotransfected as described above, and the cells were cultured in the presence of either 10^{-7} M DEX, 10^{-6} M RU486, or both. Interestingly, we found that RU486 alone was able to partially inhibit p65 activity (Fig. 8A). While DEX alone was able once again to block p65 activation, in the presence of a 10-fold excess of RU486, p65 activity was partially inhibited to an extent similar to that seen with RU486 alone, consistent with the role of RU486 as a competitive inhibitor of GR (Fig. 8A). Thus, GR-mediated transcription does not seem to be required for the inhibition of p65, consistent with our data suggesting that a physical association between GR and NF-κB is in part responsible for the inhibition of NF-κB.

Analysis of GR transactivation mutants. In order to further test the hypothesis that GR-mediated transactivation is not required for the inhibition of NF-κB, we tested several GR transactivation mutants. Figure 8B shows the structure of the GR and the two mutants analyzed. Δ428/490GR has a deletion in the zinc finger region. This protein can dimerize and translocate to the nucleus but cannot bind DNA (19). In contrast to wild-type GR, Δ428/490GR had no effect on p65 activation when cotransfected with p65 into COS cells and activated by DEX (Fig. 8B). Interestingly, this zinc finger region has been shown to be required for the physical association of GR and AP-1 (35, 40, 46, 68, 82). Thus, this mutation may have multiple effects on the properties of GR. A second transactivation mutant, Δ77/262GR, has a deletion in the N-terminal transac-

tivation domain resulting in a 10- to 20-fold drop in transactivation of a GRE. When Δ77/262GR was cotransfected with p65 into COS cells, DEX activation resulted in a significant inhibition of p65 activity (Fig. 8B). Both mutant proteins were expressed at levels similar to those of wild-type GR as measured by Western blot (data not shown). We interpret these data as showing that the transactivating potential of GR cannot fully account for the inhibition of NF-κB activity. In addition, we have found that a region encompassing the zinc finger is required for inhibition of NF-κB function.

DISCUSSION

Cross-coupling of GR and NF-κB. In this study, we have shown that NF-κB, an important regulator of immune system and inflammation genes, is inhibited by glucocorticoid administration, in part through cross-coupling with the GR and in part by a loss of nuclear NF-κB subunit protein (discussed below). Our current understanding of NF-κB suggests that GR-mediated repression of inducible NF-κB activity might play an important role in mediating the repression of cytokine transcription. Cross-coupling is suggested by the symmetrical nature of the mutual inhibition of p65 and GR (Fig. 1 and 3), by the ability of GST/NF-κB fusion proteins to bind in vitro-translated GR (Fig. 4A), by the ability to coimmunoprecipitate GR and NF-κB subunit proteins (Fig. 4B), and by the ability to uncouple GR-mediated transcription and inhibition of p65 (Fig. 1B and 8B). In addition, we have shown that DEX treatment causes the loss of NF-κB DNA binding to the *H-2K*^{b} region I enhancer, a strong NF-κB-responsive element (Fig. 7B).

The interaction of NF-κB and GR is similar to the interaction between AP-1 and GR. In both cases, a mutual repression is observed. Numerous studies have identified the zinc finger region of GR as a critical domain for the physical interaction with and repression of AP-1 (35, 40, 46, 68, 82). We have found that the zinc finger region is required for repression of p65 activity (Fig. 8B). Interestingly, the relationship between GR and these two transcription factor families is complicated by the ability of NF-κB/AP-1 synergistic complexes to overcome glucocorticoid-mediated repression (Fig. 2B). These complex interactions potentially add an additional level of regulation to this system.

DEX-mediated loss of nuclear p65. We have shown that when HeLa cells are cultured in the presence of DEX, the level of nuclear p65 protein present after TNF-α induction is decreased. Two hypotheses explaining the loss of p65 include protein destabilization and cytoplasmic sequestration. While we have no evidence for DEX inducing an increase in the degradation of p65, we cannot discount this possibility. We have, however, observed a slight increase in the amount of cytoplasmic p65, suggesting that DEX may increase the amount of p65 being sequestered in the cytoplasm, although we have shown by immunocytochemistry that the GR/NF-κB complex itself is not cytoplasmically sequestered in the presence of DEX (Fig. 6). Molecules which do sequester NF-κB in the cytoplasm have been well characterized and include the IκB family members IκBα (5, 12) and IκBβ (34), as well as the NF-κB precursors p105 (48, 59) and p100 (63). The mechanisms regulating the level of these sequestering agents are complex. NF-κB itself can induce some of these components, such as IκBα and p105, at the level of transcription (18, 21, 42, 78). In addition, NF-κB can stabilize the rapidly degraded IκBα through physical association. It is intriguing to speculate whether GR induces the transcription of any of these sequestering components.

The role of GR-mediated transactivation in the inhibition of NF-κB. We observed a partial inhibition of p65 activity both with RU486 in complex with wild-type GR and with the GR transactivation mutant, Δ77-262. One possible interpretation of these data is that the conformation of (mutant or RU486-bound) GR is compromised such that its ability to interact with p65 is decreased. Alternatively, it may be that there exists a transcriptional component to the GR-mediated inhibition which is necessary for full repression of NF-κB. Further experimentation will be required to distinguish between these possibilities.

DEX dependence of the GR-mediated inhibition of NF-κB. The data shown in Fig. 4 suggest that glucocorticoids are not required for GR interaction with NF-κB. If so, why is GR-mediated repression of NF-κB glucocorticoid dependent? The binding of hormone to the receptor induces a conformational change allowing GR to dissociate from HSP90 and translocate to the nucleus. It has been demonstrated that upon dissociation, a large pool of GR cycles between the nucleus and the cytoplasm (55). Our model would predict that this cycling hormone-GR complex is then competent to interact with released NF-κB. Alternatively, hormone binding may be required in vivo both for dissociation from HSP90 and for GR association with NF-κB. Perhaps in vitro-synthesized GR folds into a more permissive conformation, allowing DEX-independent association with NF-κB subunits.

Several physiological consequences of DEX administration can be explained by repression of NF-κB activity. Within 4 to 6 h after a single glucocorticoid dose, the human immune system undergoes a striking transformation. Monocyte as well as B- and T-lymphocyte populations redistribute from the blood to other lymphoid compartments, possibly the bone marrow, while the circulating neutrophil populations increase (23). Primary cells derived from these patients are unresponsive to mitogenic or antigenic stimulation in mixed lymphocyte reactions (23, 57). In addition, glucocorticoid treatment blocks monocyte secretion of IL-1 or IL-6 and T-cell secretion of IL-2, as well as blocking lymphocyte secretion of IL-3, gamma interferon, and GM-CSF (22, 23, 79).

Cytokines such as IL-1, IL-6, TNF-α, and GM-CSF can function in a number of ways to activate and amplify immune and inflammation responses. Cytokine secretion requires de novo cytokine gene transcription. Studies of the IL-1 and IL-6 promoters have shown that NF-κB regulatory elements play an important role in the induction of transcription (33, 45, 72, 83). IL-2 secretion is a required autocrine signal for quiescent T cells to enter the cell cycle as they progress to the activated state. Studies of the progression of cytotoxic T-lymphocyte clones or BALB/c 3T3 fibroblasts from quiescence to the cell cycle have correlated this progression with a rapid and transient induction of NF-κB activity (8, 79). The IL-2 promoter also contains an NF-κB site within the context of a tandem array of transcription factor binding sites which include NF-AT, NF-κB, AP-1, and Oct-1 (71, 79). Thus, a glucocorticoid-mediated block of NF-κB activity may function to repress the immune response at multiple levels through blocking of cytokine transcription.

Glucocorticoids have been shown to inhibit lymphocyte migration in vitro, presumably by altering their adherence properties (23). It has been hypothesized that the glucocorticoid-mediated redistribution of lymphocytes involves changes in cell surface properties regulating cell adhesion. Interestingly, NF-κB has been recently implicated in the regulation of endothelial cell adhesion molecules such as I-CAM, V-CAM, and E-selectin (37, 50). These adhesion molecules must be expressed by vascular endothelium in order to allow leukocyte

extravasation. Thus, repression of NF-κB would be expected to block leukocyte infiltration associated with inflammation.

While our manuscript was in preparation, two reports specifically implicating NF-κB in the DEX-mediated inhibition of two cytokine promoters were published. The first showed that an NF-κB-responsive element in the IL-6 promoter was necessary for DEX-mediated inhibition of IL-1 induction of this gene (58). The second report described a similar study using the IL-8 promoter (49). In both studies, DEX treatment caused a loss of NF-κB binding to its respective DNA-binding site. Our work shows similar results. In addition, our work shows that p50 (Fig. 4 and 5) and likely c-rel (Fig. 5) directly interact with GR. We have extended these observations by showing that GR interacts with endogenous NF-κB subunits to block DNA binding. Interestingly, DEX treatment causes a decrease in nuclear levels of p65, thus illustrating a novel mechanism for GR-mediated inhibition of NF-κB activity. We have shown that as with AP-1/GR interactions, the zinc finger domain is critical for inhibition of NF-κB. In addition, we show that under conditions in which AP-1 and NF-κB can synergize, GR is unable to repress NF-κB activity. Finally, we demonstrate that GR-mediated transcription may be uncoupled from the inhibition of NF-κB.

The discovery of cross-coupling between transcription factor families has altered our perceptions of how transcription is regulated. Interactions between transcription factors and their DNA-binding sites can be modulated, enhanced, or blocked by interactions with other transcription factors. Cross-coupling can result in transcriptional synergy, as seen with NF-κB/bZIP family member complexes, or transcriptional repression, as seen with AP-1/GR or NF-κB/GR complexes. We have discussed the interaction between GR and NF-κB in terms of its pharmacological implications. It will be important to determine whether other steroid hormone receptors interact with NF-κB and to determine the physiological and potential developmental consequences of this interaction.

REFERENCES

1. **Allgood, V. E., R. H. Oakley, and J. A. Cidlowski.** 1993. Modulation by vitamin B6 of glucocorticoid receptor-mediated gene expression requires transcription factors in addition to the glucocorticoid receptor. J. Biol. Chem. **268:**20870–20876.
2. **Andersson, S., D. L. Davis, H. Dahlback, H. Jornvall, and D. W. Russell.** 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. **264:**8222–8229.
3. **Arima, N., W. A. Kuziel, T. A. Grdina, and W. C. Greene.** 1992. IL-2-induced signal transduction involves the activation of nuclear NF-κB expression. J. Immunol. **149:**83–91.
4. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53:**211–217.
5. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242:**540–546.
6. **Baeuerle, P. A., and D. Baltimore.** 1989. A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3:**1689–1698.
7. **Baeuerle, P. A., and D. Baltimore.** 1991. The physiology of the NF-κB transcription factor. Hormonal control regulation of gene transcription. Mol. Aspects Cell. Regul. **6:**409–432.
8. **Baldwin, A. S., Jr., J. C. Azizkhan, D. E. Jensen, A. A. Beg, and L. R. Coodly.** 1991. Induction of NF-κB DNA-binding activity during the G_0-to-G_1 transition in mouse fibroblasts. Mol. Cell. Biol. **11:**4943–4951.
9. **Beato, M.** 1989. Gene regulation by steroid hormones. Cell **56:**335–344.
10. **Beg, A. A., and A. S. Baldwin, Jr.** 1994. Induction of multiple forms of NF-κB/rel by TNFα. Oncogene **9:**1487–1492.
11. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin, Jr.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
12. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr.** 1992. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. **6:**1899–1913.
13. **Bernstein, K. L., C. M. Jewell, M. Sar, and J. A. Cidlowski.** Intragenic sequences of the human glucocorticoid receptor complementary DNA mediate hormone inducible receptor mRNA down regulation through multiple mechanisms. Mol. Endocrinol., in press.
14. **Blank, V., P. Kourilsky, and A. Israel.** 1992. NF-κB and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. Trends Biochem. Sci. **17:**135–140.
15. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a kappa B DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature (London) **348:**76–80.
16. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
17. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκBα. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
18. **Cheng, Q., C. A. Cant, T. Moll, R. Hofer Warbinek, E. Wagner, M. L. Birnstiel, F. H. Bach, and R. de Martin.** 1994. NF-κB subunit-specific regulation of the IκBα promoter. J. Biol. Chem. **269:**13551–13557.
19. **Cidlowski, J. A.** 1994. Unpublished data.
20. **Cidlowski, J. A., D. L. Bellingham, F. E. Powell Oliver, D. B. Lubahn, and M. Sar.** 1990. Novel antipeptide antibodies to the human glucocorticoid receptor: recognition of multiple receptor forms in vitro and distinct localization of cytoplasmic and nuclear receptors. Mol. Endocrinol. **4:**1427–1437.
21. **Cogswell, P. C., R. I. Scheinman, and A. S. Baldwin, Jr.** 1993. Promoter of the human NF-κB p50/p105 gene. Regulation by NF-κB subunits and by c-REL. J. Immunol. **150:**2794–2804.
22. **Culpepper, J., and F. Lee.** 1987. Glucocorticoid regulation of lymphokine production by murine T lymphocytes. Lymphokines **13:**275–289.
23. **Cupps, T. R., and A. S. Fauci.** 1982. Corticosteroid-mediated immunoregulation in man. Immunol. Rev. **65:**133–155.
24. **Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto.** 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science **249:**1266–1272.
25. **Drouin, J., Y. L. Sun, M. Chamberland, Y. Gauthier, A. De Lean, M. Nemer, and T. J. Schmidt.** 1993. Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. EMBO J. **12:**145–156.
26. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240:**889.
27. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell **62:**1019–1029.
28. **Gillis, S., G. R. Crabtree, and K. A. Smith.** 1979. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. J. Immunol. **123:**1624.
29. **Goldfeld, A. E., C. Doyle, and T. Maniatis.** 1990. Human TNFα gene regulation by virus and lipopolysaccharide. Proc. Natl. Acad. Sci. USA **87:**9769–9773.
30. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
31. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
32. **Hansen, S. K., C. Nerlov, U. Zabel, P. Verde, M. Johnsen, P. A. Baeuerle, and F. Blasi.** 1992. A novel complex between the p65 subunit of NF-κB and c-Rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene. EMBO J. **11:**205–213.
33. **Hiscott, J., J. Marois, J. Garoufalis, M. D'Addario, A. Roulston, I. Kwan, N. Pepin, J. Lacoste, H. Nguyen, G. Bensi, and M. Fenton.** 1993. Characterization of a functional NF-κB site in the human interleukin 1β promoter: evidence for a positive autoregulatory loop. Mol. Cell. Biol. **13:**6231–6240.
34. **Inoue, J., L. D. Kerr, D. Rashid, N. Davis, H. R. Bose, Jr., and I. M. Verma.** 1992. Direct association of pp40/IκB beta with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA **89:**4333–4337.
35. **Jonat, C., H. J. Rahmsdorf, K. K. Park, A. C. Cato, S. Gebel, H. Ponta, and P. Herrlich.** 1990. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell **62:**1189–1204.
36. **Kang, S. M., A. C. Tran, M. Grilli, and M. J. Lenardo.** 1992. NF-κB subunit regulation in nontransformed CD4+ T lymphocytes. Science **256:**1452–1456.
37. **Kaszubska, W., R. H. van Huijsduijnen, P. Ghersa, A. M. DeRaemy Schenk, B. P. Chen, T. Hai, J. F. DeLamarter, and J. Whelan.** 1993. Cyclic AMP-independent ATF family members interact with NF-κB and function in the activation of the E-selectin promoter in response to cytokines. Mol. Cell. Biol. **13:**7180–7190.
38. **Kawai, S., and M. Nishizawa.** 1984. New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol. Cell. Biol. **4:**1172–1174.
39. **Kawakami, K., C. Scheidereit, and R. G. Roeder.** 1988. Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. Proc. Natl. Acad. Sci. USA **85:**4700–4704.

40. **Kerppola, T. K., D. Luk, and T. Curran.** 1993. Fos is a preferential target of glucocorticoid receptor inhibition of AP-1 activity in vitro. Mol. Cell. Biol. **13:**3782–3791.
41. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the rel oncogene product. Cell **62:**1007–1018.
42. **Le Bail, O., R. Schmidt Ullrich, and A. Israel.** 1993. Promoter analysis of the gene encoding the IκBα/MAD3 inhibitor of NF-κB: positive regulation by members of the rel/NF-κB family. EMBO J. **12:**5043–5049.
43. **Lenardo, M. J., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell **58:**227–229.
44. **Lenardo, M. J., C. M. Fan, T. Maniatis, and D. Baltimore.** 1989. The involvement of NF-κB in beta-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. Cell **57:**287–294.
45. **Libermann, T. A., and D. Baltimore.** 1990. Activation of interleukin-6 gene expression through the NF-κB transcription factor. Mol. Cell. Biol. **10:**2327–2334.
46. **Lucibello, F. C., E. P. Slater, K. U. Jooss, M. Beato, and R. Muller.** 1990. Mutual transrepression of Fos and the glucocorticoid receptor: involvement of a functional domain in Fos which is absent in FosB. EMBO J. **9:**2827–2834.
47. **Mercurio, F., J. Didonato, C. Rosette, and M. Karin.** 1992. Molecular cloning and characterization of a novel Rel/NF-κB family member displaying structural and functional homology to NF-κB p50/p105. DNA Cell Biol. **11:**523–537.
48. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. Genes Dev. **7:**705–718.
49. **Mukaida, N., M. Morita, Y. Ishikawa, N. Rice, S. Okamoto, T. Kasahara, and K. Matsushima.** 1994. Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. J. Biol. Chem. **269:**13289–13295.
50. **Neish, A. S., A. J. Williams, H. J. Palmer, M. Z. Whitley, and T. Collins.** 1992. Functional analysis of the human vascular cell adhesion molecule 1 promoter. J. Exp. Med. **176:**1583–1593.
51. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1992. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:**444–448.
52. **Nolan, G. P., and D. Baltimore.** 1992. The inhibitory ankyrin and activator Rel proteins. Curr. Opin. Genet. Dev. **2:**211–220.
53. **Nolan, G. P., S. Ghosh, H. C. Liou, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a rel-related polypeptide. Cell **64:**961–969.
54. **O'Brien, J. M., and J. A. Cidlowski.** 1981. Interaction of pyridoxal phosphate with glucocorticoid receptors from HeLa S3 cells. J. Steroid Biochem. **14:** 9–17.
55. **Orti, E., D. B. Mendel, L. I. Smith, J. E. Bodwell, and A. Munck.** 1989. A dynamic model of glucocorticoid receptor phosphorylation and cycling in intact cells. J. Steroid Biochem. **34:**85–96.
56. **Perkins, N. D., N. L. Edwards, C. S. Duckett, A. B. Agranoff, R. M. Schmid, and G. J. Nabel.** 1993. A cooperative interaction between NF-κB and Sp1 is required for HIV-1 enhancer activation. EMBO J. **12:**3551–3558.
57. **Plaut, M.** 1987. Lymphocyte hormone receptors. Annu. Rev. Immunol. **5:**621–669.
58. **Ray, A., and K. E. Prefontaine.** 1994. Physical association and functional antagonism between the p65 subunit of transcription factor NF-κB and the glucocorticoid receptor. Proc. Natl. Acad. Sci. USA **91:**752–756.
59. **Rice, N. R., M. L. MacKichan, and A. Israel.** 1992. The precursor of NF-κB p50 has IκB-like functions. Cell **71:**243–253.
60. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C. H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. Science **251:** 1490–1493.
61. **Ryseck, R. P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo.** 1992. RelB, a new Rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. **12:**674–684.
62. **Sakai, D. D., S. Helms, J. Carlstedt Duke, J. A. Gustafsson, F. M. Rottman, and K. R. Yamamoto.** 1988. Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. **2:**1144–1154.
63. **Scheinman, R. I., A. A. Beg, and A. S. Baldwin, Jr.** 1993. NF-κB p100 (Lyt-10) is a component of H2TF1 and can function as an IκB-like molecule. Mol. Cell. Biol. **13:**6089–6101.
64. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352:**733–736.
65. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10:**3805–3817.
66. **Schreck, R., and P. A. Baeuerle.** 1990. NF-κB as inducible transcriptional activator of the granulocyte-macrophage colony-stimulating factor gene. Mol. Cell. Biol. **10:**1281–1286.
67. **Schreck, R., P. Rieber, and P. A. Baeuerle.** 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J. **10:**2247–2258.
68. **Schule, R., P. Rangarajan, S. Kliewer, L. J. Ransone, J. Bolado, N. Yang, I. M. Verma, and R. M. Evans.** 1990. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell **62:**1217–1226.
69. **Sen, R., and D. Baltimore.** 1986. Inducibility of kappa immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47:**921–928.
70. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46:**705–716.
71. **Shibuya, H., and T. Taniguchi.** 1989. Identification of multiple cis-elements and trans-acting factors involved in the induced expression of human IL-2 gene. Nucleic Acids Res. **17:**9173–9184.
72. **Shimizu, H., K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto.** 1990. Involvement of a NF-κB-like transcription factor in the activation of the interleukin-6 gene by inflammatory lymphokines. Mol. Cell. Biol. **10:**561–568.
73. **Smith, K. A.** 1980. T-cell growth factor. Immunol. Rev. **51:**337–357.
74. **Stein, B., A. S. Baldwin, Jr., D. W. Ballard, W. C. Greene, P. Angel, and P. Herrlich.** 1993. Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J. **12:**3879–3891.
75. **Stein, B., P. C. Cogswell, and A. S. Baldwin, Jr.** 1993. Functional and physical associations between NF-κB and C/EBP family members: a Rel domain-bZIP interaction. Mol. Cell. Biol. **13:**3964–3974.
76. **Sun, S. C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
77. **Taniguchi, T.** 1988. Regulation of cytokine gene expression. Annu. Rev. Immunol. **6:**439–464.
78. **Ten, R. M., C. V. Paya, N. Israel, O. Le Bail, M. G. Mattei, J. L. Virelizier, P. Kourilsky, and A. Israel.** 1992. The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. EMBO J. **11:**195–203.
79. **Ullman, K. S., J. P. Northrop, C. L. Verweij, and G. R. Crabtree.** 1990. Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. Annu. Rev. Immunol. **8:**421–452.
80. **Urban, M. B., R. Schreck, and P. A. Baeuerle.** 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. EMBO J. **10:**1817–1825.
81. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19:**209.
82. **Yang Yen, H. F., J. C. Chambard, Y. L. Sun, T. Smeal, T. J. Schmidt, J. Drouin, and M. Karin.** 1990. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell **62:**1205–1215.
83. **Zhang, Y. H., J. X. Lin, and J. Vilcek.** 1990. Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence. Mol. Cell. Biol. **10:** 3818–3823.