The glucocorticoid dexamethasone inhibited the production of the rat cytokine-induced neutrophil chemotactant CINC/gro, a counterpart of human melanoma growth-stimulating activity that belongs to the interleukin-8 (IL-8) family, in the normal rat kidney epithelial cell line NRK-52E stimulated with interleukin-1β (IL-1β), lipopolysaccharide, or tumor necrosis factor α. The accumulation of CINC/gro mRNA induced by these activators was also decreased comparably by dexamethasone. A nuclear run-on assay revealed that dexamethasone decreased the IL-1β-induced transcription of the CINC/gro gene. The half-life of CINC/gro mRNA transcripts did not change significantly after exposure to dexamethasone, suggesting that this glucocorticoid acts mainly at the transcriptional level. Transfection with luciferase expression vectors containing 5′-deleted and mutated CINC/gro gene sequences demonstrated that the 5′-flanking region containing the NF-κB binding site is involved in the IL-1β- and dexamethasone-induced activation and repression of the CINC/gro gene expression, respectively. Furthermore, a tandem repeat of the NF-κB sequence in the CINC/gro gene conferred the inducibility by IL-1β and suppression of luciferase activity by dexamethasone. In an electrophoretic mobility shift assay, dexamethasone diminished the IL-1β-induced formation of NF-κB complexes, which consisted of p65 and p50. Western blotting revealed that dexamethasone inhibited the IL-1β-induced translocation of p65 from the cytoplasm into the nucleus, while the nuclear level of NF-κB p50 remained almost unchanged. In addition, the degradation of 1κB-α induced by IL-1β was not inhibited by dexamethasone. These results indicated that the suppression of the CINC/gro gene transcription by glucocorticoids occurs through the impairment of NF-κB activation, possibly by interference with the translocation of NF-κB p65 from the cytoplasm into the nucleus, thereby suppressing transactivation of the CINC/gro gene.

Glucocorticoid-mediated Gene Suppression of Rat Cytokine-induced Neutrophil Chemotactant CINC/gro, a Member of the Interleukin-8 Family, through Impairment of NF-κB Activation*

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Toshiaki Ohtsuka†, Atsushi Kubota†, Takae Hiranot, Kazuyoshi Watanabe‡, Hideaki Yoshida‡, Makoto Tsurufuji‡, Yoshio Iizuka‡, Kiyoshi Konishi‡, and Susumu Tsurufuji‡

From the †Institute of Cytosignal Research, Inc., Hiromachi 1-2-58, Shinagawa-ku, Tokyo 140 and the ‡Department of Biochemistry, Toyama Medical and Pharmaceutical University Faculty of Medicine, Sugitani, Toyama 930-01, Japan

The glucocorticoid dexamethasone inhibited the production of the rat cytokine-induced neutrophil chemotactant CINC/gro, a counterpart of human melanoma growth-stimulating activity that belongs to the interleukin-8 (IL-8) family, in the normal rat kidney epithelial cell line NRK-52E stimulated with interleukin-1β (IL-1β), lipopolysaccharide, or tumor necrosis factor α. The accumulation of CINC/gro mRNA induced by these activators was also decreased comparably by dexamethasone. A nuclear run-on assay revealed that dexamethasone decreased the IL-1β-induced transcription of the CINC/gro gene. The half-life of CINC/gro mRNA transcripts did not change significantly after exposure to dexamethasone, suggesting that this glucocorticoid acts mainly at the transcriptional level. Transfection with luciferase expression vectors containing 5′-deleted and mutated CINC/gro gene sequences demonstrated that the 5′-flanking region containing the NF-κB binding site is involved in the IL-1β- and dexamethasone-induced activation and repression of the CINC/gro gene expression, respectively. Furthermore, a tandem repeat of the NF-κB sequence in the CINC/gro gene conferred the inducibility by IL-1β and suppression of luciferase activity by dexamethasone. In an electrophoretic mobility shift assay, dexamethasone diminished the IL-1β-induced formation of NF-κB complexes, which consisted of p65 and p50. Western blotting revealed that dexamethasone inhibited the IL-1β-induced translocation of p65 from the cytoplasm into the nucleus, while the nuclear level of NF-κB p50 remained almost unchanged. In addition, the degradation of 1κB-α induced by IL-1β was not inhibited by dexamethasone. These results indicated that the suppression of the CINC/gro gene transcription by glucocorticoids occurs through the impairment of NF-κB activation, possibly by interference with the translocation of NF-κB p65 from the cytoplasm into the nucleus, thereby suppressing transactivation of the CINC/gro gene.
NF-κB-mediated Transcriptional Regulation of CINC/gro Gene

(32) and in human glioblastoma cell lines (33). In normal human embryonic lung fibroblasts, dexamethasone decreases IL-8 gene expression by reducing the stability of its mRNA (34). However, a different mechanism may be responsible in primary cultured human airway epithelial cells (35). On the other hand, although glucocorticoids inhibit GRO production in mouse (36) and rat (37) cell lines, little is known about the molecular mechanisms by which this is achieved.

As inhibition of GRO production by glucocorticoids must also have important implications for their actions as anti-inflammatory agents, we analyzed the effects of dexamethasone on CINC/gro expression at the molecular level in the normal rat kidney epithelial cell line NRK-52E. Evidence is presented here that this steroid hormone significantly decreases the transcription rate of the CINC/gro gene without affecting the stability of its mRNA in NRK-52E cells activated with IL-1. We identified the NF-κB binding site on the CINC/gro gene as the cis-element responsible for repression by dexamethasone as well as IL-1- induced CINC/gro gene activation. Moreover, an electrophoretic mobility shift assay revealed that dexamethasone decreased IL-1-induced NF-κB binding site complexes, which were recognized using antibodies against p50 and p65. Furthermore, we found that this inhibition resulted from the prevention of NF-κB p65 translocation from the cytoplasm into the nucleus after degradation of the NF-κB inhibitor IκBα in the cytoplasm. Thus, glucocorticoids can inhibit NF-κB activity by a novel mechanism involving a blockage of the cytokine-induced nuclear translocation of NF-κB.

**EXPERIMENTAL PROCEDURES**

Cell Culture—The normal rat kidney epithelial cell line NRK-52E was purchased from Flow Laboratories Inc. (Tokyo, Japan). The cells were routinely cultured in Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (Bioserum, Victoria, Australia), penicillin G (160 units/ml, Sigma), streptomycin sulfate (100 μg/ml, Sigma), and NaHCO3 (1.4 g/liter) in a 5% CO2 atmosphere at 100% humidity at 37°C.

Cytokines and Reagents—Human recombinant IL-1β was obtained from R & D Systems Inc. (Minneapolis, MN). Human recombinant TNFα was from Genzyme Corp. (Cambridge, MA), LPS, actinomycin D, and dexamethasone were from Sigma. [32P]UTP (800 Ci/mmol), and [35S]poly(dI-dC) was from Pharmacia (Uppsala, Sweden), poly(dI-dC)poly(dI-dC) was from Pharmacia (Uppsala, Sweden). Polyclonal antibodies against p50, p65, c-Rel, RelB, and MAD-3 (Santa Cruz Biotech Inc. (Santa Cruz, CA). Anti-glucocorticoid receptor polyclonal antibody was purchased from Affinity Bioreagents Inc. (Neshanic Station, NJ), Rabbit IgG was purchased from Vector Laboratory Inc.

ELISA for CINC/gro—The content of CINC/gro in the supernatants was determined by an ELISA using a rat IL-8 assay kit (Panapharm Laboratories Co., Ltd., Kumamoto, Japan), following the supplier's instructions.

Northern and Slot Blot Analyses—NRK-52E cells grown to subconfluence in medium supplemented with 10% fetal calf serum were incubated with or without indicated concentrations of dexamethasone for 3 h. Various stimulants were added, and the cells were further incubated for the indicated periods. Total cellular RNA was extracted by the acid/guanidium isothiocyanate/phenol/chloroform method (37). For Northern blotting, 10 μg of total RNA was loaded and separated on 1.5% agarose gels containing 2% formaldehyde and blotted onto nylon membranes (Hybond-N, Amersham). For slot blotting, 10 μg of total RNA was denatured in 50% formamide, 1 × SSC (1 × SSC contains 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM EDTA containing 0.1% SDS, 50% formamide, 5 × Denhardt's solution, and 100 μg/ml sonicated salmon sperm DNA. After hybridization, the membranes were washed twice with 2 × SSPE, 0.1% SDS at room temperature for 10 min, once with 1 × SSPE, 0.1% SDS at 50°C for 15 min and twice with 0.5 × SSPE, 0.1% SDS at 50°C for 10 min. The membranes were exposed to Fuji imaging plates, and the radioactivity levels were determined using a bioimage analyzer ( Fujix BAS 2000, Fuji Co., Ltd., Tokyo, Japan). Hybridization with the 0.77-kb fragment of chicken NF-κB promoter region (Clontech, Inc., Gaithersburg, MD) or the human glyceraldehyde-3-phosphate dehydrogenase (Clontech Laboratories, Inc., Palo Alto, CA) confirmed that equal amounts of RNA were applied to each blot.

Nuclear Run-on Assay—Nuclear run-on assays were performed according to Greenberg and Ziff (38) with some modifications as described previously. Cells, were harvested in phosphate-buffered saline, and lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, and 0.5% (v/v) Nonidet P-40) for 5 min on ice. After washing with the same buffer, the pelleted nuclei were resuspended in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM MgCl2, and 0.1 mM EDTA) at 0.5–1 × 106 nuclei/100 μl then mixed with 100 μl of 2 × reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 300 mM KCl, 5 mM dithiothreitol, and 1 mM each of ATP, GTP, and CTP and 50 μCi of [α-32P]UTP (800 Ci/mmol). After incubating the mixture at 30°C for 30 min, radiolabeled RNA was purified using acid/guanidium isothiocyanate/phenol/chloroform (37), and hybridized to an excess (5 μg) of linearized pBluescript II SK(+) (Stratagene, La Jolla, CA) containing full-length CINC/gro cDNA (0.9 kb) immobilized on nylon membranes (Hybond-N, Amersham) at 65°C. After washing with the membranes and exposing them to Fuji imaging plates, the radioactivity levels were determined using a bioimage analyzer ( Fujix BAS 2000). The plasmid pBluescript II SK(+) and that containing the 1.0-kbp fragment of human glyceraldehyde-3-phosphate dehydrogenase or the 0.77-kbp fragment of chicken β-actin were used as negative and positive controls, respectively.

Construction of the Luciferase Expression Vectors—The Pst–PstI fragment of the genomic CINC/gro DNA which spans nucleotides (nt) −1034 to +7 from the start of the first exon (39) was subcloned into pUC118, digested with appropriate restriction endonucleases, and further subcloned into Smal and HindIII sites in the polylinker region of pBluescript II SK(+) (Stratagene, La Jolla, CA). The promoter region upstream of the firefly luciferase gene to generate pGL2-CINC/gro (−1034). Deleted fragments of the CINC/gro gene 5′-flanking region (starting from nt −164 and −48 to +6) were prepared by means of the polymerase chain reaction. Site-directed mutagenesis of the CINC/gro promoter was performed using a U.S.E. Mutagenesis kit (Pharmacia) according to the manufacturer's protocol. The adenovirus 2 major late promoter region, which was obtained by digesting the pAD8 vector (Clontech Laboratories Inc.) with XhoI/NotI, was subcloned into pBluescript II SK(+), digested with the appropriate restriction endonucleases, and further subcloned into the pGL2-Basic vector, to place the adenovirus promoter region upstream of the firefly luciferase gene. One copy of either wild-type or NF-κB binding site-mutated (MAD-3) promoter fragment from nt −164 to −48 was inserted upstream of the adenovirus promoter in the antisense orientation with respect to the luciferase gene. Two copies of the CINC/gro NF-κB binding site (5′-GGGAAATTC-3′) or two copies of CINC/gro NF-IL6 binding site (5′-TGAGCAGA-3′) were inserted upstream of the adenovirus promoter linked to the luciferase gene. Fidelity of the constructs was verified by nucleotide sequencing.

DNA Transfection and Luciferase Assay—To isolate long term stable transfectants from NRK-52E cells, the cells were co-transfected with 15 μg of the luciferase expression vector pGL2-CINC/gro (−1034) and 5 μg of pSV3neo by calcium phosphate co-precipitation as described (40). For transient expression studies, about 2.5 × 105 NRK-52E cells were transiently transfected with 2 μg of plasmid DNA by DEAE-dextran (0.5 mg/ml), with Profec™ mammalian transfection system DEAE-dextran (Promega), following the supplier’s instructions. After 16 h, cells were incubated with or without dexamethasone and/or various stimulants for the times indicated before harvesting the transfected cells. Under our experimental conditions, the differences in transient transfection efficiencies between dishes were less than 13% (data not shown).

The luciferase activity in cell extracts was determined using either a luciferase assay system (Promega) or PicaGene™ (Toyo Ink Co., Tokyo, Japan), following the supplier's instructions. The light intensity was measured with a Lumat model LB953 luminometer (Berthold, Germany). The protein concentration was measured by the Bradford method (41) (Bio-Rad protein assay) with bovine serum albumin as a standard. To analyze stably transfected cells, the luciferase assay was performed at least three times with three different transfectants. For transiently transfected cells, results were confirmed by at least two
Results

Dexamethasone Inhibits CINC/gro Production by Rat NRK-52E Cells—Production of CINC/gro from the normal rat kidney epithelial cell line NRK-52E was examined by measuring the levels of CINC/gro protein by ELISA in the culture supernatants. As shown in Fig. 1A, CINC/gro protein production was low in unstimulated cells, but was increased markedly by treatment with IL-1β, LPS, or TNF-α for 5 h. The induced CINC/gro protein synthesis was reduced by 1 μM dexamethasone by about 50% (Fig. 1A). Dexamethasone at concentrations higher

than 1 nM significantly inhibited CINC/gro production by the cells stimulated with IL-1β (Fig. 1D).

Effects of Dexamethasone on CINC/gro mRNA Induction in NRK-52E Cells—We next examined the levels of CINC/gro mRNA in NRK-52E cells incubated with various stimuli and/or...
Dexamethasone (1 μM) significantly changed in the presence of dexamethasone (Fig. 4). The half-life of CINC/gro mRNA of less than 1 h was not significantly affected (Fig. 3). This suggested that dexamethasone inhibited CINC/gro mRNA expression mainly at the transcriptional level. Consequently, the 5′-flanking region of the CINC/gro gene to assess the mechanism of transcriptional regulation in activation and the suppression by several cytokines and dexamethasone, respectively. The 5′-upstream region (from nt -1034 to +7) of the CINC/gro gene was linked to the luciferase gene and the construct was permanently transfected into NRK-52E cells. Fig. 1, C and F, shows the results of luciferase assay using one of these transformants. As shown in Fig. 1C, dexamethasone prevented the luciferase activity induced by IL-1β, LPS, or TNF-β. Moreover, dexamethasone inhibited the luciferase activity induced by IL-1β in a dose-dependent manner similar to that of the CINC/gro protein synthesis and of mRNA expression (Fig. 1F).

The inhibition of luciferase activity induced by IL-1β, LPS, or TNF was also reversed by RU486 (1 μM) (data not shown). Similar results were obtained with LPS or TNF-β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (C) mRNAs were determined using a bioimage analyzer (Fujix BAS2000). The data are presented as the fold increase over the resting value at time 0 from three separate experiments.

dexamethasone. Since preliminary Northern blots demonstrated a specific band for CINC/gro, β-actin, or glyceraldehyde-3-phosphate dehydrogenase transcripts under our hybridization/washing conditions (data not shown), we performed slot blot analysis in these experiments. Following exposure to IL-1β, the CINC/gro mRNA transcript level increased rapidly to its maximum at 3 h and remained elevated at 8 h (Fig. 2A). Dexamethasone (1 μM) inhibited this induction by about 50%.

In contrast to CINC/gro, the levels of β-actin and glyceraldehyde-3-phosphate dehydrogenase transcripts in the same cells did not significantly change irrespective of the presence of IL-1β and/or dexamethasone (Fig. 2, B and C). Dexamethasone also reduced CINC/gro mRNA levels in the cells stimulated with LPS or TNF-α for 3 h by about 50% (Fig. 1B). Moreover, dexamethasone inhibited CINC/gro mRNA expression stimulation with IL-1β in a dose-dependent manner similar to that of CINC/gro protein production (Fig. 1E). These results indicated that the effects of dexamethasone were expected at least partly at the pretranslational level. An incubation with the specific glucocorticoid receptor antagonist RU486 (1 μM) 2 h before dexamethasone (0.1 μM) abrogated the inhibitory effect of dexamethasone on CINC/gro mRNA induction by IL-1β (100 units/ml) (data not shown), suggesting that the effect of dexamethasone is mediated through the glucocorticoid receptor.

Effect of Dexamethasone on the Transcriptional Rate of the CINC/gro Gene—To examine whether dexamethasone affects CINC/gro gene expression at the transcriptional level, we performed nuclear run-on assays. As shown in Fig. 3, IL-1β dramatically induced the transcription of the CINC/gro gene and dexamethasone reduced this enhanced [α-32P]UTP incorporation into CINC/gro mRNA by about 50%, which correlated well with the degree of inhibition of CINC/gro production by dexamethasone. In contrast, the transcription rate of the control glyceraldehyde-3-phosphate dehydrogenase gene was not significantly affected (Fig. 3). This suggested that dexamethasone specifically reduced the transcriptional rate of the CINC/gro gene.

Effect of Dexamethasone on the Stability of the CINC/gro mRNA—To directly analyze whether or not dexamethasone decreases the stability of CINC/gro mRNA, we examined the half-life of CINC/gro mRNA in NRK-52E cells stimulated with IL-1β in the presence or absence of dexamethasone (1 μM). Transcription was interrupted 3 h after stimulation by actinomycin D (10 μg/ml), an inhibitor of RNA transcription. The half-life of CINC/gro mRNA of less than 1 h was not significantly changed in the presence of dexamethasone (Fig. 4). During this period, β-actin mRNA transcripts were relatively stable and this remained unchanged in the presence of dexamethasone (Fig. 4). This suggested that the inhibition of CINC/gro mRNA—...
in the luciferase assay using another two independent stable transformants (data not shown). When the same CINC/gro promoter-luciferase construct was transiently transfected into NRK-52E cells, the results were similar (Fig. 5B).

Northern blotting analysis of total RNA from the stable transformants revealed that dexamethasone suppressed the expression of luciferase mRNA induced by IL-1β (data not shown), indicating that suppression of luciferase activities by dexamethasone was caused by transcriptional repression of the introduced fusion gene. These results provide evidence for the presence of sufficient information within the 5′-flanking sequences between nt −1034 and +7 of the CINC/gro gene for transactivation, as well as for suppression by these cytokines and dexamethasone, respectively.

**Dexamethasone Suppressed Transcriptional Activation through the NF-κB Binding Site of the CINC/gro Gene—** To delineate the sequences in the CINC/gro promoter transactivation of which by IL-1β is inhibited by dexamethasone, we studied the effects of both agents on the expression of transiently transfected luciferase expression vectors linked to deleted CINC/gro promoters (Fig. 5). IL-1β greatly increased the luciferase activity in cells transfected with −1034 and −164 luciferase constructs, but showed hardly any increase when cells were transfected with the −48 luciferase construct. Dexamethasone prevented the expression of −1034/+7 and −164/+7 luciferase constructs induced by IL-1β, suggesting that the minimal essential elements for the CINC/gro gene expression regulation by IL-1β and dexamethasone are located within the 3′ region downstream of nt −164. There are two known cis-elements, NF-IL6 (nt −119 to −111) and NF-κB (nt −62 to −53) binding sites, within the region downstream of nt −164 (Fig. 5A) (39).

The regulatory roles of the NF-IL6 and NF-κB binding sites were examined by site-directed mutagenesis of each element in the −164 luciferase plasmid (Fig. 5). The deletion of the NF-IL6 binding site had little effects on CINC/gro gene activation and repression by IL-1β and dexamethasone, respectively, indicating that this site was not essential for gene regulation by these agents. Deletion or mutation of the NF-κB binding site abolished IL-1β-induced luciferase activity, indicating the essential role of the NF-κB binding site for CINC/gro gene activation by IL-1β.

To further examine the effect of dexamethasone on the transcriptional activity through the NF-κB binding site of the CINC/gro gene, two copies of the CINC/gro NF-κB binding site were linked to the adenovirus 2 major late promoter, which was inserted upstream of the luciferase gene. As shown in Fig. 6, luciferase gene expression driven from the adenovirus promoter was not affected by treatment with IL-1β and/or dexamethasone. Dexamethasone reduced the nt −164 to +7 region of the CINC/gro promoter-driven transcription induced by IL-1β (Fig. 6). Mutation of the NF-κB target sequence of the CINC/gro gene in the same luciferase construct abolished the induction of luciferase activity by IL-1β. Furthermore, the transcriptional activity of the NF-κB motif, but not of the NF-IL6 motif, was significantly enhanced by IL-1β, and the increased activity was inhibited by dexamethasone (Fig. 6). These findings suggested that the NF-κB binding site is the target for gene repression by dexamethasone.

**Dexamethasone Suppressed NF-κB Complex Formation Induced by IL-1β—** To examine the nuclear factors binding to the NF-κB binding site of the CINC/gro promoter, we performed an electrophoretic mobility shift assay using the CINC/gro −67/−48 DNA fragment containing the NF-κB binding site (nt −62
NF-κB-mediated Transcriptional Regulation of CINC/gro Gene

FIG. 6. Requirement of NF-κB binding site for repression of transcription of CINC/gro gene by dexamethasone. One copy of the wild-type or NF-κB site-mutated CINC/gro promoter from nt –164 to –7 as shown in Fig. 5 was inserted upstream of the adenovirus promoter in the antisense orientation with respect to the luciferase gene. Two copies of the CINC/gro NF-κB site (5'-GGGAAATTCC-3') or two copies of the CINC/gro NF-κB site (5'-TGGAAGCAAG-3') were also inserted upstream of the adenovirus promoter linked to the luciferase gene. Transfection proceeded as described under “Experimental Procedures.” After transfection, the cells were incubated with or without 1 μM dexamethasone (Dex) for 3 h, then stimulated with or without 100 units/ml IL-1β for 5 h and the luciferase activity was measured. The promoter activity of each test plasmid is indicated as luciferase activity relative to that of adenovirus promoter construct in the absence of both dexamethasone and IL-1β. These values are means ± S.E. for at least three separate experiments.

FIG. 7. Effects of dexamethasone on the formation of NF-κB complexes induced by IL-1β stimulation. NRK-52E cells were incubated with or without 1 μM dexamethasone for 3 h, then stimulated with or without 100 units/ml IL-1β for 1 h. Nuclear extracts were prepared as described under “Experimental Procedures.” A, detection of NF-κB binding activity by IL-1β stimulation. An electrophoretic mobility shift assay was performed with no nuclear extracts (lane 1) or with 1 μg of nuclear extract from either unstimulated (lane 2) or IL-1β-stimulated (lanes 3-7) NRK-52E cells. Either a 4- (lanes 4 and 6) or 16-fold (lanes 5 and 7) excess of wild-type (lanes 4 and 5) or mutant (lanes 6 and 7) NF-κB oligonucleotides were added to the binding reactions as competitors. B, dexamethasone suppressed the NF-κB complex formation induced by IL-1β. An electrophoretic mobility shift assay was performed with no nuclear extract (lane 1), with 1 μg of nuclear extract from unstimulated cells (lane 2), with those incubated with IL-1β (lane 3), IL-1β plus dexamethasone (lane 4), or dexamethasone (lane 5). The positions of the IL-1β-induced bands are indicated by arrows.

FIG. 8. The factor induced by IL-1β stimulation is NF-κB bound p50 and p65. NRK-52E cells were stimulated with or without 100 units/ml of IL-1β for 1 h, and nuclear extracts were prepared as described under “Experimental Procedures.” Electrophoretic mobility shift assays were performed with no nuclear extracts (lane 1) or with 1 μg of nuclear extract from either unstimulated (lane 2) or IL-1β-stimulated (lanes 3-17) NRK-52E cells. Supershift analysis was performed using 0.5 μg of nuclear extract from either unstimulated (lanes 4 and 5) or IL-1β-stimulated (lanes 6-17) NRK-52E cells. Monoclonal antibodies against p50, p52, p65, c-Rel, and RelB. Anti-p50 and anti-p65 antibodies supershifted the complex induced by IL-1β, whereas the other antibodies did not (Fig. 8, B), indicating that the induced NF-κB complexes were composed of p50-p65 heterodimers. Dexamethasone inhibited the formation of the NF-κB complexes induced by IL-1β (Fig. 7B). The radioactivity of the bands was quantified using a PhosphorImage Analyzer (Fujix BAS2000), and we found that dexamethasone inhibited the complex formation by 30–40% (seven separate experiments), which were similar to the values of the CINC/gro production inhibited by dexamethasone. In addition, we found that the inhibited NF-κB complexes were also composed of p50 and p65 heterodimers according to the results of the supershift assay (data not shown). These results indicated that dexamethasone inhibits the binding of the NF-κB transcription factor to the NF-κB binding site of the CINC/gro promoter, leading to the suppression of CINC/gro mRNA and protein synthesis.

Dexamethasone Suppressed the Translocation of p65 from the Cytoplasm into the Nucleus—To further examine the mechanism of dexamethasone-mediated NF-κB binding inhibition, we analyzed the effect of dexamethasone on the p65 and p50 levels in NRK-52E cells by Western blotting followed by densitometric quantitation of the respective bands in fluorograms. A representative experiment is shown in Fig. 9. In the presence of IL-1β, dexamethasone, or both, the amounts of p65 in whole cell extracts were measured as 1.2-, 1.1-, or 1.2-fold that found in the absence of these agents, respectively (Fig. 9C), indicating that these agents had a little, if any, effect on the total amount of p65 protein in the cells. Under the same conditions, p65 was exclusively localized in the cytoplasmic extracts from unstimulated cells (Fig. 9, A and B). IL-1β markedly increased the amount of p65 in the nuclear extracts by about 6-fold in parallel with the decrease of that in cytoplasmic extracts by about 1/6 (Fig. 9, A and B), suggesting that IL-1β induced the translocation of p65 from the cytoplasm into the nucleus. Dexamethasone diminished the amount of translocated p65 in the nuclear extracts by 65% in IL-1β-treated cells in parallel with an increase in level of p65 in the cytoplasmic extracts from 16 to 42% of the control resting cells (Fig. 9, A and B), suggesting that dexamethasone interferes with the translocation of p65 from the cytoplasm into the nucleus. In contrast to p65 protein, we found little, if any, detectable differences in the amounts of nuclear p50 protein in control and IL-1β- and/or dexametha-
Effect of dexamethasone on the levels of the p65 or p50 subunits of NF-κB. NRK-52E cells were incubated with or without 1 μM dexamethasone for 3 h, then stimulated with or without 100 units/ml IL-1β for 1 h. SDS-polyacrylamide gel electrophoresis was performed using 25 μg of nuclear (A and D), 30 μg of cytoplasmic (B), or 30 μg of whole cell (C and E) extracts from unstimulated cells (lane 1) or those treated with IL-1β (lane 2), IL-1β plus dexamethasone (lane 3), or dexamethasone (lane 4). After electrophoresis, the proteins were electroblotted onto Immobilon-P® polyvinylidene difluoride membranes. Transblotted membranes were incubated with either anti-p65 (A-C) or anti-p50 (D and E) antibody, followed by a reaction with horseradish peroxidase-conjugated anti-rabbit IgG. Proteins detected by the primary antibody were visualized using an ECL assay kit (Amersham) and by exposure to x-ray film. The degradation of IκB-α coincided with both the increase in nuclear p65 levels (Fig. 10A) and the appearance of NF-κB DNA-binding activity in nuclear extracts (Fig. 7), suggesting a causal relationship between the three events.

To determine whether dexamethasone inhibits the release of NF-κB from IκB-α after exposure to IL-1β, we examined the amount of IκB-α protein in cytoplasmic extracts prepared 1 h after exposure to IL-1β in the presence or absence of dexamethasone. As shown in Fig. 10B, the amount of IκB-α in IL-1β-activated cells was markedly reduced to 21% of that in resting cells. Dexamethasone, however, had no effect on the IL-1β-induced degradation of IκB-α (Fig. 10B). These results suggested that dexamethasone inhibits the activation of NF-κB by interfering with a pathway after the degradation of IκB-α.

**DISCUSSION**

Glucocorticoid hormones are highly immunosuppressive and reportedly inhibit the gene expression of several cytokines, particularly those with proinflammatory actions, such as IL-1 (25, 26), IL-2 (27), TNFα (28), interferon-β (29), interferon-γ (27), IL-8 (31), and monocyte chemotactic and activating factor (30). The results of this study demonstrated the inhibitory effect of the synthetic glucocorticoid dexamethasone on CINC/gro mRNA production in rat NRK-52E cells and its mechanism. We found that dexamethasone suppressed IL-1β-induced CINC/gro gene expression (Figs. 1 and 2) by inhibiting the transcriptional rate of the CINC/gro gene (Fig. 3) without affecting the stability of CINC/gro mRNA (Fig. 4). The inhibition of CINC/gro gene expression by dexamethasone was reversed by the specific glucocorticoid receptor antagonist RU486 (data not shown), suggesting the involvement of the glucocorticoid recep-
tor in transcriptional repression. Furthermore, we showed that the 5′-flanking region of the CINC/gro gene (extending from nt –1034 through +7) is sufficient to confer responsiveness to IL-1β and dexamethasone, since dexamethasone inhibited the IL-1β-induced expression of the CINC/gro promoter-driven luciferase vector stably transfected into NRK-52E cells (Fig. 1, C and F).

Functional analysis of the regulatory sequences of the CINC/ gro gene demonstrated that the minimally essential elements for the induction by IL-1β and repression by dexamethasone of the CINC/gro gene were present within the 3′ region downstream of nt –164, which contains the two known cis-elements, NF-IL6 (nt –119 to –111) and NF-κB (nt –62 to –53) binding sites (Fig. 5). Deletion of the NF-IL6 binding site did not abolish the inhibition of IL-1β-induced luciferase activity by dexamethasone (Fig. 5B), suggesting that dexamethasone does not inhibit CINC/gro gene transcription through interference with the NF-IL6 binding site. Although either a deletion or mutation of the NF-κB binding site abolished luciferase activity induced by IL-1β (Fig. 5B), a tandem repeat of the NF-κB sequence in the CINC/gro gene conferred inducibility by IL-1β and suppression of luciferase activity by dexamethasone (Fig. 6), suggesting that this site is the element responsible for dexamethasone-mediated gene repression. In addition, an electrophoretic mobility shift assay demonstrated that dexamethasone significantly diminished the IL-1β-induced formation of NF-κB complexes (Fig. 7B), which were identified immunochemically to consist of p50 and p65 (Fig. 8). Our results suggested that the NF-κB binding site is responsible for CINC/gro gene repression by dexamethasone.

Western blotting of cytoplasmic and nuclear extracts from NRK-52E cells demonstrated that IL-1β treatment induced the translocation of p65 from the cytoplasm into the nucleus (Fig. 9, A and B). Dexamethasone diminished the amount of p65 translocated to the nuclear extracts from cells exposed to IL-1β (Fig. 9A). We also found that the loss of nuclear p65 was paralleled by an increase in cytoplasmic p65 (Fig. 9, A and B) without affecting the total amount of p65 in the cells (Fig. 9C), suggesting that dexamethasone interfered with its translocation from the cytoplasm into the nucleus. Under our experimental conditions for Western blotting however, we detected neither the IL-1β-induced translocation of p50 protein into the nucleus nor the inhibition of translocation by dexamethasone (Fig. 9D). It is possible that the amount of p50 protein translocated from the cytoplasm into the nucleus was much less than that of the nuclear p50 protein constitutively present even in unstimulated cells.

We surmised that dexamethasone prevented the release of NF-κB from IκB after exposure to IL-1β. Since the phosphorylation and degradation of IκB is necessary for the activation of NF-κB (46–49), we examined the amount of IκB protein by Western blotting. Time course experiments showed that the activation of NF-κB by IL-1β in NRK-52E cells is correlated closely with the degradation of IκB-α, a member of the IκB family (Fig. 10A). We found that dexamethasone did not prevent the IL-1β-induced degradation of IκB-α (Fig. 10B), suggesting that it interfered with the translocation of NF-κB into the nucleus after dissociation of NF-κB/IκB-α complexes in the cytoplasm. However, we cannot exclude the possibility that dexamethasone has some effects on the synthesis and stability of other members of the IκB family, including IκB-β (50), as well as the NF-κB precursors p105 (51, 52) and p100 (53).

Mechanisms of glucocorticoid receptor-mediated repression of transcription have been proposed involving the physical interaction of the glucocorticoid receptor and NF-κB (33, 54, 55). This interaction may result in the glucocorticoid-mediated blockage of nuclear NF-κB binding to NF-κB binding sites. We showed that glucocorticoid decreased the levels of nuclear NF-κB of IL-1β-stimulated cells (Fig. 9A) while the glucocorticoid receptor level in the nucleus was markedly increased under the conditions where dexamethasone inhibited the NF-κB binding to DNA in the nucleus (Fig. 7B). Thus, although we could not immunochemically detect the glucocorticoid receptor in the NF-κB complexes formed in the presence of IL-1β and dexamethasone by means of an electrophoretic mobility shift assay, it is likely that a combination of decreasing the nuclear NF-κB protein level and blocking the binding of nuclear NF-κB to DNA by protein–protein interaction accounts for the total repression of CINC/gro gene expression by dexamethasone. The interaction of a glucocorticoid receptor with NF-κB complexes in the nucleus is now under study to determine whether the interaction also involves suppression of NF-κB activation in NRK-52E cells.

The AUUUA sequence in the 3′-untranslated region may be involved in the rapid degradation of mRNAs for some inflammatory cytokines and proto-oncogenes (56, 57). Moreover, several reports have shown that dexamethasone decreases the stability of mRNAs containing the AUUUA sequence in the 3′-untranslated region (25, 29). A specific protein binding to RNAs containing AUUUA has been identified (58, 59), and it is thought that formation of this complex may target susceptible mRNAs for rapid cytoplasmic degradation. CINC/gro mRNA contains similar AUUUA sequences in the 3′-untranslated region (39). However, we found that dexamethasone does not significantly decrease the half-life of entire CINC/gro mRNA, although its half-life was much shorter than that of the β-actin transcript which does not contain this sequence (Fig. 4). Thus, the present findings suggest that the AUUUA sequence is responsible for destabilization of CINC/gro mRNA, but not for CINC/gro gene repression by dexamethasone in NRK-52E cells.

Early studies showed that glucocorticoid inhibits the transcription of human IL-8 gene, either by binding of the glucocorticoid receptor to the glucocorticoid responsive element in the 5′-flanking region of the gene (32) or by interference with the binding of NF-κB to DNA without inhibiting the nuclear translocation of the factor (33). We demonstrated here that dexamethasone interfered with the binding of NF-κB to its cis-element on the rat CINC/gro gene that lacks a glucocorticoid responsive element in its promoter region. Unlike human IL-8, dexamethasone decreased the nuclear level of p65, perhaps by sequestering p65 in the cytoplasm after dissociation from IκB-α in NRK-52E cells. Thus, it is likely that glucocorticoids can inhibit NF-κB activity by two novel mechanisms involving blocks of cytokine-induced nuclear translocation and DNA binding of NF-κB. Given the role of NF-κB in the transcriptional activation of many inflammatory cytokine genes, we propose that these types of inhibition of NF-κB activation represent an important mechanism for the immunosuppressive properties of glucocorticoids.

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