Transcriptional Regulation of the Interleukin-6 Gene in Mesangial Cells

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Abstract. Cytokine secretion by mesangial cells (MC) plays a major role in the pathogenesis of glomerulonephritis. To define signaling events that occur during the activation of MC, the cell-specific transcriptional regulation of the interleukin-6 (IL-6) gene was studied. Stimulation with lipopolysaccharide and IL-1β resulted in the full induction of IL-6 expression only if the cells were coincubated with cAMP agonists; this effect was attenuated by protein kinase A inhibitors. In reporter gene experiments, the IL-6 promoter showed a stimulation pattern comparable to that of the endogenous gene. Elimination of individual transcription factor binding sites provided evidence for functional roles for four cis-acting elements, i.e., activator protein-1, cAMP response element-binding protein (CREB), nuclear factor for IL-6 expression (NF-IL6), and nuclear factor-κB (NF-κB). Electrophoretic mobility shift assays using nuclear extracts from MC revealed that the DNA-binding activities of activator protein-1 and NF-κB were inducible, whereas no change could be observed for CREB and NF-IL6. The presence of several transcription factor proteins, including JunB, JunD, c-Fos, Fra-1, CREB-1, activating transcription factor-2, NF-κB p50, p52, and p65, and CAAT/enhancer-binding protein-δ, was demonstrated by supershift analysis. Of particular interest was the novel finding of the participation of NF-κB p65 in the NF-IL6 complex. In summary, a signal transduction pathway in MC that requires protein kinase A activation in addition to a second signal provided by lipopolysaccharide or IL-1β was identified.

The generation of glomerulonephritis involves a number of mediators of inflammation and cytokines. One hallmark of the inflammatory response in the progression of glomerulonephritis is mesangial cell (MC) activation, which is mainly characterized by proliferation and secretion of various factors, including many cytokines and chemokines. Activation of MC is thought to be intimately associated with the pathogenesis of glomerulonephritis, ultimately leading to irreversible functional damage of the filtering apparatus (1).

Interleukin-6 (IL-6) has been implicated in the cytokine network controlling glomerular inflammation since Horii et al. (2) first reported, in 1989, that MC are able to express IL-6 and that IL-6 can be detected in kidney biopsies and in the urine of patients with mesangial proliferative glomerulonephritis. Several in vitro studies subsequently confirmed that IL-6 is a secretory product of MC and that its expression can be stimulated by serum, bacterial lipopolysaccharide (LPS), IL-1, IL-6, tumor necrosis factor-α (TNF-α), platelet-derived growth factor, granulocyte inhibitory protein, aggregated IgG and IgA, immune complexes, angiotensin II, fibronectin, and FK506 (3). Although the exact role of IL-6 in various forms of glomerulonephritis remains to be determined, cell-specific regulation of the IL-6 gene in MC is of special interest for understanding the involvement of IL-6 in glomerular diseases. At the level of transcriptional regulation, the function of IL-6 as a target gene for several different signal transduction pathways is reflected by the fact that various transcription factors can interact with the IL-6 promoter to initiate mRNA synthesis. Previous work by several groups showed that nuclear factor-κB (NF-κB) (4,5), nuclear factor for IL-6 expression (NF-IL6) (5,6), activator protein-1 (AP-1), cAMP response element (CRE)-binding protein (CREB) (7,8), interferon-regulatory factor-1, and specificity protein 1 (9) are involved. Furthermore, IL-6 is generally not constitutively expressed in resting nontransformed cells. These properties (inducibility by a wide variety of different stimuli and responsiveness to several transcription factors) make the IL-6 gene an ideal model for the study of transcription factor activation.

The cAMP-mediated signal transduction cascade is a prototypical second messenger pathway that involves stimulus-dependent cAMP generation, binding of cAMP to the regulatory subunits of protein kinase A (PKA), dissociation and nuclear translocation of the catalytic subunits of PKA, and phosphorylation of the transcription factor CREB. Genes regulated by cAMP contain a CRE, with a conserved palindromic 8-bp sequence, and include many cellular enzymes, hormones, and developmental genes (10). CAMP has also been implicated in the differential regulation of immune responses, particularly by inhibiting IL-2 expression. In a more general context, it has
been proposed that cAMP may modulate the balance between T_{H}1 (IL-2, IL-12, interferon-γ, and TNF-α) and T_{H}2 (IL-4, IL-5, IL-6, and IL-10) cytokine patterns in favor of the T_{H}2-type response (11). The transcriptional regulation of IL-6 expression with respect to cAMP seems to be cell-specific. Human lung fibroblasts show cAMP-dependent inhibition of IL-6 expression (12), whereas the ability of cAMP to stimulate IL-6 expression has been observed in fibroblasts, monocytes/macrophages (8), osteoblasts, anterior pituitary cells, and recently also MC (13). Studies of glomerular inflammation have led to the conclusion that elevation of intracellular cAMP levels in MC exerts anti-inflammatory effects by downregulating the expression of several genes, such as TNF-α, colony-stimulating factor-1, monocyte chemoattractant protein-1 (14), RANTES, and intercellular adhesion molecule-1 (15), and suppressing proliferation (16). It could thus be hypothesized that cAMP redirects gene expression in MC toward an inflammation-repressing pattern. The role of different transcription factors has been studied in a variety of cell types, but the specific regulation of IL-6 in MC has not been explored thus far. Therefore, in this study we attempted to characterize in some detail the binding sites in MC for the transcription factors AP-1, CREB, NF-κB, and NF-IL6, which are not only involved in IL-6 gene expression. In addition, we addressed the mechanisms that could account for the role of cAMP in cell-specific IL-6 regulation at the transcriptional level.

Materials and Methods

Cell Culture

An SV40-transformed murine MC line (17) was grown in Dulbecco’s modified Eagle’s medium supplemented with 100 U/ml penicillin/streptomycin (all from Biochrom, Berlin, Germany) and 2.5% Serum Supreme with very low LPS content (BioWhittaker, Walkersville, MD). Potential LPS contamination of the medium was removed with Ultrafilter U2000 columns (Gambro, Hechingen, Germany) before the addition of serum. Reagents used for stimulation of the MC included LPS from *Escherichia coli* (10 µg/ml; Sigma, Deisenhofen, Germany), 8-bromo-cAMP and cGMP (0.5 mM; BioLog, Bremen, Germany), forskolin (10 µM; Biomol, Hamburg, Germany), prostaglandin E_{2} (PGE_{2}) (1 µM) and 3-isobutyl-1-methylxanthine (IBMX) (100 µM) (both from Sigma), and the murine recombinant cytokine IL-1β (1 ng/ml; Boehringer, Mannheim, Germany). The PKA inhibitors H-89 and KT-5720 were from Calbiochem (Schwalbach, Germany) and Biomol, respectively.

IL-6 Enzyme-Linked Immunosorbent Assay

IL-6 levels in MC supernatants were determined using a pair of specific monoclonal antibodies and recombinant murine IL-6 from Pharmingen (San Diego, CA), according to a protocol supplied by the manufacturer.

Northern Blotting

RNA was prepared from cultured MC by the guanidinium thiocyanate/acid phenol technique. Total RNA (20 µg) was separated on a formaldehyde-containing agarose gel and transferred to a GeneScreen membrane (NEN Research Products, Boston, MA). A cDNA probe was synthesized, using the Prime-It II random primer labeling kit (Stratagene, Heidelberg, Germany), from a murine IL-6 cDNA provided by Genetics Institute (Cambridge, MA). Hybridization was performed overnight in 5× SSC, 1% sodium dodecyl sulfate (SDS), 5× Denhardt’s solution, 100 µg/ml fish sperm DNA, at 65°C, and the filters were then washed in 4× SSC, 1% SDS, 1× Denhardt’s solution, at 65°C for two more 30-min periods.

Transfection and Reporter Gene Assays

The pGL3-basic vector (Promega, Madison, WI) was used to construct the IL-6 promoter-driven reporter gene system. A 1.2-kb DNA fragment containing the human IL-6 promoter was inserted into the luciferase reporter vector (yielding pGL3-IL6-wt). Several mutants of pGL3-IL6-wt, corresponding to the mutant oligonucleotides used in gel shift experiments, were generated as described previously (8). The cells were transfected, in 6-cm tissue culture dishes, for 2 h with 50 ng/ml diethylaminomethyl-dextran (Sigma) containing 1 µg DNA/well, shocked for 2 min with 30% DMSO (Sigma), and grown for 12 h. After stimulation for 24 h, the cells were lysed in situ with reporter lysis buffer (120 µl/6-cm tissue culture dish). The luciferase assay (Promega) was performed according to the protocol supplied by the manufacturer. Each test was performed in triplicate. To control for transfection efficiency, the cells were cotransfected with 200 ng/well β-galactosidase reporter vector (Galacto-Light; Boehringer, Ingelheim, Germany).

Nuclear Extracts

The cells were first cultured under serum-free conditions for 24 h and then stimulated for 3 h in 15-cm tissue culture dishes. Nuclear extracts were prepared according to a method reported by Schreiber et al. (18), with minor modifications. Briefly, cells were washed once in ice-cold phosphate-buffered saline and then lysed by the addition of 1 ml of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl_{2}, 10 mM KCl, 0.5 mM dithiothreitol [DTT]) containing 0.6% Nonidet P-40 and protease inhibitors (200 µM Pefabloc™ SC [Boehringer, Mannheim, Germany], 1 µg/ml leupeptin, and 1 µg/ml aprotinin). After 15 min, the cells were scraped off, transferred to an Eppendorf tube, and vortex-mixed. Nuclei were washed once in buffer A without Nonidet P-40 and were collected by centrifugation. Proteins were extracted in 50 µl of high-salt buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl_{2}, 0.2 mM ethylenediaminetetraacetate [EDTA], 0.5 mM DTT, with protease inhibitors) by vigorous shaking for 20 min at 4°C. The protein solution was concentrated in microcentrifugation (molecular weight cutoff, 10,000; Amicon, Witten, Germany) and resuspended in buffer D (20 mM Hepes, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, with protease inhibitors). Samples were divided into aliquots and stored at −80°C.

Electrophoretic Mobility Shift Assays

The sequences of the double-stranded synthetic oligonucleotides used as probes in the gel shift experiments, corresponding to the binding sequences of the human IL-6 promoter, were as follows: AP-1: wild-type, 5'-TGACAGTCGTTCTGACTAAC-3' and 3'-CACGACTTCGATTGACCT-5'; mutant, 5'-TGACAGTCGTTCCAGACTAACC-3' and 3'-GCAGACCTCGATTGACCT-5'; CREB: wild-type, 5'-TGGACATGCTAAAGGACGTCACATTC-3' and 3'-TGGCTAGTCTTCGATTGACATCG-5'; mutant, 5'-TGGACATGCTAAAGGATTTGACATCG-3' and 3'-TGGCTAGTCTTCGATTGACATCG-5'; TES, and intercellular adhesion molecule-1 (15), and suppress-
GTGTACCTTTGCTGCT-5′; mutant, 5′-TCGACACTATAGTTAGGTATGCT-5′. Core sequences for transcription factor binding are underlined. Mutated nucleotides are marked in bold.

The oligonucleotides were 5′-radiolabeled with T4 polynucleotide kinase and [γ-32P]ATP, annealed, and purified on Sephadex G-50 columns (Pharmacia Biotech, Freiburg, Germany). Ten femtomoles of probe (corresponding to 5,000 to 30,000 cpm) were incubated with 4 µg of nuclear proteins for 20 min at 4°C. The binding conditions were 20 mM Hepes (pH 7.9), 5 mM spermidine, 5 mM KCl, 1 mM MgCl2, 2 mM EDTA, and 5% glycerol for AP-1 and CREB, 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 5% glycerol for NF-κB, and 2 mM Hepes (pH 7.9), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM KCl, 1 mM EDTA, and 7% glycerol for NF-IL6. In addition, all binding buffers contained 1 mM DTT, 50 ng/ml poly(dI:dC)/dI:dC), and 1 mg/ml bovine serum albumin. For supershift analysis, rabbit polyclonal anti-peptide antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear extracts prepared from LPS-plus cAMP-stimulated cells were preincubated with 1 µg of the corresponding antisera for 30 min at room temperature. Separation of protein-DNA complexes from the unbound probe was performed on 4% nondenaturing polyacrylamide gels that had been prerun at 100 V for 1 h. Electrophoresis was performed at 250 V for 2 to 3 h at 4°C, in 1 × Tris/borate/EDTA (TBE) for AP-1 and CREB, 0.25 × TBE for NF-κB, and 0.5 × TBE for NF-IL6.

Western Blotting

After stimulation for 30 min, the cells were washed and then lysed in boiling SDS sample buffer (300 µl/3-cm dish; 62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol). The lysate was boiled for 5 min, and 20 µl was loaded onto a 10% Laemmli minigel. The proteins were transferred to nitrocellulose membranes (Hybond ECL; Amersham, Freiburg, Germany) using an electroblotting system (XCell II; Novex, San Diego, CA). The filters were blocked with 5% dry milk and 0.1% Tween 20 in Tris-buffered saline and were incubated with an antisera specific for phosphorylated CREB (PhosphoPlus CREB [Ser133] antibody, at a 1:1000 dilution; New England Biolabs, Schwalbach, Germany). Visualization of bound antibody was performed with the Phototope-HRP detection system (New England BioLabs).

Statistical Analyses

Statistical comparisons of multiple experimental groups were performed using the nonparametric Kruskal-Wallis and Friedman tests. Significant differences (P < 0.05) are indicated.

Results

IL-6 Secretion Is Synergistically Induced by cAMP Agonists

We began by testing whether MC in culture would secrete IL-6 into the supernatant after stimulation. LPS and IL-1β are typical inducers of the IL-6 gene in several experimental systems (7,19) and are able to act on MC via specific surface receptors (20). After a 24-h incubation period, the IL-6 concentration in the supernatant from unstimulated cells was below the detection limit of the enzyme-linked immunosorbent assay (ELISA) (20 pg/ml). After stimulation with LPS and IL-1β, we were able to induce low levels of IL-6 in MC (Figure 1A). When the membrane-permeable cAMP analogue 8-bromo-cAMP was added simultaneously with the stimulants, a dramatic increase in IL-6 secretion was observed (Figure 1B). The synergistic effect of cAMP was approximately 50-fold with LPS and 30-fold with IL-1β. Addition of 8-bromo-cAMP alone did not lead to enhanced IL-6 generation. To confirm that the effect observed with 8-bromo-cAMP was indeed attributable to cAMP, we wanted to use a different agonist for cAMP-dependent signaling. Forskolin increases intracellular cAMP levels by activating adenylate cyclase. After exposure to forskolin at three different concentrations and costimulation with LPS or IL-1β, the MC secreted large quantities of IL-6, in a dose-dependent manner (Figure 1C). The maximal amounts that could be obtained with IL-1β and forskolin at 10 µM were still approximately threefold lower than those obtained with 8-bromo-cAMP. In the next series of experiments, the cells were treated with the phosphodiesterase inhibitor IBMX at 0.1 mM; IL-6 expression was enhanced by a factor of 9, compared with LPS and IL-1β alone (Figure 1D). This suggests that phosphodiesterases are continually acting to remove cAMP from the intracellular pool, even in unstimulated cells. Furthermore, a natural adenylate cyclase agonist, PGE2, was used to stimulate cAMP production via a receptor-dependent mechanism. PGE2 at a concentration of 1 µM enhanced LPS/IL-1β-induced IL-6 secretion six- to sevenfold. Coincubation with PGE2 and IBMX further upregulated IL-6 accumulation by a factor of 2. To confirm that the effect of increased intracellular cAMP levels on IL-6 expression involved activation of PKA, two specific PKA inhibitors, H-89 and KT-5720, were tested (at concentrations of 5 and 10 µM, respectively) (Figure 1E). IL-6 production was stimulated by 8-bromo-cAMP combined with LPS or IL-1β in this series of experiments. H-89 reduced IL-6 levels to 47 and 38% for LPS and IL-1β stimulation, respectively, and KT-5720 reduced levels to 24 and 33% of the baseline values. Therefore, inhibition of PKA led to a marked decrease in IL-6 expression, although it could not be blocked completely. Finally, cGMP was used to examine whether the observed effects might be shared between different cyclic nucleotides (Figure 1E). At a concentration of 0.5 mM, there was no effect of cGMP, either alone or in combination with LPS and IL-1β, on IL-6 production.

These experiments were repeated three times in triplicate; means ± SD were calculated for the three experiments combined. We conclude from the data that an increase in intracellular cAMP levels, in a PKA-dependent manner, synergistically enhances the production and release of IL-6 protein by a murine MC line.

Costimulation of IL-6 Gene Expression by LPS, IL-1β, and cAMP Is Reflected at the mRNA Level

Northern blot analysis was performed to verify that IL-6 secretion by MC in response to inducers and cAMP is regulated at the mRNA level (Figure 2). No IL-6 mRNA could be detected in unstimulated cells. IL-6 gene expression was weakly activated by 8-bromo-cAMP. For stimulation with LPS and IL-1β alone, steady-state mRNA levels were low but clearly above background levels. Addition of 8-bromo-cAMP together with LPS or IL-1β to the culture medium resulted in
a dramatic enhancement of IL-6 mRNA, corresponding to the IL-6 protein data from the ELISA. These results were confirmed in three series of independent experiments.

**Reporter Gene Assays Reveal the Function of at Least Four Promoter Elements**

Our earlier studies with macrophages showed that intact binding sites for at least four transcription factors, i.e., AP-1, CREB, NF-IL6, and NF-κB, are required for full inducibility of the IL-6 gene (8). To determine whether the binding sites for the four transcription factors present in the IL-6 promoter are functional in MC, we performed reporter gene experiments. MC were transiently transfected with a plasmid containing a 1.2-kb fragment of the 5' flanking region of the human IL-6 gene cloned upstream of a luciferase reporter gene (Figure 3). Transfected cells were exposed for 24 h to LPS or IL-1β alone or together with 8-bromo-cAMP. Expression of luciferase activity by pGL3-IL6-wt after stimulation was unaffected by 8-bromo-cAMP or LPS alone, whereas with IL-1β a slight induction (approximately twofold) was seen. Stimulation of the cells with IL-1β or LPS in combination with 8-bromo-cAMP resulted in a seven- to ninefold induction of luciferase activity (Figure 4A). Therefore, the synergistic effects of cAMP seen in the ELISA and Northern blot experiments were also observed in the reporter gene assay for IL-6. In additional experiments, we investigated the role of individual transcription factor binding sites in the inducibility of the IL-6 gene. Plasmids in which the binding sites for a single transcription factor had been eliminated by site-directed mutagenesis were used. Mutations in the binding sites for AP-1, CREB, NF-IL6, and NF-κB in the IL-6 promoter constructs each produced a large decrease in the maximal inducibility by LPS or IL-1β in combination with

three sets of experiments are shown as means ± SD. Each experimental condition was statistically compared with the unstimulated (unstim.) group. (B) The effects of 8-bromo-cAMP on IL-6 protein production by MC were assessed. Induction of IL-6 secretion was performed in the presence (gray bars) or absence (black bars) of 0.5 mM 8-bromo-cAMP. Statistical comparisons with the control were performed within the untreated and cAMP-treated groups. (C) MC were cultured with the indicated inducers and forskolin at increasing concentrations of 0 (black bars), 1 (dark gray bars), 5 (medium gray bars), and 10 μM (light gray bars). Statistical analyses were performed within each dose group (n = 3). *P < 0.05 or better for each experiment. (D) The effects of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the natural adenylate cyclase agonist prostaglandin E2 (PGE2) were assessed. Cells were either left untreated (black bars) or incubated with 100 μM IBMX (bars with horizontal stripes), 1 μM PGE2 (bars with vertical stripes), or both reagents (hatched bars), in combination with LPS and IL-1β as indicated. (E) IL-6 induction was repressed by specific protein kinase A (PKA) inhibitors. 8-Bromo-cAMP, with or without LPS or IL-1β, was used to stimulate the cells (light gray bars); the inhibitor H-89 was added to a final concentration of 5 μM (bars with horizontal stripes) and KT-5720 was added at 10 μM (bars with vertical stripes). Black bars, control without 8-bromo-cAMP. In this series of experiments, MC were also incubated with cGMP at 0.5 mM (dark gray bars), to demonstrate the specificity of cAMP action.

**Figure 1.** Secretion of interleukin-6 (IL-6) by murine mesangial cells (MC) after incubation with various stimulants. (A) MC were cultured for 24 h in the presence of lipopolysaccharide (LPS) (10 μg/ml) or IL-1β (1 ng/ml). The IL-6 concentrations in the supernatants were determined by enzyme-linked immunosorbent assays. Results from
8-bromo-cAMP (Figure 4B). The maximal inducibility of luciferase reporter gene activity after stimulation with LPS and 8-bromo-cAMP in the absence of a functional AP-1 or CREB binding site was approximately 30 to 40% of the IL-6 promoter wild-type control values. When the NF-κB or NF-IL6 sites were mutated, only 15 to 25% of the maximal inducibility was retained (Figure 4B). These findings confirm data on IL-6 gene regulation in monocytes, showing the functional importance of all four cis-acting elements in the IL-6 promoter (8). Figure 4A shows the mean of three independent experiments, and Figure 4B shows the mean of four independent experiments. The inducibility of the constructs was the same for cAMP and IL-1β (data not shown). The AP-1 and CRE sites in the IL-6 promoter show a high degree of sequence similarity. The sequence of the CRE differs by only two nucleotides, compared with the AP-1 site, so the two sites may compete for the same transcription factors (as shown in the gel shift experiments; Figures 5 and 6). Mutation of both the AP-1 site and the CRE in the IL-6 promoter reduced luciferase activity to basal levels under all stimulation conditions (Figure 4B).

Figure 2. Northern blot analysis of steady-state IL-6 mRNA levels in MC. Cells were grown for 24 h in serum-free medium and then stimulated for 4 h with the indicated inducers. Total RNA was prepared, blotted, and hybridized with a random-priming probe synthesized from mouse IL-6 cDNA. The filter was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to demonstrate equal loading of the lanes. Data from one of three independent experiments are shown.

Figure 3. IL-6 promoter. Functional involvement in the activation of IL-6 gene expression has been demonstrated for activator protein-1 (AP-1), cAMP response element (CRE)-binding protein (CREB), nuclear factor for IL-6 expression (NF-IL6), and nuclear factor-κB (NF-κB) (8). This 1.2-kb fragment of the IL-6 promoter was used to construct the pGL3-IL6-wt reporter gene vector.

Figure 4. Reporter gene assay using an IL-6 promoter-driven luciferase vector. (A) MC were transfected with the reporter gene vector pGL3-IL6-wt (containing a 1.2-kb fragment of the human IL-6 promoter) and stimulated for 24 h. Luciferase activity was measured in the presence (gray bars) or absence (black bars) of 8-bromo-cAMP. The means ± SD of three transfection experiments are shown. Luciferase activity was statistically compared with the basal activity of unstimulated cells. The control was the promoterless pGL3-basic vector. (B) The effect of the elimination of individual binding sites on the maximal inducibility of luciferase activity with LPS and cAMP costimulation was assessed. Values are shown as percentages, using the basal activity without stimulation of the cells as the reference. Each bar represents the mean ± SD of four experiments. mut., mutant.
Inducibility and Subunit Composition of the AP-1 Transcription Factor Complex Can Be Demonstrated

To investigate the binding of transcription factors to individual promoter elements, single binding sites were analyzed by electrophoretic mobility shift assays (EMSA). To examine AP-1 activity in MC, nuclear protein extracts were prepared and incubated with a double-stranded oligonucleotide containing an AP-1 element, and sequence-specific DNA binding was analyzed by nondenaturing polyacrylamide gel electrophoresis (Figure 5A). The AP-1-oligonucleotide complex was represented in untreated cells by a single, very weak, shifted band (Figure 5A, arrow); this band was markedly enhanced by addition of unlabeled wild-type (wt) and mutated (mut) oligonucleotides, at 10- and 100-fold molar excess, to the binding reaction showed that the wild-type oligonucleotide competed much more efficiently for protein binding, confirming the sequence specificity of the interaction. (C) Supershift analysis of AP-1 binding activity. Preincubation with polyclonal antibodies against the indicated transcription factor proteins showed the presence of JunB, JunD, c-Fos, and possibly Fra-1 proteins in the protein-DNA complex. In this and all subsequent experiments, nuclear extracts from cells that had been costimulated with LPS and cAMP were used for the supershift assays.

Figure 5. Electrophoretic mobility shift assay (EMSA) of AP-1 binding activity in the nuclei of MC. (A) Demonstration of a single protein-DNA complex (arrow) after stimulation with 8-bromo-cAMP, LPS, and IL-1β. (B) Competition analysis of AP-1 binding activity. Addition of unlabeled wild-type (wt) and mutated (mut) oligonucleotides, at 10- and 100-fold molar excess, to the binding reaction showed that the wild-type oligonucleotide competed much more efficiently for protein binding, confirming the sequence specificity of the interaction. (C) Supershift analysis of AP-1 binding activity. Preincubation with polyclonal antibodies against the indicated transcription factor proteins showed the presence of JunB, JunD, c-Fos, and possibly Fra-1 proteins in the protein-DNA complex. In this and all subsequent experiments, nuclear extracts from cells that had been costimulated with LPS and cAMP were used for the supershift assays.
incubation with LPS and IL-1β. 8-Bromo-cAMP alone, which did not increase IL-6 protein secretion, caused a clear induction of the AP-1 complex. An additive effect of 8-bromo-cAMP could be observed for stimulation with IL-1β but not with LPS.

Competition analysis was used to investigate the sequence specificity of the protein-DNA interaction (Figure 5B). Unlabeled oligonucleotide was added to the binding reaction at 10- or 100-fold molar excess. Displacement of the radioactive probe was almost 10-fold stronger for the wild-type competitor, compared with the mutant. To define the subunit composition of the DNA-protein complex, supershift assays were performed using polyclonal antisera specific for certain transcription factor proteins from the Jun and Fos families (Figure 5C). “Supershifted” bands could be detected with JunB-, JunD-, and c-Fos-specific antibodies. For the anti-Fra-1 antibody, the supershifted complex showed only moderate intensity but was consistently present in three experiments. Antibodies against c-Jun, FosB, Fra-2, CREB-1, and CREB-2 yielded negative results in supershift experiments (data not shown). EMSA for AP-1 were performed three times, with the same results. In summary, proteins with sequence-specific binding properties for the AP-1 element of the IL-6 gene could be demonstrated in nuclear extracts from MC. DNA-binding activity was very low in unstimulated cells and could be induced by 8-bromo-cAMP, LPS, and IL-1β; 8-bromo-cAMP had an additive effect when combined with IL-1β. The AP-1 complex in MC contained JunB, JunD, c-Fos, and small amounts of Fra-1 proteins, as shown by supershift analysis.

**CREB Transcription Factor Shows cAMP-Independent Constitutive Binding**

Next, we evaluated properties of CRE-binding factors in nuclear extracts from uninduced and stimulated MC (Figure 6A). Incubation of the proteins with the CRE oligonucleotide from the IL-6 promoter (GGACGTCAGA) resulted in the formation of a single complex (Figure 6A, arrow). The slight decrease of the shifted band after 8-bromo-cAMP treatment of the cells was not significant in repeated experiments. Overall, there were no consistent changes in the intensity of the complex in response to different stimulation conditions, as shown for cAMP and LPS as examples. Competition analysis with unlabeled mutant and wild-type oligonucleotides at increasing concentrations demonstrated the sequence specificity of the interaction. In the supershift assays, complexes with decreased electrophoretic mobility were observed with antibodies against activating transcription factor-2 (ATF-2), JunB, and JunD (Figure 6B). When the nuclear proteins were incubated with the anti-CREB-1 antisum, the intensity of the complex was markedly reduced; this suggests that CREB-1 is a constituent of the DNA-protein complex as well. Antibodies against several other members of the CREB/ATF family of transcription factors (CREB-2, ATF-1, ATF-3, and ATF-4) and against c-Jun, c-Fos, Fra-1, and Fra-2 had no effect.

Transcriptional activation by CREB occurs when the protein is phosphorylated at serine residue 133. Whether this phosphorylation affects DNA binding has been a matter of debate in the literature; our results provide no evidence that the DNA-binding activity of CREB is modulated after stimulation of MC. We wondered whether any changes in CREB phosphorylation could be demonstrated in our experimental system. This question is addressed by means of an antibody reacting only with phosphorylated CREB (p43) (21). Supershift assays using the anti-phospho-CREB antibody showed no changes in the shifted complexes of MC, under all stimulation conditions. This was confirmed by Western blot analysis of nuclear extracts (data not shown). However, the phosphorylation of CREB might be lost during purification of the nuclear proteins. We therefore used a protocol for preparation of whole-cell extracts that included immediate inactivation of phosphatas (by boiling in 1% SDS) as the first step. MC were stimulated for 30 min, and total cellular proteins were evaluated by Western blotting (Figure 7). Phosphorylated CREB was present in cells that had been treated with cAMP and was also present at low concentrations in IL-1β-induced cells. LPS had no effect on CREB phosphorylation (data not shown), but phospho-CREB levels were markedly diminished by the addition of the PKA inhibitors H-89 and KT-5720. The antibody cross-reacts with the phosphorylated form of ATF-1, because of the close homology between the two proteins.

In conclusion, elevation of intracellular cAMP levels resulted in phosphorylation of CREB at serine 133. However, in the nuclear extracts used for EMSA, the phosphorylation was lost, i.e., the anti-phospho-CREB antibody no longer recognized CREB. Therefore, the failure to show a change in CREB EMSA results might be attributable to dephosphorylation occurring during the preparation of nuclear extracts. In contrast to LPS, IL-1β alone also caused CREB phosphorylation to a certain extent, indicating a potential involvement of the cAMP pathway in IL-1-dependent signal transduction.

**NF-κB DNA-Binding Activity Is Inducible But Is Attenuated by cAMP**

Many genes that are important in immune and inflammatory reactions contain a recognition sequence for the transcription factor NF-κB. When nuclear extracts from MC were incubated with an NF-κB oligonucleotide, the appearance of multiple shifted bands was noted (Figure 8A). As can be seen from the competition experiment (Figure 8B), the three upper bands (Figure 8B, filled arrows) represent specific binding, whereas the lower band (Figure 8B, open arrow) represents nonspecific binding. There was a significant amount of sequence-specific DNA-binding activity in nonstimulated cells, which was decreased after pretreatment with 8-bromo-cAMP. LPS and IL-1β increased the intensity of the complex. The addition of 8-bromo-cAMP to these agents led to a reduction of all three bands. Comparable results for aggregated IgG and cAMP were previously reported from our laboratory (22). Incubation with antibodies against NF-κB p52 and p65 caused supershifting of one of the upper bands, whereas antibodies against p50 caused supershifting of all three bands. Anti-c-Rel and anti-RelB antibodies had no effect (Figure 8C). These data show that NF-κB DNA-binding activity in MC could be stimulated by LPS and IL-1β, whereas increased cAMP levels inhibited...
inducible NF-κB DNA binding. MC contained the three NF-κB subunits, i.e., p50, p52, and p65.

**CAAT/Enhancer-Binding Protein-δ (C/EBP-δ) and NF-κB p65 Bind Constitutively to the NF-IL6 Element**

The NF-IL6 transcription factor was cloned as a protein binding to an IL-1-responsive element in the IL-6 promoter (6). Analysis of the protein sequence revealed that NF-IL6 was a member of the C/EBP family of transcription factors. Therefore, it was renamed C/EBP-β (23). Nuclear extracts from MC displayed one specific band with the isolated NF-IL6 element from the IL-6 promoter; no alterations of the complex were observed with any of the stimulation conditions applied (Figure 9A, filled arrow). This was surprising, because the regulatory
mechanisms for NF-IL6 postulated to date include nuclear translocation (24) and modulation of DNA-binding activity (25). The specificity of the shifted band was verified by competition analysis (Figure 9B). In the supershift assay, the NF-IL6 band was eliminated by incubation with an anti-C/EBP-δ antibody (Figure 9C), indicating that C/EBP-δ rather than C/EBP-β actually represented the NF-IL6-binding activity in MC. The second protein that was part of the complex binding to NF-IL6 sequences was the NF-κB subunit p65. Heterodimer formation between members of the C/EBP and NF-κB protein families has been described by several authors (26–28) but, to our knowledge, the combination of C/EBP-δ and NF-κB p65 has not been reported to date.

All results presented in this report are summarized in Table 1. This overview emphasizes the correlations among data obtained by different methods.

### Discussion

We report here that elevation of intracellular cAMP levels synergistically upregulates IL-6 protein expression in a mouse MC line in combination with stimulants such as LPS and IL-1β. On the basis of results obtained with selective inhibitors, the effects could be assigned to cAMP-dependent PKA activation. These ELISA findings were confirmed by Northern blot and transient transfection experiments. The synergistic efficacy of cAMP measured in IL-6 protein secretion experi-

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**Table 1.** Overview of the regulatory influences of LPS, IL-1β, and cAMP on IL-6 protein secretion, mRNA expression, and transcription factor activation in murine MC

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control</th>
<th>LPS</th>
<th>IL-1β</th>
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<tr>
<td></td>
<td>− cAMP</td>
<td>+ cAMP</td>
<td>− cAMP</td>
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<tr>
<td>IL-6 ELISA</td>
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<td>Northern blotting</td>
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<td>(+)</td>
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<td>Reporter gene assay</td>
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<td>AP-1 EMSA</td>
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<td>CREB EMSA</td>
<td>+</td>
<td>(+)</td>
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<tr>
<td>pCREB Western blotting</td>
<td>−</td>
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<tr>
<td>NF-κB EMSA</td>
<td>+</td>
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<td>NF-IL6 EMSA</td>
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*This synopsis summarizes the results obtained in the study by applying a semiquantitative scheme with five gradations (−, (+), +, ++, and +++). Correlations among secreted IL-6 protein levels (ELISA), steady-state mRNA levels (Northern blotting), promoter activity (transient transfections), nuclear DNA-binding activities of the transcription factors AP-1, CREB, NF-κB, and NF-IL6 (EMSA), and the cellular content of phosphorylated CREB protein (pCREB) (Western blotting), are shown.*
ments seems greater than the promoter activity determined in reporter gene assays. This could indicate the additional involvement of cis-regulatory elements located outside our 1.2-kb promoter fragment or of posttranscriptional mechanisms such as variations in mRNA stability. The cAMP-dependent signal transduction pathway seems to be an essential coactivator for full induction of the IL-6 gene in MC. IL-6 expression by glomerular MC has been evaluated in numerous studies (3). However, the role of cAMP as a second messenger for IL-6 activation was only recently investigated by Robson et al. (13). None of these studies addressed the molecular basis of the changes in IL-6 gene expression. Using promoter constructs that lack individual binding sites, we showed that at least four binding sites in the IL-6 promoter are required for full inducibility. The identification of transcription factors involved in the regulation of a particular gene is a prerequisite for understanding intracellular signal transduction, because the transcription factors represent the ultimate targets integrating understanding intracellular signal transduction, because the involved in the regulation of Fos and Jun by cAMP.

The transcription factor AP-1 is a dimer composed of members of the Jun and Fos families of proto-oncoproteins, and it has a major role in the control of cell growth, cell differentiation, and expression of numerous genes (29). We were able to detect AP-1 DNA-binding activity in EMSA experiments. This binding activity was enhanced after treatment with cAMP, LPS, and IL-1β, suggesting either nuclear translocation, increased binding affinity, or de novo synthesis of the corresponding DNA-binding proteins. The pattern of AP-1 induction was in accordance with the additive effects of the stimulants and cAMP in the ELISA, Northern blot, and reporter gene experiments. The mechanism thought to be mainly responsible for the regulation of AP-1 trans-activation involves the mitogen-activated protein (MAP) kinase cascade (30), whose role in IL-6 induction was recently addressed (31). How could cAMP interact with the function of the AP-1 complex? There is now clear evidence for crosstalk between the MAP kinase (activated by Ras) and cAMP/PKA signaling pathways. On the basis of results obtained in various systems, a general role for cAMP as a molecular switch or gate modulating the activity of other signal transduction pathways has been postulated (32). Regulation of the MAP kinase cascade, however, is not the only way in which cAMP could potentially induce an AP-1-mediated transcriptional response. An inhibitor of Fos/Jun whose function is blocked by phosphorylation through PKA has been identified (33). Furthermore, several additional mechanisms, including transcriptional induction, nuclear translocation, phosphorylation, and targeted degradation, have been implicated in the regulation of Fos and Jun by cAMP.

Of special interest in the context of cAMP-mediated gene regulation is the CREB/ATF family of transcription factors, because they are the prototypical targets of the cAMP second messenger system (34). In our EMSA experiments, we have found a constitutive DNA-protein complex formed between the MC nuclear extracts and the IL-6 CRE oligonucleotide. The binding activity of nuclear proteins was not significantly altered after cellular stimulation, and costimulation with cAMP had no additive effect on CRE protein binding. However, CREB modification after cAMP elevation was demonstrated in a Western blot that specifically detected serine 133-phosphorylated protein only in cells stimulated with cAMP. The hypothesis that the effect of cAMP is mediated by PKA activation is supported by the antagonism of two different PKA inhibitors. These findings, together with the reporter assay results, argue in favor of the participation of CREB in cAMP-dependent IL-6 induction. The failure to show a change in CREB EMSA results might be attributable to dephosphorylation occurring during the preparation of nuclear extracts. This is an important point, because it calls into question the validity of EMSA for proteins that can be phosphorylated, thus altering their binding affinities.

Not only CREB-1 but also JunB and JunD proteins from MC interact with the CRE, as determined in the supershift assays. Interestingly, CREB-1 does not bind to the AP-1 oligonucleotide. This obvious overlap between the binding characteristics of the AP-1 element and CRE is not unexpected, because these elements differ by only 2 bp. Furthermore, the Jun, Fos, CREB/ATF, and C/EBP families of transcription factors all belong to the class of basic leucine zipper proteins, which can form heterodimers because of their close structural relationships. It is noteworthy that ATF-2 can be regulated by the c-Jun amino-terminal kinase signal transduction pathway (35) and that CREB can be activated by a Ras-dependent protein kinase (36). It is therefore conceivable that communication between the cAMP/PKA and Ras/Raf/MAP kinase pathways is also involved in transcriptional activation through the CRE site in the IL-6 promoter. The functional relevance of the AP-1 site and the CRE for IL-6 transcription in MC has been proven in transient transfection studies.

We and others previously described the inducibility of the NF-κB transcription factor in MC by IL-1β, LPS, TNF-α, and aggregated IgG, as well as the inhibitory effects of 8-bromo-cAMP, forskolin, and PGE2 on DNA-protein complex formation (22,37). The data presented here confirm these observations and extend them by showing that the three major NF-κB subunits (p50, p52, and p65) are present in nuclear extracts of stimulated MC, whereas RelB and c-Rel are absent. The major regulatory molecule for NF-κB activity is the inhibitor IkB, which retains NF-κB in the cytoplasm. After phosphorylation and degradation of IkB, free NF-κB is translocated into the nucleus and binds to its target sites (38). Assuming that activation of NF-κB occurs mainly via nuclear translocation, there is an apparent discrepancy between the requirement for an intact NF-κB site in our transfection studies and the attenuation of NF-κB DNA-binding activity in nuclear extracts after incubation with 8-bromo-cAMP. Although decreased DNA-binding activity of NF-κB proteins after cAMP stimulation was also shown by others, the reasons for this effect are still unclear. On the other hand, PKA regulates not only the transcriptional activity of AP-1 and CREB, but also that of NF-κB p65 (reviewed in reference (39)). This subunit binds to the NF-κB and NF-IL6 sites in the IL-6 promoter in MC. How the decrease in NF-κB binding is compensated for under conditions of elevated intracellular cAMP levels remains to be
clarified but could involve interactions with other binding proteins, such as CREB-binding protein/p300 (40,41).

With respect to the NF-IL6 binding site in the IL-6 promoter, we observed a constitutive, sequence-specific, protein complex whose intensity was not altered by any of the stimulation conditions applied to the cells. The only member of the C/EBP family that could be identified in this complex by supershift analysis was C/EBP-δ; C/EBP-β was absent. This could explain why we found no evidence for cAMP-induced nuclear translocation or activation of DNA binding, as described for C/EBP-β in previous reports (24,25). Furthermore, the anti-NF-κB p65 antibody reacted strongly with the specific complex, indicating physical association and cooperation between NF-κB p65 and C/EBP-δ. This is a new finding, because this observation had previously been made for only NF-κB p50 and C/EBP-β (26,27) and C/EBP-δ (28). The capacity for a physical interaction between NF-κB p65 and C/EBP-β in vitro was demonstrated by Stein et al. (27).

The transcriptional regulation of the IL-6 gene seems to be cell-specific. In some cell lines, cAMP is required for expression of the IL-6 gene, whereas expression is independent of cAMP in others (42). The synergistic effect of cAMP on IL-6 induction seen in our MC line resembled the effect previously described for the monocytic cell line Pu5-1.8. Even if the inducibility of the complexes seen in EMSA is different (8), this could explain why we found no evidence for cAMP-induced nuclear translocation or activation of DNA binding, as described for C/EBP-β in previous reports (24,25). Furthermore, the anti-NF-κB p65 antibody reacted strongly with the specific complex, indicating physical association and cooperation between NF-κB p65 and C/EBP-δ. This is a new finding, because this observation had previously been made for only NF-κB p50 and C/EBP-β (26,27) and C/EBP-δ (28). The capacity for a physical interaction between NF-κB p65 and C/EBP-β in vitro was demonstrated by Stein et al. (27).

The specificity of a cellular response to a stimulus is produced by the cooperation of different transcription factors. Therapeutic inhibition of one transcription factor could possibly block the activation of several cytokine genes and thus influence the progression of inflammation (43). Elegant studies exploring the potential of transcription factors as targets for novel therapeutic approaches have already been presented (44,45). Further elucidation of signal transduction pathways and gene regulatory mechanisms that become activated after immunologic injury may eventually enable us to prevent the deleterious clinical consequences of diverse inflammatory processes.

Acknowledgments

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (De 467/3-1).

References