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c-Rel and p65 *trans*-activate the monocyte chemoattractant protein-1 gene in interleukin-1 stimulated mesangial cells

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c-Rel and p65 *trans*-activate the monocyte chemoattractant protein-1 gene in interleukin-1 stimulated mesangial cells.

Background. The chemokine monocyte chemoattractant protein-1 (MCP-1) is secreted by human glomerular mesangial cells in response to interleukin-1 (IL-1) and has a central role in amplifying the inflammatory response during glomerulonephritis. However, the mechanism by which IL-1 regulates its transcription is not understood. Specific members of the nuclear factor κB/rel (NF-κB) proteins may regulate MCP-1 expression in a stimulus- and tissue-specific manner.

Methods. Electrophoretic mobility shift assays and Western blot analysis characterized the members of the NF-κB family that bound the two NF-κB sites of the MCP-1 enhancer (A1 and A2) *in vitro*. *Trans*-activation of the MCP-1 gene was investigated by transfer of the MCP-1 enhancer DNA to mesangial cells.

Results. Primary human mesangial cells contained in addition to p50 (NF-κB1) and p65 (Rel A) NF-κB proteins, the oncoprotein c-rel, and Rel B, but not p52 (NF-κB2). IL-1 induced c-rel to form a complex with p65, which bound the MCP-1 A2 site but not the A1 or IL-6 NF-κB sites *in vitro*. IL-1 up-regulated transfected MCP-1 enhancer activity. Cotransfer of the MCP-1 enhancer together with individual members of the NF-κB family showed that the heterodimer c-relp65 or $(p65)_2$ can selectively *trans*-activate the MCP-1 gene via its A1 and A2 sites in mesangial cells.

Conclusions. This study demonstrates for the first time that the c-rel oncoprotein can enhance MCP-1 transcription in mesangial cells and suggests that it may have an important role in amplifying gene expression in the inflamed glomerulus.

Chemokines are critical to the establishment of chronic inflammatory diseases such as glomerulonephritis [1]. Persistent chemokine secretion leads to inflammatory tissue damage, scarring, and loss of tissue function, as exemplified by glomerulonephritis in which the all-too-frequent failure to resolve the inflammation culminates in glomerulosclerosis and renal failure [2–4].

One chemokine, the monocyte chemoattractant protein-1 (MCP-1), has been shown to mediate recruitment of monocytes to the inflamed site [1, 5, 6]. *In situ* hybridization analysis of renal biopsies has demonstrated activated levels of chemokine expression in a number of inflammatory nephropathies (abstract; Cockwell et al, *J Am Soc Nephrol* 7:1694, 1996) [5, 6] and a rodent model of glomerulonephritis showed a significant reduction in glomerular monocyte/macrophage infiltration following administration of a neutralizing anti–MCP-1 antiserum [5].

The glomerular mesangial cell actively participates in the inflammatory response to glomerular injury [2–4]. In the inflamed glomerulus, it is activated by interleukin-1 (IL-1) to proliferate, secrete extracellular matrix, and synthesize MCP-1 [7], the neutrophil chemoattractant IL-8 [8, 9] and the cytokine IL-6 [9].

The molecular mechanism by which IL-1 regulates the expression of inflammatory genes is poorly understood [10]. The cytokine induces a spectrum of cell-type–specific genes that is responsible for the pleiotropic effects of IL-1. However, the ability of these IL-1–induced signaling pathways to regulate gene transcription selectively or cooperatively in a cell- or gene-specific manner remains unclear.

A crucial regulator of many IL-1–responsive genes is the nuclear factor (NF) κB or rel family of transcription factors [11, 12]. These proteins have two regulatory sites (A1 and A2) in the enhancer region (−2640 to −2632 and −2612 to −2603, respectively) and one in the promoter region (−90 to −80) of the MCP-1 gene [13, 14]. The enhancer sites A1 and A2 are vital regulatory elements in the inducible transcription of the gene, and the A2 site is critical for IL-1–enhanced MCP-1 activity in mouse carcinoma cells. In addition, the MCP-1 gene encodes regulatory sites for a number of other transcription factors, including Sp1, which controls basal transcription [13].

Key words: primary cells, MCP-1 gene, nuclear factor-κB, interleukin-1, glomerulonephritis.

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The importance of the NF-κB activators of transcription is apparent from the extent to which immune and inflammatory responses are compromised in mice with targeted disruption of NF-κB subunits [reviewed in 11, 12, 15, 16]. In its simplest, most common form, NF-κB comprises a heterodimer of the most abundant, ubiquitous members of the family: the 50 (p50/NF-κB1) and 65 kDa (p65 or RelA) proteins. When inactive, it resides as a heterodimer in the cytoplasm of resting cells complexed to an inhibitory protein I-κBα. After appropriate stimulation of cells, I-κB dissociates from the complex, and the active heterodimer translocates to the nucleus to bind its target promoter [11, 12, 17]. IL-1 can rapidly induce NF-κB via its type I but not type II receptor [18] by phosphorylating and degrading I-κBα/MAD3 [19]. Several workers have reported on the importance of I-κB phosphorylation in targeting the inhibitor for ubiquitin-mediated proteolysis [reviewed in 17].

Recent evidence suggests that specific dimeric combinations of the NF-κB family control the transcription of particular genes. For example, homodimers of p50 and p65 can bind target DNA motifs in lymphoid cells and can repress or activate transcription, respectively [12]. Furthermore, specificity may also be conferred by differential activation of NF-κB proteins in different cell types.

Thus, complexes such as c-rel/p65 can be activated in monocytes HeLa and THP1 cells [20, 21], but are found only in an inactive form in HepG2 cells [22].

Previous studies of MCP-1 transcription in tumor cell lines have shown that lipopolysaccharide, tetradecanoyl phorbol acetate (TPA), and IL-1 can induce human MCP-1 mRNA synthesis [13, 14, 23]. One previous study has reported that IL-1β increases MCP-1 mRNA in mesangial cells, although only p50 and p65 NF-κB were studied and the role of these in *trans*-activation of the MCP-1 gene in this cell type remains unknown [23]. Interestingly, the mouse homologue of MCP-1, termed *JE*, appears to be regulated in a stimulus-specific manner; for example, regulation by tumor necrosis factor (TNF) appears to require both distal and proximal NF-κB regions [24], whereas platelet-derived growth factor (PDGF) invokes an additional remote NF-κB site to activate MCP-1 transcription [25].

This study set out to define the importance of individual members of the NF-κB family in regulation of transcription of the MCP-1 gene in primary mesangial cells, and to assess whether or not IL-1 enhances MCP-1 gene expression via selective activation of the NF-κB proteins. The presence of the c-rel oncoprotein is shown, and a role for it in binding to the A2 NF-κB site but not to that of the A1 MCP-1 or the IL-6 promoter is demonstrated. Moreover, to our knowledge for the first time, IL-1 is shown to up-regulate MCP-1 transcription via both the A1 and A2 sites in transfected mesangial cells, and a role for c-rel in MCP-1 *trans*-activation is demonstrated.

METHODS

Materials

Tissue culture medium RPMI 1640 containing standard supplements (100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine) were obtained from GIBCO (Uxbridge, UK). Insulin 25 mg/ml, selenium 25 mg/ml, transferrin 25 μg/ml, and growth supplement were obtained from Sigma (Dorset, UK), fetal calf serum (FCS) from Advance Protein Products Ltd. (West Midlands, UK). IL-1α and -β were a gift from Professor Jeremy Saklatvala (Kennedy Institute, Charing Cross Hospital, London, UK). 3,4-Dichloroisocoumarin (DCI), 1-trans-epoxysuccinyl-leucylamide (4-guanidino) butane (E64), NaF, Na_3VO_4, β-glycerophosphate, deoxycholate (DOC), Nonidet P40 (NP40), and goat antirabbit peroxidase-conjugated whole IgG were all supplied by Sigma. T4 polynucleotide kinase was from Stratagene (Cambridge, UK); [γ^{32}P] ATP (6000 Ci/mmol) was from DuPont NEN (Herts, UK), and poly (dI-dC) was from Pharmacia (Milton Keynes, UK). All oligonucleotides were synthesized and high-performance liquid chromatography (HPLC) purified by Oswel DNA Service (Southampton, UK). NF-κB p50 antibody was a gift of Dr. H. Holmes (MRC AIDS Reagent project, NIBSC, South Mimms, UK). Antisera to c-rel together with their cognate peptides were obtained from Serotec (AHP 289; Oxford, UK) or Santa Cruz Biotechnology (sc-71x; Heidelberg, Germany). The c-rel antisera were each raised against a peptide taken from the C terminus of c-rel (sequence undisclosed by manufacturer). Antisera to p65 NF-κB (raised against aa 531-550 in C terminus of human p65), Rel B (raised against aa 540-558), its corresponding peptide, anti-p52 (aa 298-324) were all supplied by Santa Cruz Biotechnology. All tissue culture plastic ware was purchased from Costar (Bucks, UK).

Cell culture

Mesangial cells. Human mesangial cells were prepared by standard methods of serial culture/trypsinization of adherent outgrowths from glomeruli obtained by sieving of kidney cortex, and their identity and purity were confirmed as described previously [26, 27]. The normal cortex from human kidneys removed from patients with renal cell carcinoma were used. Cells were cultured in RPMI 1640 with 10% FCS supplemented with 1000 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and a growth supplement containing 25 mg/ml insulin and transferrin and 25 μg/ml sodium selenite at 37°C in a humidified 95% air/5% CO_2 atmosphere. Cells were used between passages 3 and 5 after subculture into 162 cm^2 flasks.

A clone of mesangial cells isolated from normal rat glomeruli (gift of Dr. Masanori Kitamura, UCL, London, UK) was cultured in DMEM/F12/Nut Mix supplied with Glutamax and supplemented with 1000 U/ml penicillin, 100 μg/ml streptomycin, and 5% vol/vol FCS. Cells were used between passages 4 and 18.

Preparation of mesangial cells for nuclear factor-κB activation. Confluent mesangial cells were washed twice in phosphate-buffered saline and placed in growth medium containing 0.1% FCS for 48 to 72 hours prior to the experiment. Cell cultures were stimulated with 10 or 20 ng/ml IL-1α or IL-1β. At the end of the period of stimulation, cells were placed on ice and nuclear and cytosolic extracts were prepared as follows.

Nuclear and cytosolic extract preparation. Cell extracts were prepared by modification of previously described standard procedures [28, 29]. Protein estimations were performed by a dye-binding assay [30]. Briefly, cells were washed in ice-cold phosphate-buffered saline and scraped, on ice, in a hypotonic buffer containing additional proteinase and phosphatase inhibitors [buffer A: 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 100 μM phenanthroline, 1 μg/ml pepstatin, 100 μM E64, 100 μM DCI, 100 mM NaF, 100 μM Na_3VO_4, and 25 mM β-glycerophosphate]. Mesangial cells were then lyzed by incubating for 10 minutes on ice in 60 to 80 μl hypotonic buffer containing 0.2% NP40. Lysates were then centrifuged (10,000 *g* at 4°C) for 10 minutes, and the supernatants were discarded. The pelleted nuclei were resuspended in 60 to 80 μl of a lysis buffer [buffer C: 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 25% glycerol, and 100 μM DCI] and were incubated at 4°C for 15 minutes. Lyzed nuclei were vortexed then centrifuged (10,000 *g* at 4°C) for 10 minutes, and supernatants were snap frozen and stored at −80°C.

Electrophoretic mobility shift assay

Nuclear factor-κB binding activity was detected with synthetic oligonucleotide probes containing either the A1 (−2640 to −2632, "TGGGAACTTCC") or A2 NF-κB binding site of the MCP-1 gene (−2611 to −2603, "TGG GAATTTCC") [13, 14], the NF-κB site of the IL-6 promoter (−125 to −135, "GGGATTTTCC") [31], or a consensus mutant site (CTCACTTTCC) [18]. These were radiolabeled with [γ^{32}P]ATP by T4 polynucleotide kinase for 10 minutes at 37°C.

DNA-binding assays were essentially as previously described [18]. All reactions were performed in a total volume of 20 μl containing the binding buffer [10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% (vol/vol) glycerol, 5 mM DTT, 100 μg/ml bovine serum albumin (BSA)], the sample, 2 μl of [^{32}P]-labeled oligonucleotide and 3 μg of poly (dI-dC). After incubation for 15 minutes at room temperature, samples were electrophoresed in a 5% polyacrylamide gel/0.25 × Tris borate EDTA, ph 8.0 (TBE). For competition experiments, unlabeled oligonucleotides were incubated with extracts for five minutes prior to the addition of radiolabeled probe. After electrophoresis, gels were dried and then autoradiographed by exposure to medical x-ray film.

Supershift analysis

Polyclonal antibodies to p50, c-rel, p65, Rel B, or p52 were incubated with cell nuclear extracts for either two hours at room temperature or at 4°C overnight. Poly (dI-dC, 3 μg) and 2 μl radiolabeled NF-κB probe were then added, and the samples were incubated and electrophoresed as described for the electrophoretic mobility shift assay (EMSA).

Western blot analysis

Cell extracts (100 to 300 μg) were mixed with twice-concentrated sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, heated (100°C for 3 min), and electrophoresed on gels containing 12.5% acrylamide and 0.33% bis-acrylamide. The gels were electrotransferred to nitrocellulose (250 mA for 4 hr) in a buffer containing 25 mM Tris base, 192 mM glycine, and 20% methanol (vol/vol). The membrane was incubated for one hour at 37°C in blocking buffer [10 mM Tris HCl, pH 7.4, 154 mM NaCl, and 8% (wt/vol) milk powder], then in blocking buffer supplemented with antiserum (1:500, vol/vol) for 16 hours at 4°C. After five washes in Tris-buffered saline (TBS; 10 mM Tris HCl, pH 7.4, 154 mM NaCl), the membrane was incubated in TBS containing an antirabbit peroxidase-conjugated IgG for a further two hours at room temperature, washed as before, and air dried. Proteins were detected by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK).

Cell transfection and luciferase assays

Rat mesangial cells (50 to 70% confluent) cultured in DMEM/F12/Nut mix containing 0.1% FCS in a 24-well plate were transiently transfected with 0.2 μg of pGL3 basic plasmid (*firefly* luciferase reporter vector; Promega, Southampton, UK) containing the MCP-1 enhancer [14] per well by incubation with a liposome preparation (Transfast; Promega) for 24 hours at 37°C. The effect of individual NF-κB subunits on MCP-1 expression was investigated by cotransfection of pRSV expression vectors (25 ng) containing the cDNAs of p50, p65, or c-rel or the empty expression vector (pRSPA; gifts of Dr. Nancy Rice, NCI, Frederick, MD, USA) as previously described [30]. The cells were then incubated in growth medium containing 0.1% FCS or IL-1β (20 ng/ml) for a further 22 to 24 hours. Transfection efficiency was determined by cotransfer of 250 pg of a *Renilla* luciferase gene under the control of an SV40 promoter (pRL SV40; Promega). At the end of this period, the *Firefly* and *Renilla* luciferase activities were determined using separate detection buffers consecutively on the same sample according to the protocol provided by the manufacturer.

Statistical analysis

The data presented are the mean ± SEM of N experiments. Statistical comparisons between multiple groups were performed by analysis of variance, with a minimum value of $P < 0.05$ considered to represent statistical significance.

RESULTS

Human mesangial cells contain interleukin-1–inducible NF-κB activity against the NF-κB sites of the monocyte chemoattractant protein-1 and interleukin-6 promoters

Human mesangial cells are known to secrete MCP-1 and IL-6 in response to IL-1 [7, 9], but how the cytokine selectively regulates the expression of inflammatory genes is not known. One previous study in human mesangial cells has reported IL-1–inducible NF-κB activity with a tandem repeat of an NF-κB consensus sequence [23]. In our current study, induction of NF-κB by IL-1α was demonstrated by employing oligonucleotides containing the region of the MCP-1 enhancer that included either the A1 or the A2 NF-κB site (shown to be crucial to IL-1 activation of the MCP-1 gene in tumor cells) and, for comparison, the NF-κB site from the IL-6 promoter. Confluent cultures were incubated in growth medium containing 0.1% vol/vol FCS for 48 to 72 hours and then stimulated with 20 ng/ml IL-1α for 1 hour, and nuclear and cytosolic extracts were prepared as described in the **Methods** section. Oligonucleotides containing the MCP-1 A1, A2, or IL-6 promoter binding sites for NF-κB were added to the extracts and NF-κB activity measured by EMSA (a minimum of three independent experiments). Induction of NF-κB activity was observed in nuclear extracts following stimulation with IL-1α for 15 minutes. The A1 and A2 MCP-1 probes detected four DNA–protein complexes (Fig. 1 a–d, lanes 1–4). The two inducible complexes labeled (a) and (b) and the third complex (c) were also observed with the IL-6 probe (Fig. 1, lanes 7 and 8), but the fourth constitutive complex (d) was not detected. When extracts were mixed with radiolabeled mutant probe, complexes (a), (b), and (d) were not observed, indicating that these were NF-κB specific. One nonspecific complex was detected with the mutant probe (complex c; Fig. 1, lanes 5 and 6). The level of binding of complexes (c) and (d) varied between experiments and is likely to be caused by interdonor variation. Incubation of extracts with unlabeled MCP-1 A1, A2, or IL-6 probes as competitors abrogated the formation of (a) and (b) and diminished d (Fig. 1, lanes 9–11). The unlabeled mutant probe did not affect the binding of any of the specific complexes to the A1 (Fig. 1, lane 12) or A2 (Fig. 1, lane 13) probes, confirming that these consisted of members of the NF-κB family of transcription factors. Thus, two IL-1–inducible complexes were detected at consistent levels with both the MCP-1 and the IL-6 probes, and a constitutive complex was detected only by the A1 and A2 MCP-1 probes.

c-Rel oncoprotein and Rel B are present in human mesangial cell extracts

Having established the ability of IL-1α to induce NF-κB activity against the MCP-1 and IL-6 oligonucleotides, it was necessary to identify whether members of the NF-κB family other than p50 and p65 [23] were present in human mesangial cell extracts. Western blot analysis revealed the presence of two of the ubiquitous members of the NF-κB family, p50 and p65 (Fig. 2 A, B, panels 1–3), in both the nuclear and cytosolic compartments of human mesangial cells. In addition, both cell fractions contained the oncoprotein c-rel (Fig. 2, panels 4 and 5) and also Rel B (Fig. 2, panels 6 and 7) but not another member of the NF-κB family p52 (NF-κB2; Fig. 2B, panel 8). IL-1 consistently increased the level of c-rel in the nucleus, as observed by Western blot analysis of nuclear extracts. To confirm the identity of the p65, c-rel, and Rel B antigens, peptides containing the sequences to which each antibody was raised were included as competitors in the Western blot analysis (Fig. 2, panels 3, 5, and 7). The p65, c-rel, and Rel B peptides abrogated binding of their respective antigens by the antibodies but did not "cross-compete" with other antibodies (data not shown), confirming the identity of the proteins detected. The minor bands of lower molecular weight detected by the p65 antiserum appear to be specific or immunologically related to the antibody, as they were abrogated by peptide competition or they may be proteolytic fragments of p65. The specificity of the c-rel antibody was confirmed by immunoprecipitation of lysates prepared from confluent rat mesangial cells with 2 different antisera to c-rel (AHP289 c-rel antibody; Serotec) and a C-terminal peptide antibody (sc 71; Santa Cruz Biotechnology), each of which detected a protein of approximately 80 kDa, further verifying that the antigen detected was c-rel (data not shown).

Interleukin-1 induces c-rel binding to the A2 but not the A1 or interleukin-6 nuclear factor-κB binding sites *in vitro*

The level of expression of c-rel and Rel B is tissue dependent [33–35]. The same antibodies used in the Western blot analysis were employed to test the immunoreactivity of the DNA-protein complexes detected by EMSA. Incubation with antibodies to p50 and p65 resulted in either the abrogation of binding or the formation of supershifted complexes, demonstrating that both proteins were present in the complexes detected by the A1 and A2 probes (Fig. 3A, B). p50 and p65 appear to be constituents of the fastest migrating, inducible com-

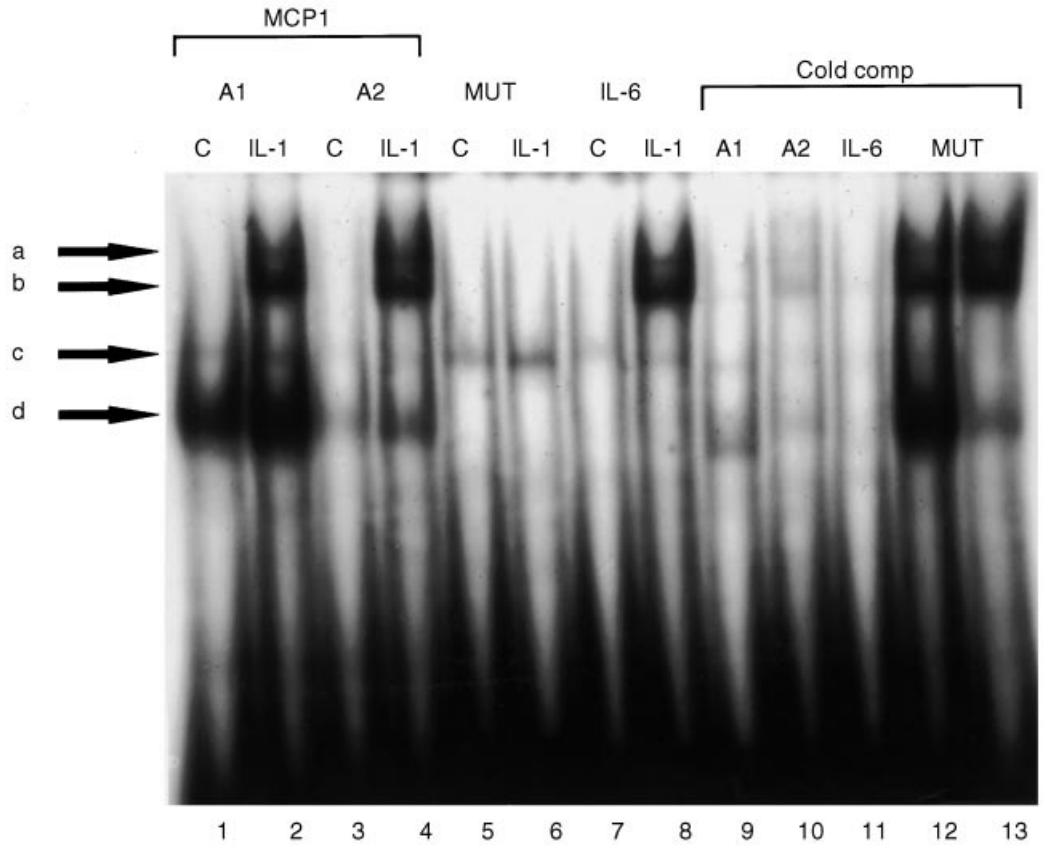

Fig. 1. Interleukin (IL)-1α induces nuclear factor (NF)-κB–specific activity against the monocyte chemoattractant protein-1 (MCP-1) and IL-6 NF-κB sites in human mesangial cells. Nuclear extracts from human mesangial cells incubated with 20 ng/ml IL-1α (IL-1, lanes 2, 4, 6, and 8–13) or without (C, lanes 1, 3, 5, 7) were incubated with radiolabeled oligonucleotides containing the NF-κB site from the MCP-1 A1 (lanes 1–2, 9, 12), A2, (lanes 3, 4, 10, and 13) or IL-6 (lanes 7, 8, and 11) NF-κB sites and EMSAs performed. The four DNA-protein complexes detected (arrows a, b, c, d) and the affect of incubation of nuclear extracts with a mutant NF-κB probe (MUT, lanes 5 and 6) or competition with unlabeled A1 (lane 9), A2 (lane 10), IL-6 (lane 11), or mutant probes on A1 (lane 12) or A2 (lane 13) binding are shown.

plex b, whereas p65 is present in the complexes a and b. Complexes a and b were NF-κB specific (Fig. 1). c-Rel was found to be present in the complex detected by the A2 (Fig. 3B, lane 7) but not the A1 (Fig. 3A, lane 5) nor the IL-6 probes (Fig. 3C, lane 4). The c-rel antibody did not alter the extent of NF-κB binding to the A1 or the IL-6 probes. Rel B binding was not detected (Fig. 3 A and C, lanes 6 and 5, respectively) and was always absent from the MCP-1 and IL-6 sites. c-Rel binding to the MCP-1 A2 probe was reproducibly detected in seven experiments, with cells from four donors. Thus, c-rel may be involved in regulating the MCP-1 A2, but not the A1 or IL-6 NF-κB, sites in human mesangial cells. To further test the validity of the c-rel antibody, supershift assays were performed on primary human fibroblasts and on HeLa cells. The latter are known to express c-rel. Interestingly, human gingival fibroblasts (HUGI) contained p50 and p65 but did not contain c-rel (Fig. 4A, compare lanes 1–5), whereas the A2 MCP-1 NF-κB complexes in HeLa cells appeared to contain active oncoprotein (Fig. 4B, compare lanes 6–10), in agreement with previous studies [21].

p65c-rel and $(p65)_2$ *trans*-activate the monocyte chemoattractant protein-1 enhancer when transfected in mesangial cells

To determine whether specific NF-κB subunits interacted with the MCP-1 enhancer, mesangial cells were first transfected with the MCP-1 enhancer (0.2 μg) containing the A1 and A2 NF-κB sites. Because of the difficulty in transfecting human mesangial cells, primary rat cells were employed for these experiments. IL-1 up-regulated MCP-1 activity in rat mesangial cells, as measured by an increase in the reporter *firefly* luciferase activity (Fig. 5). IL-1 had no effect on the transfected empty pGL3 basic vector (data not shown). To study the ability of homodimeric and heterodimeric combinations of NF-κB subunits to enhance MCP-1 transcription, p50, p65 or c-rel were individually or in heterodimeric combinations, co-transfected with the MCP-1 enhancer (Fig. 5). In order

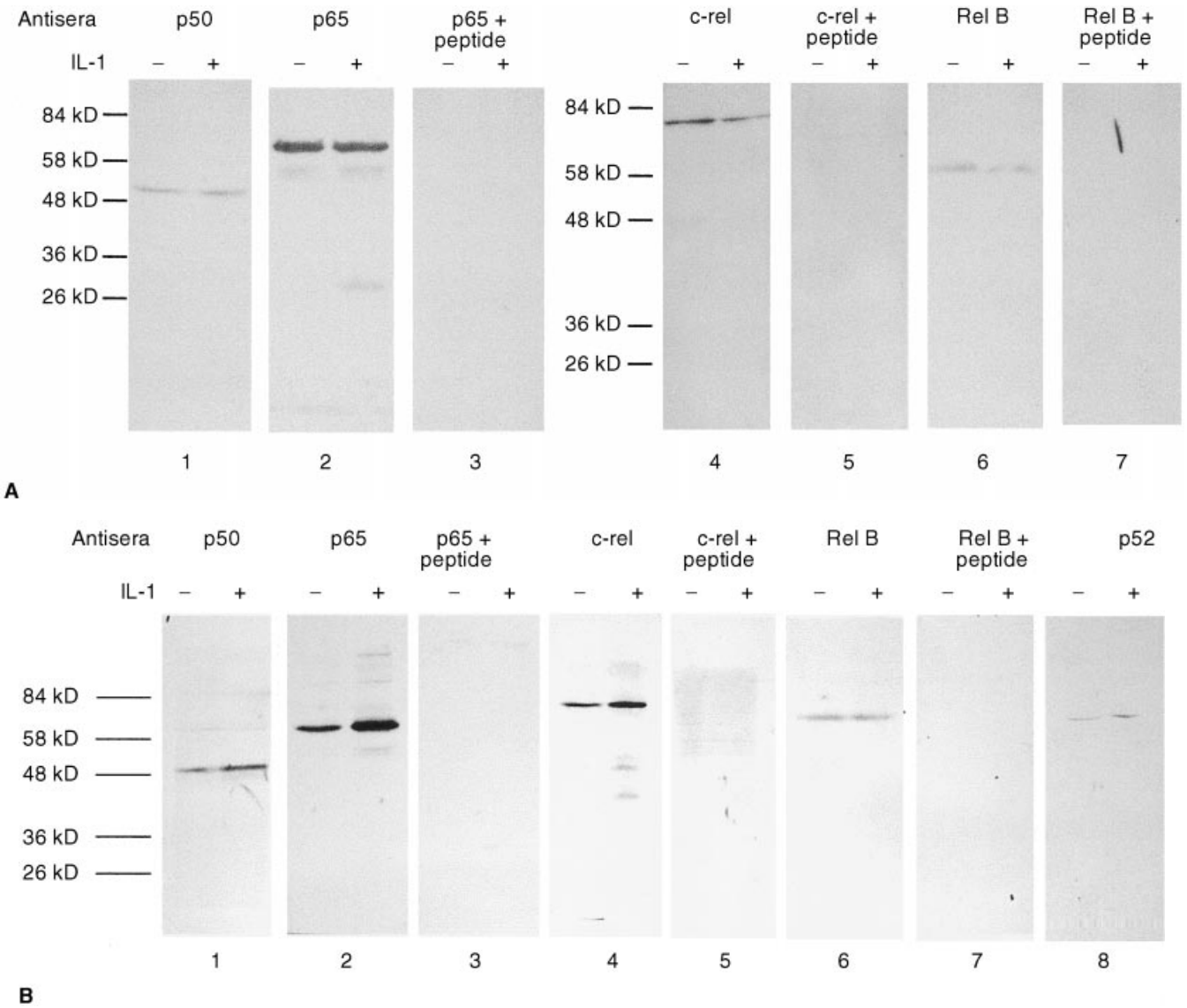

Fig. 2. Western blot analysis of constituent NF-κB proteins in human mesangial cell extracts. Cytosolic (*A*) and nuclear extracts (*B*) were subjected to SDS-PAGE and electrotransferred to nitrocellulose as described in the **Methods** section. Membranes were probed with antibodies to NF-κB p50 (panel 1), p65 (panels 2 and 3), c-rel (panels 4 and 5), Rel B (panels 6 and 7), and p52 (B panel 8). p65, c-rel, and Rel B antibodies were coincubated with peptides containing the sequences to which each antibody was raised (A and B, panels 3, 5, and 7, respectively). NF-κB antibody binding was detected with a peroxidase-conjugated IgG followed by enhanced chemiluminescence.

to ensure that identical quantities of DNA were added to the cells in all conditions, empty expression vector was added to supplement the level of DNA. All data were normalized against the level of *renilla* luciferase activity obtained by cotransfer of pRL SV40 in all conditions. Coexpression of different combinations of the NF-κB subunits with the MCP-1 enhancer showed that p65-crel and p65 homodimer (but not c-rel homodimer, p50p65, nor p50crel) selectively and significantly increased MCP-1 transcription. An identical though enhanced effect was observed following IL-1β (1 ng/ml, 24 hr) stimulation.

Mutation of A1 and or A2 nuclear factor-κB sites abrogates interleukin-1–induced monocyte chemoattractant-1 enhancer activity

To determine which of the NF-κB sites in the MCP-1 enhancer was mediating IL-1α transcriptional activity, the MCP-1 A1 and A2 sites were mutated individually or in combination, and the effect on enhancer activity in mesangial cells transfected with the MCP-1 enhancer was examined in both the absence (Fig. 6A) or presence of IL-1 (20 ng/ml, 24 hr) stimulation (Fig. 6B). Mutation of either the A1 or the A2 site or both abrogated MCP-1 enhancer activity in both control and IL-1–stimulated

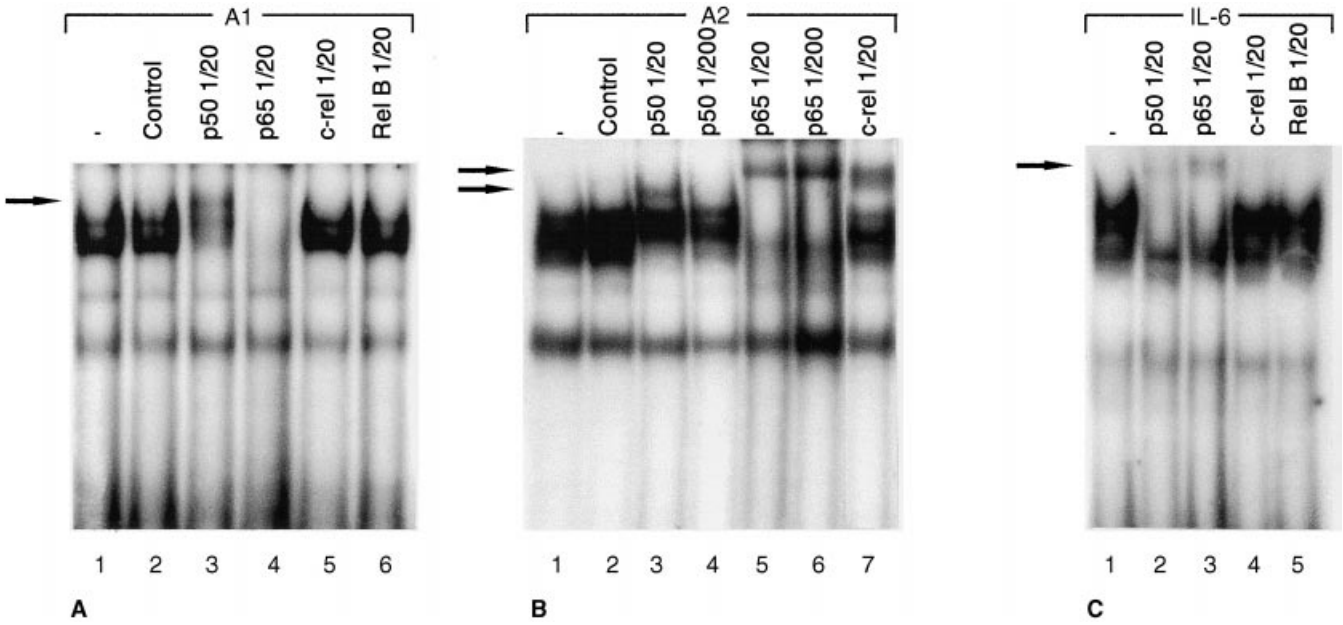

Fig. 3. Supershift analysis of human mesangial cell NF-κB complexes detected by the monocyte chemoattractant protein-1 (MCP-1) and interleukin (IL)-6 probes. Nuclear extracts from human mesangial cells stimulated with 20 ng/ml IL-1α for one hour at 37°C (*A–C*, lanes 1–6, 1–7, and 1–5, respectively) were preincubated in the absence (lane 1, A–C) or presence (lane 2, A and B) of control serum, an antibody to p50 (A, lane 3; B, lanes 2 and 3; C lane 2) or p65 (A, lane 4; B, lanes 5 and 6; C, lane 3), c-rel (A, lane 5; B, lane 7; C, lane 4), or Rel B (A, lane 6; C, lane 5) overnight at 4°C at the dilutions shown. EMSAs were performed with the A1 (A), A2 (B), or IL-6 (C) probes prior to autoradiography. Arrows indicate positions of supershifted complexes.

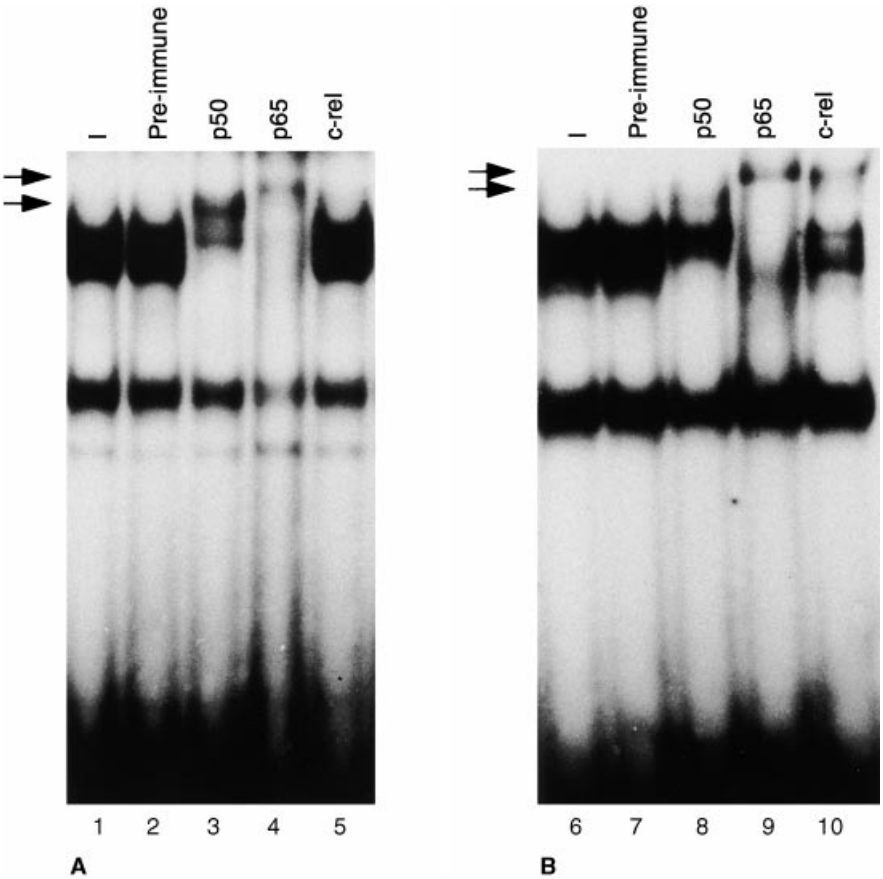

Fig. 4. Supershift analysis of the NF-κB complexes detected by the monocyte chemoattractant protein-1 (MCP-1) probe in HUGI and HeLa cells. Nuclear extracts from HUGI (*A*, lanes 1–5) or HeLa cells (*B*, lanes 6–10) were stimulated with 20 ng/ml IL-1α for one hour at 37°C (lanes 1–10) and preincubated in the absence (lanes 1 and 6) or the presence (lanes 2 and 7) of preimmune serum or an antibody to p50 (lanes 3 and 8), p65 (lanes 4 and 9), or c-rel (lanes 5 and 10) overnight at 4°C. EMSAs were performed with the MCP-1 probe (A and B, lanes 1–10) prior to autoradiography. Arrows indicate positions of supershifted complexes.

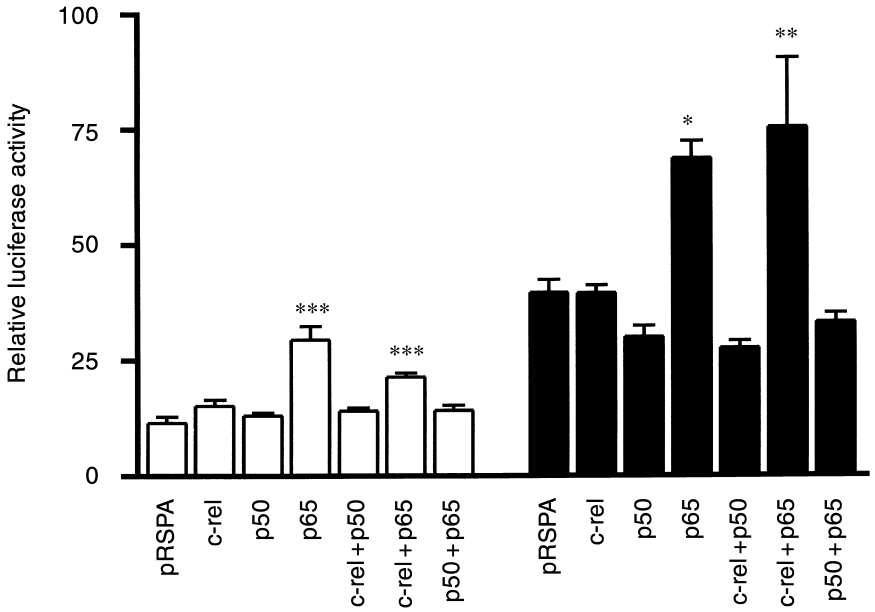

Fig. 5. Effect of different combinations of NF-κB subunits on monocyte chemoattractant protein-1 (MCP-1) transcription in mesangial cells before and after stimulation with IL-1. Cells were transfected with the MCP-1 enhancer (0.2 μg) together with 25 ng of empty vector pRSP, p50, p65, or c-rel or 25 ng total of p50p65, p50crel, or p65c-rel. pRL SV40 *renilla* luciferase reporter (0.25 ng) was cotransfected to measure transfection efficiency. Incubations were performed in medium containing 0.1% fetal calf serum for 24 hours and then incubated for a further 24 hours in the presence (■) or absence (□) of IL-1 20 ng/ml. Turner light units represent *firefly* luciferase activity, as measured on a Turner luminometer (normalized against the level of *renilla* luciferase activity detected). Results shown are the means ± SEM of four independent experiments. $^*P < 0.05$.

mesangial cells. It also inhibited the up-regulation of MCP-1 enhancer activity observed in the presence of c-relp65 or p65 homodimer, indicating that both sites are required for *trans*-activation by these NF-κB complexes.

DISCUSSION

This study investigated the mechanism by which IL-1 regulates NF-κB–mediated MCP-1 gene expression in primary mesangial cells. The cytokine is known to activate the NF-κB family of transcription factors, which is crucial to the inducible activation of MCP-1 [13], a chemokine critical to the pathogenesis of glomerulonephritis [1, 5, 6].

To identify the effects of IL-1 on specific complexes of the NF-κB family, it was necessary first to characterize the NF-κB proteins that were present and active against the MCP-1 enhancer in mesangial cells. The experiments also demonstrate that NF-κB subunits can selectively *trans*-activate the MCP-1 gene *in vivo.* We report here that in addition to p50 and p65, the oncoprotein c-rel and Rel B are present in primary human mesangial cells. Studies in mice and transformed cell lines suggest that the level of expression of c-rel and Rel B is tissue dependent and that they have important cell- and gene-specific effects [reviewed in 12]. Evidence obtained from our antibody supershift assay experiments shows that c-rel bound to the A2 MCP-1 but not the A1 or IL-6 NF-κB sites, and suggests that c-rel and p65 are constituents of the same complex bound as a heterodimer to the A2 MCP-1 region of the enhancer. Although we detected c-rel activity in HeLa cells (Fig. 4B) in agreement with others [36, 37], this was not found in primary human fibroblasts (HUGI; Fig. 4A). Taken together, these data indicate that in mesangial cells, NF-κB proteins may regulate the MCP-1 gene in a cell- and/or gene-specific manner.

Recent work in tumor cell lines has shown that two NF-κB sites in the MCP-1 enhancer (A1 and A2) are crucial to the induction of MCP-1 transcription [14]. Moreover, both p65 homodimers and p65c-rel heterodimers overexpressed in mouse embryonic carcinoma cells increased expression of the MCP-1 transcript *in vivo* [14]; however, the relevance of these findings to mesangial cells has hitherto been unclear. In our current study, transfection of a primary clone of mesangial cells with the MCP-1 enhancer containing both the A1 and A2 sites together with different combinations of p50, p65, or c-rel showed interestingly that the c-relp65 heterodimer or p65 homodimer selectively up-regulated MCP-1 transcription. These are the same complexes that were detected by supershift analysis (Fig. 3) as binding the A2 MCP-1 probe *in vitro.* Transfection experiments performed with mutant A1 or A2 or both showed the requirement for both sites in order to obtain maximal induction of the MCP-1 enhancer both in resting and IL-1–stimulated cells. Our inability to detect c-rel binding to the A1 site in the supershift assay may be due to the fact that in this assay, the NF-κB sites are isolated and out of context of the whole enhancer. It may be that neighboring sequences are important in determining which proteins bind to these motifs *in vivo.* Further studies on the role of c-rel are underway involving immunodepletion of nuclear extracts employing a c-rel antibody

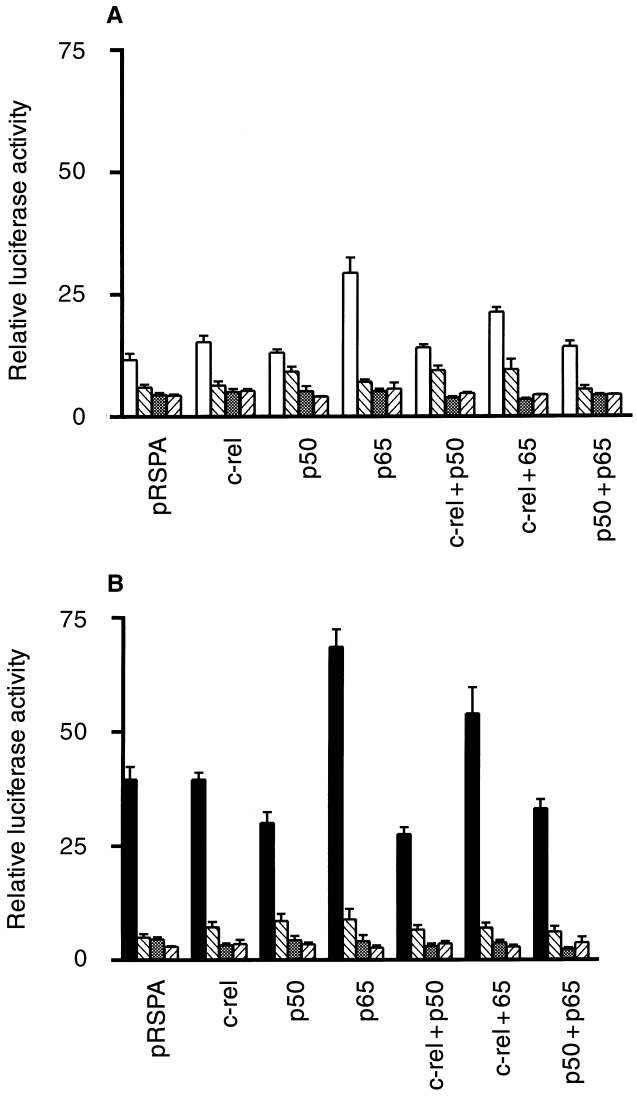

Fig. 6. Effect of mutations of the A1 and or A2 sites in the MCP-1 enhancer. Cells were transfected with 0.2 μg intact MCP-1 enhancer (□), or MCP-1 enhancer with mutated A1 site (▧; MA1), mutated A2 site (■; MA2), or both (▨; MA1A2) together with 25 ng of empty vector pRSPA, p50, p65, or c-rel or 25 ng total of p50p65, p50crel, or p65c-rel. pRL SV40 *renilla* luciferase reporter (0.25 ng) was cotransfected to measure transfection efficiency. Incubations were performed in medium containing 0.1% fetal calf serum for 24 hours and then incubated for a further 24 hours in the presence (*B*) or absence (*A*) of IL-1 20 ng/ml. Turner light units represent *firefly* luciferase activity as measured on a Turner luminometer (normalized against the level of *renilla* luciferase activity detected). Results shown are the means ± SEM of four independent experiments. $^*P < 0.05$.

with subsequent EMSA and also demonstration of c-rel binding by DNAse I footprinting analysis.

It is also worth mentioning that the NF-κB subunits are likely to be only part of the transcription factor requirement for expression of the MCP-1 gene because there are a number of other binding motifs present in the MCP-1 promoter and enhancer regions, including sites for AP1 and Sp1 [13, 14].

The detection of p50 and p65 in the complexes binding the IL-6 site is in agreement with that reported previously in U937 cells stimulated with lipopolysaccharide [38]. Interestingly, a previous study in lymphoid cells has shown binding of c-rel homodimers to an IL-6 NF-κB site [39]. Further experiments are required to reveal whether complex *trans*-activates the IL-6 gene.

Rel B activity was not detected against either NF-κB sequence investigated, which is not surprising, as it does not bind with high affinity to NF-κB sites [12]. Rel B has been reported to be constitutively active in primary lymphoid cells of transgenic mice [34], and Rel B knockout mice are characterized by multiorgan inflammation caused by infiltration of mixed populations of inflammatory cells [40]. Rel B can form heterodimers with p50 (NF-κB1), p52 (NF-κB2), and their respective precursors but not with p65 (Rel A) or c-rel (Rel), suggesting that it too may be involved in regulating the expression of a particular subset of genes, which excludes IL-6 and MCP-1. The inability of Rel B to form homodimers [41] and the presence of p50 suggest that Rel B might form heterodimers with p50 in human mesangial cells. The function of this complex in these cells awaits further investigation.

Characterization of the NF-κB family in human mesangial cells in this study has identified the candidate proteins that regulate the inducible transcription of MCP-1 by IL-1. The role of c-rel, in particular, merits further investigation given its variable level and gene-specific function in different tissues. Studies of the mechanism by which IL-1 exerts its effects on c-rel as well as on other members of the NF-κB family, for example, p65, with which it is transcriptionally active, will provide the basis for defining the events specific to the cytokine that regulate chemokine transcription in mesangial cells and ultimately may identify targets for therapeutic manipulation of mesangial cell function in the inflamed kidney *in vivo*.

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REFERENCES

1. Furie MB, Randolph GJ: Chemokines and tissue injury. *Am J Pathol* 146:1287–1301, 1997
2. Ooi BS, Cohen DJ, Veis JH: Biology of the mesangial cell in glomerulonephritis: Role of cytokines. *Proc Soc Exp Biol Med* 213:230–237, 1996
3. Kashgarian M, Sterzel RB: The pathobiology of the mesangium. *Kidney Int* 41:524–529, 1992
4. Abboud HE: Growth factors in glomerulonephritis. *Kidney Int* 43:252–267, 1993
5. Wenzel U, Schneider A, Valente AJ, Abboud HE, Thaiss F, Helmchen UM, Stahl RAK: Monocyte chemoattractant protein-1 mediates monocyte/macrophage influx in anti-thymocyte antibody-induced glomerulonephritis. *Kidney Int* 51:770–776, 1997
6. Tam FWK, Karkar AM, Smith J, Yoshimura T, Steinkasserer A, Kurrle R, Langner K, Rees AJ: Differential expression of macrophage inflammatory proein-2 and monocyte chemoattractant protein-1 in experimental glomerulonephritis. *Kidney Int* 49:715–721, 1996
7. Rovin BH, Yoshimura T, Tan L: Cytokine induced production of monocyte chemoattractant protein-1 by cultured human mesangial cells. *J Immunol* 148:2148–2153, 1992,
8. Brown Z, Strieter RM, Chensue SW, Ceska M, Lindley I, Neild GH, Kunkel SL, Westwick J: Cytokine-activated human mesangial cells generate the neutrophil chemoattractant, interleukin 8. *Kidney Int* 40:86–90, 1991
9. Abbott F, Ryan JJ, Ceska M, Matsushima K, Sarraf CE, Rees AJ: Interleukin-1β stimulates human mesangial cells to synthesize and release interleukins-6 and -8. *Kidney Int* 40:597–605, 1991
10. Stylianou E, Saklatvala J: Molecules in focus: Interleukin 1. *Int J Biochem Cell Biol* 30:1075–1079, 1998
11. Perkins N: Achieving transcriptional specificity with NFκB. *Int J Biochem Cell Biol* 29:1433–1448, 1997
12. Siebenlist U, Franzoso G, Brown K: Structure, regulation and function of NF-κB. *Annu Rev Cell Biol* 10:405–455, 1994
13. Ueda A, Okuda K, Ohno S, Shirai A, Igarashi T, Matsunaga K, Fukushima J, Kawamoto S, Ishigatsubo Y, Okubo T: NFκB and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *J Immunol* 153:2052–2063, 1994
14. Ueda A, Ishigatsubo Y, Okubo T, Yoshimura T: Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. *J Biol Chem* 272:31092–31099, 1997
15. Koentgen F, Grumont RJ, Strasser A, Metcalf D, Li R, Tarlinton D, Gerondakis S: Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes Dev* 9:1965–1977, 1995.
16. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D: Embryonic lethality and liver degeneration in mice lacking the Rel A component of NFκB. *Nature* 376:167–170, 1995.
17. Finco TS, Baldwin AS: Mechanistic aspects of NFκB regulation: The emerging role of phosphorylation and proteolysis. *Immunity* 3:263–272, 1995
18. Stylianou E, O'Neill LAJ, Rawlinson L, Edbrooke MR, Woo P, Saklatvala J: Interleukin 1 induces NFκB through its type I but not type II receptor in lymphocytes. *J Biol Chem* 267:15836–15841, 1992
19. Guesdon F, Ikebe I, Stylianou E, Warwick-Davies J, Haskill S, Saklatvala J: Interleukin 1 induced phosphorylation of MAD3, the major inhibitor of nuclear factor κB of HeLa cells. *Biochem J* 307:287–295, 1995
20. Cordle SR, Donald R, Read MA, Hawiger J: Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-rel and related NFκB proteins in human monocytic THP-1 cells. *J Biol Chem* 268:11803–11810, 1993
21. Hansen SK, Nerlov C, Zabel U, Verde P, Johnson M, Baeuerle PA, Blasi F: A novel complex between the p65 subunit of NFκB and c-rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene. *EMBO J* 11:205–213, 1992
22. Hansen SK, Baeuerle PA, Blasi F: Purification, reconstitution, and IκB Association of the c-rel-p65 (Rel A) complex, a strong activator of transcription. *Mol Cell Biol* 14:2593–2603, 1994
23. Rovin BH, Dickerson JA, Tan LC, Herbert CA: Activation of nuclear factor-κB correlates with MCP-1 expression by human mesangial cells. *Kidney Int* 48:1263–1271, 1995
24. Ping D, Jones PL, Boss JM: TNF regulates the in vivo occupancy of both distal and proximal regulatory regions of the MCP-1/JE gene. *Immunity* 4:455–469, 1996
25. Freter RR, Alberta JA, Lam KK, Stiles CD: A new platelet-derived growth factor-regulated genomic element which binds a serine/threonine phosphoprotein mediates induction of the slow immediate early gene MCP-1. *Mol Cell Biol* 15:315–325, 1995
26. Lovett DH, Sterzel RB: Cell culture approaches to the analysis of glomerular inflammation. *Kidney Int* 30:246–254, 1986
27. Savill JS, Smith SJ, Ren Y, Sarraf C, Abbott F, Rees AJ: Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis. *Kidney Int* 42:924–936, 1992
28. Dignam JD, Lebovitz RM, Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11:1475–1489, 1983
29. Osborn L, Kunkel S, Nabel GJ: Tumour necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. *Proc Natl Acad Sci USA* 86:2336–2340, 1989
30. Bradford MM: 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, 1976
31. Yasukawa K, Hirano T, Watanabe Y, Muratani K, Matsuda T, Nakai S, Kishimoto T: Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. *EMBO J* 6:2939–2945, 1987
32. Latimer M, Ernst MK, Dunn LL, Drutskaya M, Rice NR: The N-terminal domain of IκBα masks the nuclear localization signal(s) of p50 and c-rel homodimers. *Mol Cell Biol* 18:2640–2649, 1998
33. Carrasco D, Weih F, Bravo R: Developmental expression of the c-rel proto-oncogene in haematopoietic organs. *Development* 120:2991–3004, 1994
34. Lernbecher T, Muller U, Wirth T: Distinct NF-κB/rel transcription factors are responsible for tissue-specific and inducible gene activation. *Nature* 365:767–770, 1993
35. Carrasco D, Ryseck R-P, Bravo R: Expression of *relB* transcripts during lymphoid organ development: Specific expression in dendritic antigen-resenting cells. *Development* 118:1221–1231, 1993
36. Mackman N: Protease inhibitors block lipopolysaccaride induction of tissue factor gene expression in human monocytic cells by preventing induction of c-rel/p65 heterodimers. *J Biol Chem* 269: 26363–26367, 1994
37. Oeth PA, Parry GCN, Kunsch C, Nantermet P, Rosen CA, Mackman N: Lipopolysaccaride induction of tissue factor gene expression in monocytic cells is mediated by binding of c-rel/p65 heterodimers to a κB-like site. *Mol Cell Biol* 13:3772–3781, 1994
38. Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S: Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc Natl Acad Sci USA* 90:10193–10197, 1993
39. Nakayama K, Shimizu H, Mitomo K, Watanabe T, Okamoto S-I, Yamamoto K-I: A lymphoid cell-specific nuclear factor containing c-rel-like proteins preferentially interacts with interleukin-6 κB-related motifs whose activities are repressed in lymphoid specific cells. *Mol Cell Biol* 12:1736–1746, 1992
40. Weih F, Carrasco D, Durham SK, Barton DS, Rizzo CA, Ryseck R-P, Lira SA, Bravo R: Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of Rel B, a member of the NFκB/Rel family. *Cell* 80:331–340, 1995
41. Ryseck R-P, Bull P, Takamiya M, Bours V, Siebenlist U, Dobrzanski P, Bravo R: Rel B, a new rel family transcription activator that can interact with p50-NFκB. *Mol Cell Biol* 12:674–684, 1992