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# Persistent Interleukin-1β Signaling Causes Long Term Activation of NFκB in a Promoter-specific Manner in Human Glial Cells*

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**Bryan D. Griffin and Paul N. Moynagh**[1]

*From the UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland*

**Nuclear factor-κB (NFκB) is an inducible transcription factor that plays a key role in regulating the expression of a wide range of immune and inflammatory response genes. The activity of NFκB is controlled at multiple levels, including cytoplasmic retention with inhibitor of κB (IκB) proteins in the basal state. Persistent activation of the transcription factor is seen in numerous chronic inflammatory disease states, and we have previously demonstrated sustained activation of NFκB in human glial cells upon stimulation with interleukin (IL)-1β. In these cells, NFκB retains DNA binding activity for up to 72 h despite the presence of resynthesized IκBα and in the absence of IκBβ. Here we characterized the apparent inability of newly synthesized IκBα to terminate activation of NFκB in glial cells. We showed unexpectedly that newly synthesized IκBα can enter the nucleus, interact with the NFκB subunit p65, and export it to the cytoplasm. However, *in vitro* analysis of enzyme activity demonstrates that IL-1β causes the long term activation of the IκB kinase complex leading to chronic phosphorylation of the newly synthesized IκBα signal response domain and persistent activation of NFκB. Such sustained activation of NFκB is dependent on the continuous presence and activity of IL-1β. Interestingly, the sustained nature of NFκB activity is promoter type-specific. Chromatin immunoprecipitation studies revealed that p65 is detected at the promoters of both intercellular adhesion molecule-1 and IL-8 1 h following IL-1β stimulation but is only found at the latter at 24 h. The functional significance of this finding is indicated by the transient induction of intercellular adhesion molecule-1 mRNA, but more sustained induction of *IL-8* expression, by IL-1β. These studies thus demonstrated that persistent IL-1 signaling causes sustained activation of NFκB in a promoter-specific manner in human glial cells, leading to prolonged induction of selective pro-inflammatory genes. This is likely to make a key contribution to chronic inflammatory conditions of the brain.**

Nuclear factor κB (NFκB)[2] is an inducible transcription factor that plays a key role in regulating the expression of numerous immune and inflammatory response genes (1, 2). It is a dimeric factor consisting of subunits from the Rel family of proteins, some members of which are transcriptionally active (p65, c-Rel, and RelB), although others lack transactivation domains (p50 and p52) (3). The members of the family share a conserved region termed the Rel homology domain, containing the dimerization and site-specific DNA binding domains as well as the nuclear localization signal (NLS) (4). The activity of NFκB is controlled at multiple levels, most notably by regulation of its subcellular localization. In resting cells, NFκB is retained in the cytoplasm through its interaction with inhibitor of κB (IκB) proteins. The most extensively studied members of this family are IκBα and IκBβ. They associate with NFκB via a conserved ankyrin repeat domain. By interacting with the Rel homology domain, IκB serves to occlude the DNA binding domain and one (IκBα) or both (IκBβ) NLSs of NFκB (5, 6).

Activators of NFκB include pro-inflammatory cytokines, bacterial and viral products, and UV light (2). Exposure of cells to this diverse range of stimuli results in a common mechanism of NFκB activation. It is mediated by IκB kinase (IKK) complex phosphorylation of the N terminus of IκB on two specific serines, *e.g.* Ser-32 and Ser-36 on IκBα (7). The IKK complex consists of a regulatory subunit, IKKγ, and two catalytic subunits IKKα and IKKβ, the latter being most important for signaling from pro-inflammatory cytokines. The phospho-IκB protein is then polyubiquitinated by the SCF-βTrCP ubiquitin ligase complex, which in turn tags it for degradation by the 26 S proteasome (8). With the NLS and DNA binding domains unmasked, NFκB can translocate to the nucleus and bind to the κB sites in the promoters of its target genes. IκBα and IκBβ differ considerably in their degradation profile and activity (9). All known inducers of NFκB cause IκBα degradation, and *IκBα* gene expression is then rapidly induced by NFκB (10). Free IκBα can enter the nucleus, remove NFκB from DNA, and export it back to the cytoplasm (11). A leucine-rich nuclear export sequence in the C-terminal region of IκBα is responsible for this function by recognizing the nuclear export receptor CRM1 (12). This inducible autoregulatory feedback mechanism accounts for the transient nature of NFκB activation in response to certain agents. However, long term activation of NFκB can occur in a cell type- and stimulus-dependent manner. Those stimuli that induce this persistent activation cause degradation of IκBβ in addition to IκBα (13).

Sustained activation of NFκB is associated with many chronic inflammatory disease states such as multiple sclerosis (MS), where the entry of activated T-lymphocytes into the central nervous system (CNS) is a key pathogenic step (14–16). The repertoire of pro-inflammatory genes expressed upon NFκB activation includes interleukin-8 (IL-8), a *CXC* chemokine primarily known for its chemoattractant effects on neutrophils, and intercellular cell adhesion molecule-1 (ICAM-1), an adhesion molecule of the immunoglobulin superfamily (17–19). These proteins play an important role in the transendothelial migration of T-lympho-

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[1] To whom correspondence should be addressed: UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland. Tel.: 353-1-7166761; Fax: 353-1-2692749; E-mail: P.Moynagh@ucd.ie.

[2] The abbreviations used are: NFκB, nuclear factor κB; IκB, inhibitor of κB; IL-1β, interleukin-1β; IKK, IκB kinase; ICAM-1, intercellular adhesion molecule-1; NLS, nuclear localization signal; PBS, phosphate-buffered saline; DTT, dithiothreitol; DAPI, 4,6-diamidino-2-phenylindole; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; CHX, cycloheximide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LMB, leptomycin B; MS, multiple sclerosis; EAE, experimental allergic encephalitis; CNS, central nervous system; ChIP, chromatin immunoprecipitation.

cytes required to breach the specialized vasculature separating the CNS from the peripheral circulation, the blood-brain barrier (20). The expression levels of IL-8 and ICAM-1 are elevated in MS and experimental allergic encephalitis (EAE), an animal model of MS (21, 22). Furthermore, both are expressed by astrocytes, a resident CNS cell that directly underlies the blood-brain barrier (23, 24). This group has previously shown that interleukin-1β (IL-1β) stimulation of 1321N1 astrocytoma, a model astrocyte cell system, results in persistent, long term activation of NFκB (25). Analysis of the accompanying IκB degradation profile revealed that IκBα was transiently degraded and rapidly resynthesized, whereas disappearance of IκBβ was sustained. This signified a notable departure from other models of long term NFκB activation. In pre-B cells treated with LPS, the initial degradation of IκBβ is followed by its resynthesis in a hypophosphorylated form. This form of the protein can bind to NFκB but does not inhibit nuclear translocation or binding to target promoters. Furthermore, although hypophosphorylated IκBβ remains bound, IκBα cannot gain access to NFκB to terminate transcriptional activation (26). However, in the case of astrocytes, IL-1β causes sustained activation of NFκB under conditions where IκBα is resynthesized and IκBβ is absent. This study thus explored the apparent inability of IL-1β-induced IκBα to terminate sustained activation of NFκB in human astrocytes.

We show that global NFκB activation is sustained despite the ability of resynthesized IκBα to enter the nucleus, interact with NFκB subunit p65, and return it to the cytoplasm in IL-1β-treated astrocytes. This is because of persistent IL-1β-dependent activation of the IKK complex and subsequent phosphorylation and degradation of IκBα, leading to continuous activation of NFκB. Furthermore, analysis of the *IL-8* and *ICAM-1* promoters by chromatin immunoprecipitation studies reveals that the sustained activation of NFκB is a not a universal feature at all promoters of NFκB-responsive genes. This study advances our appreciation of the mechanism regulating the temporal activation of NFκB by pro-inflammatory cytokines in astrocytes, and more importantly demonstrates that its sustained activation occurs only in the context of specific promoters. Such findings increase our understanding of the molecular basis underlying the chronic inflammatory condition of the brain.

## EXPERIMENTAL PROCEDURES

*Materials*—The human astrocytoma cell line 1321N1 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Dulbecco's modified Eagle's medium, fetal calf serum, penicillin/streptomycin, and trypsin were from Invitrogen. Human recombinant IL-1β and recombinant IL-1 receptor antagonist were purchased from R & D Systems Europe (Oxon, UK). T4 polynucleotide kinase and the 22-bp oligonucleotide containing the NF-κB consensus sequence (underlined) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were supplied by Promega (Madison, WI). [γ-$^{32}$P]ATP was purchased from Amersham Biosciences. Rabbit polyclonal antibodies against IκBα (sc-203), IκBβ (sc-945), p65 (sc-372), and protein A/G-agarose (sc-2003) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal against phospho-IκBα (Ser-32/36) and rabbit polyclonal antibody against IKKβ were purchased from Cell Signaling Technology (MA01915). Texas Red-conjugated anti-rabbit polyclonal antibody was obtained from Molecular Probes (OR97402). Anti-β-actin mouse monoclonal antibody, MG132, leptomycin B, and cycloheximide were purchased from Sigma. Primers and probes for quantitative real time analysis of ICAM-1 (Hs00164932_m1), IL-8 (4309885P), and 18 S rRNA (4310893E) were from Applied Biosystems (CA94404). The C-terminal histidine-tagged recombinant form of IκBα was a gift from Helmut Sparrer (Novartis Pharma AG, Switzerland) and has been described previously (27).

*Cell Culture*—The human astrocytoma cell line, 1321N1, was cultured in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) of fetal calf serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$. All cells were passaged using 1% (w/v) of trypsin in PBS. IL-1β stimulation was performed on cells in serum-containing medium at 37 °C for all experiments.

*Generation of Cell Extracts*—1321N1 astrocytomas were seeded into 25-cm$^2$ flasks (5 ml; 2 × 10$^5$ cells/ml) and allowed to adhere for 72 h before stimulation. Cells were exposed to IL-1β for various times. Stimulation was terminated by removal of medium followed by washing of the adherent cells with 3 ml of ice-cold PBS. For preparation of subcellular fractions, washed cells were scraped into 1 ml of hypotonic buffer (10 mM HEPES-NaOH buffer, pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF). Cells were pelleted in hypotonic buffer by centrifugation at 20,000 × *g* for 10 min and were then lysed for 10 min on ice in hypotonic buffer (35 μl) containing 0.1% (v/v) of Nonidet P-40. Lysates were centrifuged at 20,000 × *g* for 10 min. The resulting supernatants constituted cytosolic extracts and were removed to new minifuge tubes and stored at −20 °C. The resulting pellets were resuspended in 20 mM HEPES-NaOH buffer, pH 7.9 (25 μl), containing 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (w/v) glycerol, and 0.5 mM PMSF and incubated for 15 min on ice. Incubations were then centrifuged at 20,000 × *g* for 10 min, and the supernatants were removed into 10 mM HEPES-NaOH buffer, pH 7.9 (75 μl), containing 50 mM KCl, 0.2 mM EDTA, 20% (w/v) glycerol, 0.5 mM PMSF, and 0.5 mM DTT. Such samples constituted nuclear extracts. All of the above steps were carried out at 4 °C. Protein concentrations of the cytosolic and nuclear extracts were determined by the method of Bradford (28). For the generation of whole cell extracts, cells were scraped into 1 ml of PBS before being pelleted by centrifugation at 20,000 × *g* for 10 min. After discarding the supernatant, cells were resuspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromphenol blue; 100 μl per sample) and incubated on ice for 10 min. Resuspended cells were then boiled for 5 min. Cell debris was pelleted by further centrifugation at 20,000 × *g* for 10 min. The supernatant containing the cell extract was removed to fresh Eppendorf tubes.

*Electrophoretic Mobility Shift Assay*—Nuclear extracts (10 μg of protein) were incubated with 30,000 dpm of a 22-bp oligonucleotide containing the NF-κB consensus sequence that had been labeled previously with [γ-$^{32}$P]ATP (10 mCi/mmol) by T4 polynucleotide kinase (29). Incubations were performed for 30 min at room temperature in 10 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 4% (w/v) glycerol, 4 μg of poly(dI-dC), and 1 mg/ml nuclease-free bovine serum albumin. In the supershift analysis, polyclonal antibody (1 μg) against the NF-κB subunit p65 was added to the extracts and incubated for 30 min on ice prior to incubation with labeled oligonucleotide. All incubations were subjected to electrophoresis on 4% nondenaturing polyacrylamide gels, which were subsequently dried and autoradiographed.

*Western Immunoblotting*—Cytosolic extracts (20 μg of protein) in 2× sample buffer (0.125 M Tris-HCl buffer, pH 6.8, containing 20% (w/v) glycerol, 4% (w/v) of SDS, 1.4 M 2-β-mercaptoethanol, and 0.0025% (w/v) bromphenol blue) or whole cell extracts (20 μl) were boiled for 5 min prior to loading. Extracts were separated by SDS-PAGE using a 12% (w/v) resolving gel, and proteins were transferred electrophoretically to nitrocellulose. Immunodetection of proteins was con-

ducted as described previously (25). Blots were blocked in 20 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl (TBS) and 5% (w/v) powdered milk (Marvel). Incubation with primary mouse monoclonal anti-phospho-IκBα (Ser-32/36) antibody (1:2000 dilution) was performed overnight at 4 °C, although incubations with primary antibodies against p65, IκBα, or IκBβ (all 1 μg/ml) were for 2 h at room temperature. Horseradish peroxidase-conjugated sheep anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG (both from Upstate) were used at concentrations of 1 and 0.5 μg/ml respectively. Immunoreactive bands were detected using the ECL detection system from Pierce according to the instructions of the manufacturers.

*Immunofluorescence*—Cells were seeded in 4-well chamber slides (Lab-Tek) and grown to confluency. Following the appropriate treatment the medium was removed, and cells were washed once in ice-cold PBS. For detection of endogenous p65, cells were first fixed with 3.7% (v/v) paraformaldehyde in PBS (500 μl per chamber) for 20 min at room temperature. Cells were then washed twice with PBS, before being permeabilized by incubation with 0.2% (v/v) Triton-X-100 (in PBS) for 30 min on an orbital shaker at room temperature. For detection of IκBα, cells were incubated for 10 min at −20 °C with a 1:1 mixture of 100% methanol and acetone (500 μl per chamber) after the initial PBS wash. This served to both fix and permeabilize the cells. After the suitable fixation/permeabilization step, cells were again washed twice with PBS. This was followed by blocking the cells in 0.5% bovine serum albumin/PBS for 20 min at room temperature. After further washes, primary antibody was added (150 μl per well; IκBα or p65 rabbit polyclonal antibody at a working concentration of 2 μg/ml), and the chamber slides were placed at 4 °C with rocking overnight. Cells were next incubated with Texas Red-conjugated goat anti-rabbit antibody (10 μg/ml; 150 μl per well), and the chamber slides were incubated for 1 h in the dark at room temperature with rocking. Cells were washed twice with PBS before being mounted with Slowfade antifade reagent (DAPI containing medium (1.5 μg/ml)) (Molecular Probes, Inc.). Confocal images were captured using the ×63 objective (oil immersion) on the UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets and analyzed using the LSM 5 browser imaging software.

*Co-immunoprecipitation*—Cells were seeded in 6-well plates, grown to confluency, and treated with or without IL-1β for the various time periods. Stimulation was terminated by the removal of medium. Cells were washed once with ice-cold PBS, scraped into 1 ml of PBS, and pelleted by centrifugation at 20,000 × *g* for 10 min at 4 °C. Cells were lysed by incubation with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Igepal, 50 mM NaF, 1 mM $Na_3VO_4$, 1 mM DTT, and 1 mM PMSF (500 μl per sample). 50 μl of supernatant was retained for Western blot analysis, and the remainder was used for immunoprecipitation. Lysates were precleared by incubation with 1 μg of IgG antibody and 10 μl of protein A/G-agarose for 30 min. Anti-p65 antibody (1 μg) was added to precleared lysates and incubated overnight at 4 °C with rocking. This was followed by the addition of protein A/G-agarose (20 μl per sample). Incubations were placed at 4 °C with rocking for 1 h. Immunoprecipitates were collected by centrifugation, and the beads were then washed four times with lysis buffer. The pellet was resuspended in 2× sample buffer. Samples were then boiled for 5 min before analysis by SDS-PAGE and Western immunoblotting for p65 and IκBα.

*In Vitro Kinase Assay*—Cells were seeded in 6-well plates, cultured for 72 h, and treated with or without IL-1β for the various time periods. Stimulation was terminated by the removal of medium. Cells were washed once with ice-cold PBS, scraped into 1 ml of PBS, and pelleted by centrifugation at 20,000 × *g* for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in lysis buffer (20 mM HEPES, pH 7.5, containing 1% Igepal, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 2 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 100 μM $Na_3VO_4$, and 20 mM β-glycerol phosphate). Cells were incubated on ice for 10 min, after which the lysates were centrifuged at 20,000 × *g* for 10 min at 4 °C. The resulting supernatant was transferred to a new microcentrifuge tube. 1.5 μl of rabbit polyclonal anti-IKKβ antibody was added, and the incubations were placed on a roller at 4 °C overnight. A 50% slurry comprising equal volumes of dry protein G-Sepharose beads (Sigma) and lysis buffer was next added (35 μl per sample). The incubations were placed on a roller at 4 °C for 1 h. The immunoprecipitates were pelleted by centrifugation at 1000 × *g* for 2 min at 4 °C. The beads were then washed twice in lysis buffer and a further three times in kinase buffer (20 mM HEPES, pH 7.5, containing 2 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 100 μM $Na_3VO_4$, and 20 mM β-glycerol phosphate). After each wash, the beads were pelleted by centrifugation at 1000 × *g* for 2 min. The washes and subsequent centrifugation steps were performed at 4 °C. After the final wash the beads were drained dry. The beads were then incubated for 45 min at room temperature with kinase buffer including 1 μg of recombinant IκBα and 20 μM ATP per sample. Following addition of sample buffer, samples were boiled for 5 min and subjected to SDS-PAGE and Western immunoblotting to detect phospho-IκBα.

*Chromatin Immunoprecipitation*—1321N1 astrocytoma cells were grown to confluency in 90-mm dishes and stimulated with IL-1β (10 ng/ml) for the appropriate time period. Chromatin immunoprecipitation assays were performed as described previously with some modifications (30). Following stimulation cells were cross-linked with 1% formaldehyde for 10 min at 37 °C. Isolated nuclei were subjected to seven 10-s sonication pulses from a Sanyo/MES Soniprep 150 at one-third of total power. Separate aliquots from each chromatin preparation were incubated overnight at 4 °C with anti-p65 antibody and rabbit IgG antibody. An aliquot was also retained as an input sample to normalize PCRs and analyze shearing efficiency. Chromatin used had an average size of 750 bp. After reversion of cross-links by overnight incubation at 65 °C, DNA was extracted using the QIAquick purification kit (Qiagen) according to the manufacturer's instructions.

Standard PCR was performed using 1 μl (~3% of total) of template DNA, 500 nM primers, and 0.2 units of *Taq* DNA polymerase (Invitrogen) per 50-μl reaction. Quantitative real time PCRs were performed in duplicate with 2 μl of template DNA, 50 nM primers, and the SYBR Green Jumpstart Taq Readymix (Sigma) in a total volume of 20 μl, using the Mx3000P QPCR System (Stratagene). Dissociation curve analysis and gel electrophoresis of the final products confirmed that only the expected specific amplicon of correct size was generated for each target promoter. Real time PCR data analysis was performed as described previously (31). Results for each treatment are expressed as fold differences between DNA enrichment in the p65-ChIP sample relative to IgG-ChIP sample. The sequences of primers used are as follows: ICAM-1, 5′-CTCCACTCTCCGGGGAAGTTG-3′ and 5′-GCTGCAGTTATTTCCGGACTGAC-3′; IL-8, 5′-GGAAGTGTGATGACTCAGGTTTGC-3′ and 5′-GATGGTTCCTTCCGGTGGTTTCTTC-3′; and GAPH, 5′-CTACTAGCGGTTTTACGGGCG-3′ and 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′.

*Quantitative Real Time PCR Analysis of ICAM-1 and IL-8 Expression*—1321N1 astrocytomas ($2 \times 10^5$ cells/ml) were plated into 6-well plates and grown for 48 h. Cells were treated with IL-1β (10 ng/ml) for various time periods. Cells were washed with PBS, and RNA was extracted using Tri-Reagent (Sigma). After DNase I digestion, cDNA was generated from normalized RNA using Superscript II reverse transcriptase. Samples were assayed by quantitative real time PCR for levels of ICAM-1 and IL-8 cDNA using the ABI PRISM 7900HT thermal cycler. Reactions were performed using pre-validated primers and probes (Applied Biosystems).

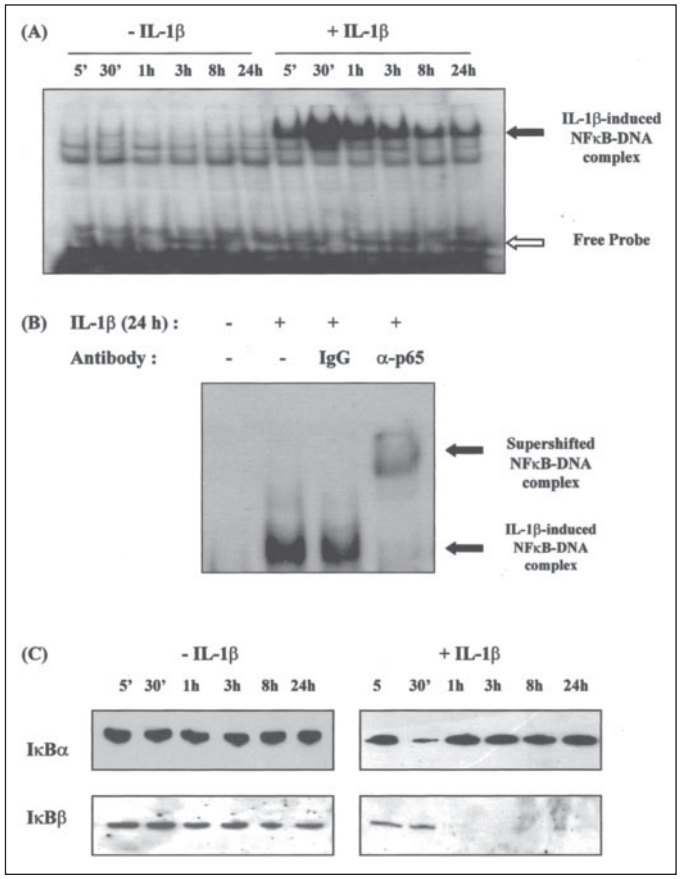


FIGURE 1. **IL-1β induces long term persistent activation of NFκB in 1321N1 astrocytomas.** 1321N1 astrocytomas were treated with or without IL-1β (10 ng/ml) for the indicated time periods. *A,* nuclear extracts (10 μg of protein) were assessed for the presence of NFκB DNA binding activity by EMSA. *B,* nuclear extracts from cells, treated for 24 h with IL-1β, were preincubated with anti-p65 or nonimmune (IgG) antibody before assessing NFκB DNA binding activity. *C,* cytosolic extracts (20 μg protein) were prepared and analyzed by Western immunoblotting for levels of IκBα and IκBβ. These results are each representative of three independent experiments.

## RESULTS

*IL-1β Stimulates the Rapid and Persistent Activation of NFκB in Astrocytes*—To confirm and characterize the sustained activation of NFκB by IL-1β in astrocytes, 1321N1 astrocytomas were treated with or without IL-1β for various time periods. Nuclear extracts were generated, and the presence of NFκB-DNA binding activity was assessed by EMSA (Fig. 1*A*). A low level of basal NFκB activation was detected in unstimulated cells. Treatment with IL-1β induced activation of NFκB, observed as the increased presence of a complex of slow electrophoretic mobility. Activation was rapid and sustained, detectable at 5 min, peaking at 30 min but with significant levels of complex remaining for at least 24 h. Previous studies on this cell type had demonstrated that p65/p50 heterodimers and p65/p65 homodimers display DNA binding activity after 1 h of stimulation with IL-1β (25). To address whether the later phase complexes might be capable of promoting transcription, the complexes were assessed by supershift analysis for the presence of the most common transactivating NFκB subunit, p65. Nuclear extracts from cells treated with or without IL-1β for 24 h were incubated with antisera against p65, prior to EMSA (Fig. 1*B*). The IL-1β-induced complex was unaffected by preincubation with the nonimmune IgG isotype control. In contrast, anti-p65 antibody caused the appearance of a complex of slow electrophoretic mobility, which coincided with a significant decrease in the levels of the IL-1β-induced complex. It is therefore likely that the composition of NFκB detected throughout the 24-h period was unaltered. The consistent electrophoretic mobility of the IL-1β-induced NFκB-DNA complexes over time in Fig. 1*A* supports this proposition. This demonstrates that IL-1β stimulation causes the persistent activation of a form of NFκB in glial cells that has transactivation potential.

The degradation profile of the IκB proteins was next investigated. Cytosolic extracts from unstimulated and IL-1β-treated cells were probed for IκBα and IκBβ by Western immunoblotting (Fig. 1*C*). IL-1β caused a transient degradation of IκBα, with a decrease evident at 30 min but returning to control levels by 1 h. In contrast, IκBβ was degraded with slower kinetics, disappearing by 1 h. This slower rate of proteolysis has been observed in numerous cell types. It has been proposed that although the same degradation pathway is utilized, IκBβ is a poorer substrate for IKK-mediated phosphorylation and polyubiquitination by SCF-βTrCP (32). Also, unlike IκBα, IκBβ protein failed to manifest at detectable levels up to 24 h post-IL-1β stimulation. This clearly eliminated the possibility that IκBβ was resynthesized in a hypophosphorylated form and acting as a molecular chaperone to sustain NFκB activity. Given the absence of IκBβ at later time points of post-IL-1β stimulation, we next assessed the apparent failure of newly synthesized IκBα to terminate the activation of NFκB. More specifically the ability of newly synthesized IκBα to translocate to the nucleus and bind to p65 was probed.

*Resynthesized IκBα Can Enter the Nucleus and Interact with NFκB Following IL-1β Treatment of Glial Cells*—CRM1-dependent nuclear export of IκBα is inhibited by leptomycin B (LMB) (33). Thus, to facilitate an investigation into whether IκBα, resynthesized after IL-1β treatment, can enter the nucleus of 1321N1 astrocytoma, cells were treated with IL-1β for 30 min to deplete existing IκBα stocks before addition of LMB for a further 2.5 h. The subcellular localization of IκBα was therefore assessed after a total of 3 h of stimulation in the presence or absence of IL-1β. The ability of newly synthesized IκBα to enter the nucleus would be indicated by the nuclear accumulation of IκBα under conditions where its export was blocked. Cytoplasmic and nuclear extracts were prepared, and Western immunoblotting was performed to examine the subcellular localization of IκBα (Fig. 2*A*). IκBα was detected in both compartments in astrocytes treated with or without IL-1β, but considerably higher levels were observed in the cytoplasm (Fig. 2*A*, *lanes 1–4*). In unstimulated cells, LMB treatment caused the accumulation of IκBα in the nucleus (Fig. 2*A*, *lane 6*). This most likely represents NFκB-complexed IκBα undergoing nucleocytoplasmic shuttling that becomes sequestered in the nucleus following inhibition of CRM1-dependent nuclear export. Resynthesized IκBα also accumulates in the nucleus of IL-1β-treated cells in response to LMB (Fig. 2*A*, *lane 8*), indicating that newly formed IκBα can enter the nucleus. This was further confirmed by confocal microscopy-based detection of endogenous IκBα using anti-IκBα rabbit polyclonal antibody and Texas Red-conjugated anti-rabbit secondary antibody. Nuclear chromatin was stained with DAPI. Untreated cells, or cells treated with IL-1β for 3 h, showed predominant cytoplasmic localization of IκBα (Fig. 2*B*, *upper panels*). However treatment with LMB resulted in nuclear accumulation of basal and newly synthesized IκBα, as reflected by coincident staining with DAPI-stained nuclei (Fig. 2*B*, *lower panels*). The combined approaches of subcellular fractionation and confocal microscopy strongly indicate that IL-1β can induce IκBα that is capable of translocating to the nucleus.

Although newly synthesized IκBα is capable of nuclear accumulation, NFκB remains bound to DNA thus raising the possibility that newly made IκBα undergoes some *in vivo* modification that prevents an interaction with DNA-bound NFκB. Co-immunoprecipitation studies were

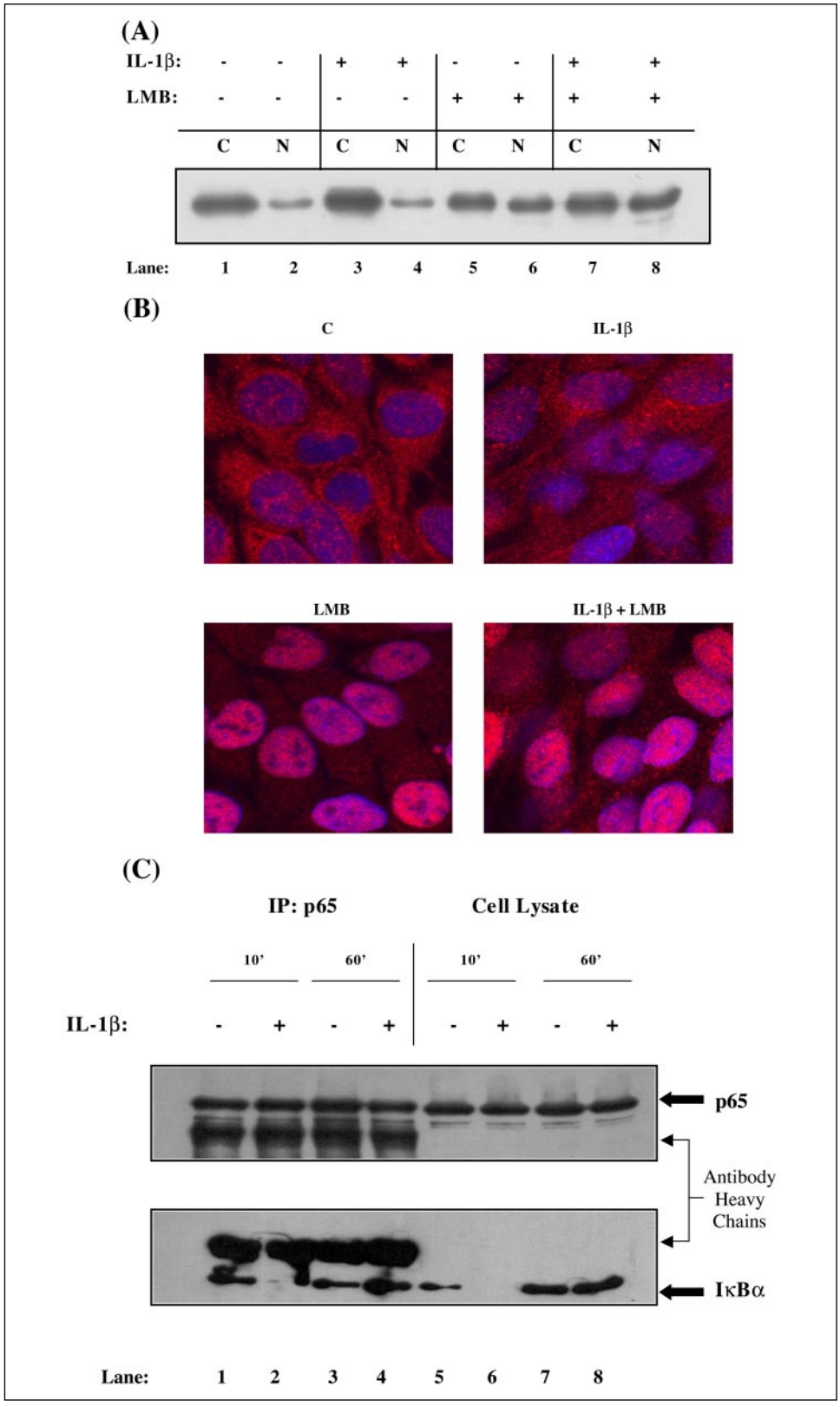


FIGURE 2. **IκBα, resynthesized following IL-1β stimulation, can enter the nucleus and interact with p65 in 1321N1s.** 1321N1 astrocytomas were stimulated with or without IL-1β (10 ng/ml) for 30 min before treatment with LMB (10 nM) for a further 3 h. *A,* cytosolic (*C*) and nuclear (*N*) extracts (20 μg) were prepared and analyzed by Western immunoblotting for levels of IκBα. *B,* the subcellular localization of IκBα was assessed by indirect immunofluorescence using anti-IκBα rabbit polyclonal antibody and Texas Red-conjugated anti-rabbit secondary antibody. Nuclei were stained with DAPI, and confocal images were captured using the ×63 objective (oil immersion) on the UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets. Data analysis was performed using the LSM 5 browser imaging software. *C,* 1321N1 astrocytomas were stimulated with or without IL-1β (10 ng/ml) for 10 min or 1 h. Cell lysates were generated and immunoprecipitated (*IP*) with anti-p65 antibody. Cell lysates and corresponding immunoprecipitates were subjected to Western immunoblotting to measure levels of p65 and IκBα. The above results are each representative of three independent experiments.

employed to evaluate this possibility. 1321N1 astrocytomas were treated with or without IL-1β for 10 min or 1 h. Cell lysates and the corresponding anti-p65 immunoprecipitates were analyzed by SDS-PAGE and subsequent Western immunoblotting for p65 and IκBα. Levels of p65 were constant in both cell lysates and immunoprecipitates (Fig. 2*C, upper panel*). IκBα was fully degraded after 10 min of treatment with IL-1β (Fig. 2*C, lower panel, lanes 2* and *6*). After 1 h of treatment with IL-1β, IκBα had returned to control levels (Fig. 2*C, lower panel, lane 8*) and, significantly, was capable of interacting with p65 as evidenced by co-immunoprecipitation of p65 and newly synthesized IκBα

(Fig. 2*C, lower panel, lane 4*). This finding strongly indicates that resynthesized IκBα can enter the nucleus and interact with NFκB complexes containing p65. However, the sustained activation of NFκB, as evidenced by the persistent presence of p65 complexes in EMSA analysis, suggests that although newly synthesized IκBα can translocate to the nucleus and interact with p65, it is apparently incapable of terminating the prolonged DNA binding activity of NFκB. Thus, newly synthesized IκBα was next examined for its ability to promote relocation of p65 from the nucleus to the cytoplasm.

*IL-1β-induced IκBα Mediates the Nuclear Export of NFκB in 1321N1 Astrocytoma*—To monitor the localization of p65, a confocal microscopy-based approach was developed. This was initially used to characterize the time-dependent distribution of p65 in response to IL-1β stimulation. 1321N1 astrocytomas were stimulated with IL-1β for various time periods, and the localization of endogenous p65 was assessed by indirect immunofluorescence. In resting cells, p65 is predominantly cytoplasmic, as evidenced by the detection of Texas Red-conjugated immunocomplexes outside of the DAPI-stained regions (Fig. 3*A*). The stimulation of cells with IL-1β for 30 min caused a pronounced accumulation of p65 in the nucleus, and this remained for at least 1 h. However, 3 h following stimulation, p65 had undergone significant redistribution to the cytoplasm, although residual levels were still detectable in the nuclei of some cells. This predominant cytoplasmic distribution with residual presence in the some nuclei was still observed 8 and 24 h after IL-1β treatment. To assess a potential role for resynthesized IκBα in the export of p65, the sensitivity of the CRM1 receptor to LMB was again exploited. 1321N1 astrocytomas were treated with IL-1β for 30 min prior to addition of LMB for a further 2.5 h. LMB alone caused the partial accumulation of p65 in the nucleus (Fig. 3*B, lower left panel*). As mentioned above, IκBα-NFκB complexes undergo nucleocytoplasmic shuttling in the resting state, as IκBα only masks one of the two available NLSs of the NFκB dimer. Thus, this accumulation most likely resulted from the export of shuttling complexes being inhibited. When LMB treatment was preceded by IL-1β stimulation, a more complete nuclear accumulation of p65 was observed (Fig. 3*B, lower right panel*). The effect of LMB treatment on p65 localization, post-IL-1β stimulation, indicates that the redistribution of p65 to the cytoplasm following IL-1β treatment is CRM1-dependent and likely IκBα-dependent.

Further evidence supporting the ability of newly synthesized IκBα to remove NFκB from the nucleus was obtained using cycloheximide (CHX), a potent inhibitor of protein synthesis (34). 1321N1 astrocytomas were treated with or without CHX for 1 h before stimulation in the presence or absence of IL-1β for an additional 3 h. Inhibition of protein synthesis prior to IL-1β stimulation blocks the resynthesis of IκBα without affecting levels of p65, as shown by Western immunoblotting of whole cell extracts (Fig. 4*A*). The lack of new IκBα synthesis in the presence of CHX was also demonstrated by indirect immunofluorescence (Fig. 4*B*). The localization of p65 in the presence and absence of resynthesized IκBα was then examined. As shown above, p65 largely redistributes to the cytoplasm 3 h after IL-1β treatment (Fig. 4*C, upper right panel*). However no such relocation is seen when IL-1β addition is preceded by treatment with CHX. In the absence of IκBα to mediate export, p65 remains in the nucleus (Fig. 4*C, lower right complex*). It should be noted that the shorter half-life of IκBα relative to Rel subunits can result in NFκB activation with prolonged inhibition of protein synthesis. Although a small amount p65 was detected in the nuclei of some CHX-treated cells compared with control cells, CHX itself was not believed to have had a major effect on NFκB activation over the 4-h time course of this experiment.

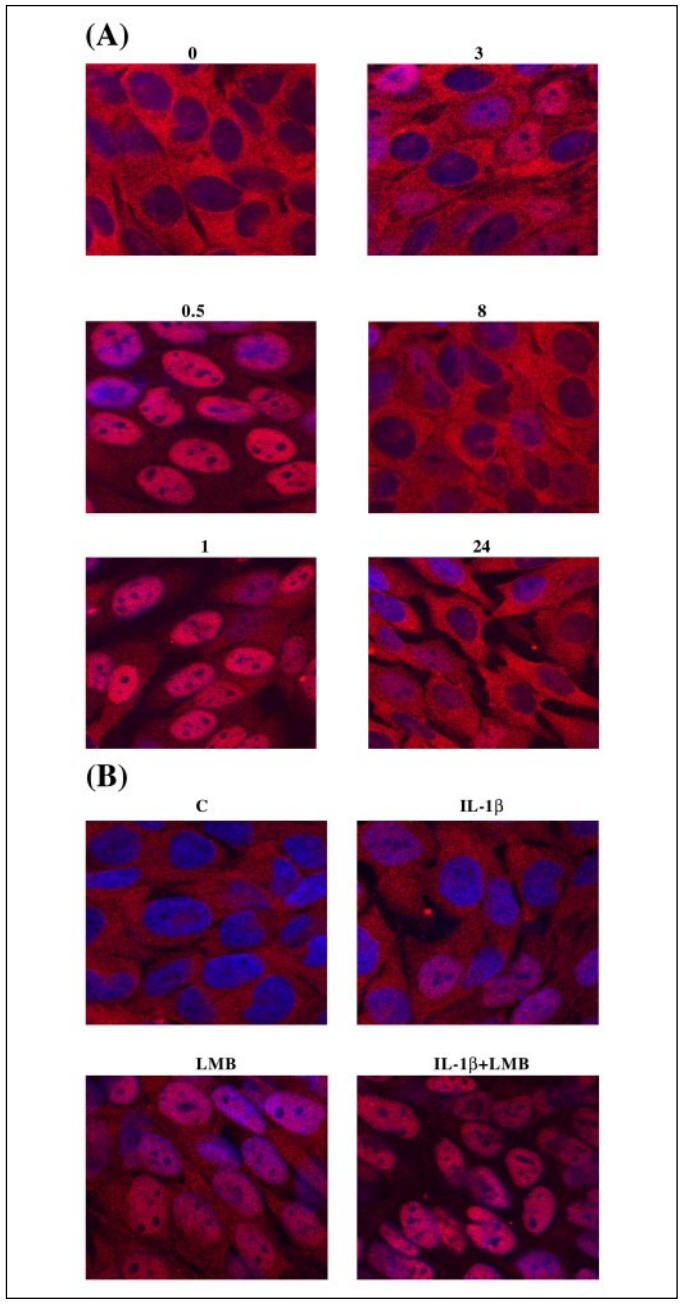


FIGURE 3. **p65 undergoes partial redistribution to the cytoplasm following IL-1β stimulation of 1321N1 astrocytomas.** Indirect immunofluorescence was conducted to assess the subcellular localization of p65 in 1321N1 astrocytomas following treatment with or without IL-1β (10 ng/ml) for the indicated time periods (*A*), or stimulation with or without IL-1β (10 ng/ml) for 30 min prior to treatment with LMB (10 nM) for a further 2.5 h (*B*). Nuclei were stained with DAPI, and confocal images were captured using the ×63 objective (oil immersion) on the UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets. Data analysis was performed using the LSM 5 browser imaging software. The above results are each representative of three independent experiments.

These data indicate that IL-1β can induce resynthesis of IκBα that translocates to the nucleus, interacts with p65, and promotes the nuclear export of p65 complexes. However, the newly synthesized IκBα fails to fully terminate the activation of NFκB as indicated by persistent residual levels of nuclear p65 in the EMSA and confocal microscopy analysis. We envisaged that the sustained nuclear presence of p65 may be because of some p65 complexes that are refractory to inhibition by IκBα or alternatively that continuous flux through the IL-1 signaling

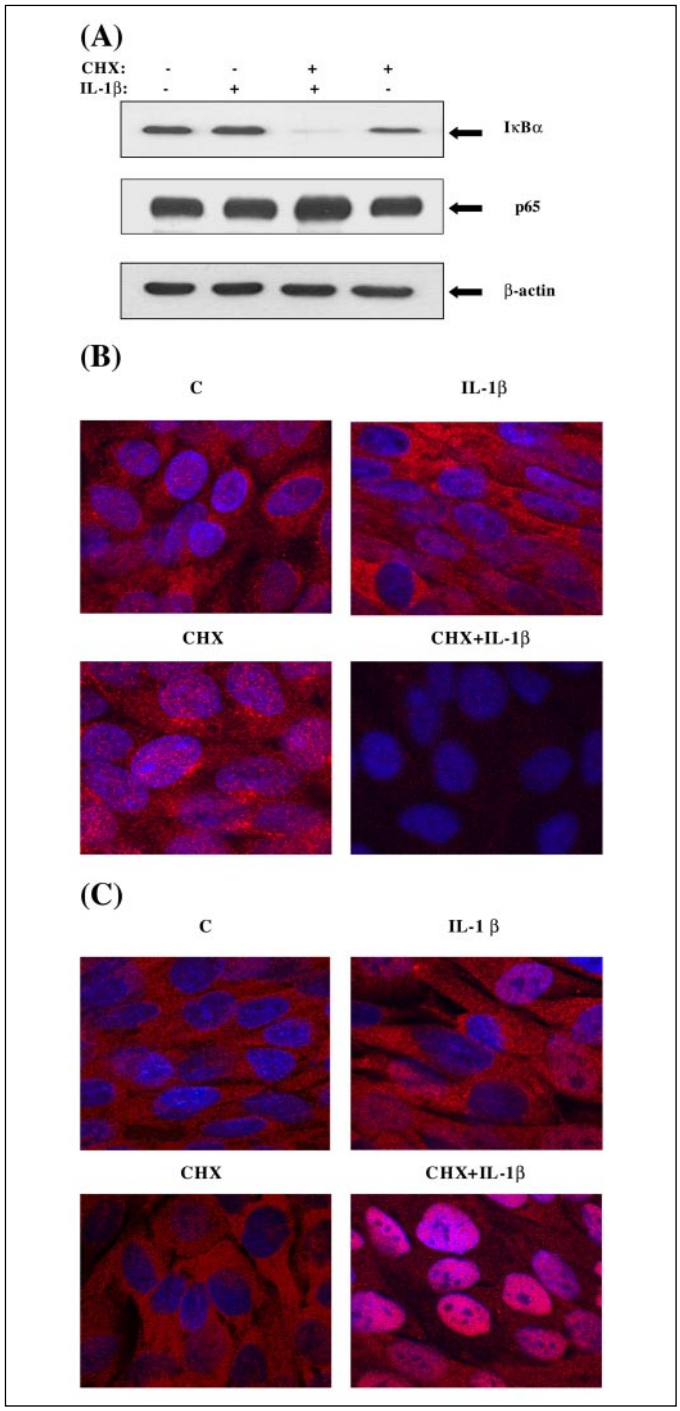


FIGURE 4. **Nuclear export of p65 requires IκBα resynthesis in 1321N1 astrocytomas following IL-1β stimulation.** 1321N1 astrocytomas were treated with or without IL-1β (10 ng/ml) for 3 h in the presence or absence of CHX (10 μg/ml). Western immunoblotting was performed on whole cell extracts to measure levels of IκBα, p65, and β-actin (*A*). The subcellular localization of IκBα (*B*) and p65 (*C*) was determined by indirect immunofluorescence. Nuclei were stained with DAPI, and confocal images were captured using the ×63 objective (oil immersion) on the UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets. Data analysis was performed using the LSM 5 browser imaging software. The above results are each representative of three independent experiments.

pathway leads to sustained degradation of some of the newly synthesized IκBα and allows for persistent translocation of NFκB into the nucleus. The latter hypothesis was next addressed.

*Long Term Activation of NFκB in 1321N1 Astrocytoma Is Caused by Persistent Signaling from the IL-1 Receptor*—To measure the flux through the IL-1 signaling pathway over time, the phosphorylation status of IκBα during the course of sustained NFκB activity in 1321N1 cells was examined. Whole cell extracts were generated, and IκBα was probed for phosphorylation status at serine residues 32 and 36. (Ser-32/36) (Fig. 5*A*). Phosphorylated IκBα was detected 5 min following treatment with IL-1β. IκBα was absent from cells at 30 min but reappeared by 1 h. This resynthesized IκBα was subject to phosphorylation, and phospho-IκBα was detected up to 24 h after IL-1β stimulation, albeit at lower levels than at 1 h. To assess the activity of the IKK complex at these time points, IKKβ was immunoprecipitated from 1321N1 astrocytoma previously stimulated with or without IL-1β, and incubated in kinase buffer with recombinant IκBα. Phosphorylation of the substrate on serines 32 and 36 was then measured (Fig. 5*B*). Activity of the enzyme was increased after a 5-min treatment of cells with IL-1β and remained elevated for 1 h. By 3 h, IKKβ activity had declined. However, at 8 and 24 h, IKKβ activity was higher than basal levels. The detection of endogenous phospho-IκBα and IKKβ activity 24 h following IL-1β stimulation strongly indicates that IL-1β causes activation of its signal transduction pathway in a persistent manner, leading to long term activation of the IKK enzyme complex, continuous phosphorylation and degradation of IκBα, and sustained activation of NFκB.

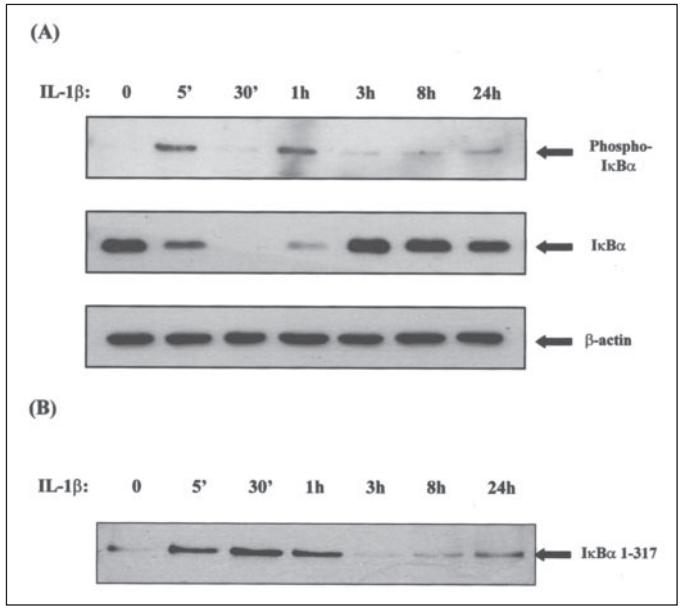


FIGURE 5. **The IL-1 signaling pathway is persistently activated by IL-1β stimulation in 1321N1 astrocytomas.** 1321N1 astrocytomas were stimulated with or without IL-1β (10 ng/ml) for the indicated time periods. *A,* whole cell extracts were generated and probed for levels of phospho-IκBα (Ser-32/36), total IκBα, and β-actin by Western immunoblotting. *B,* cell lysates were immunoprecipitated with anti-IKKβ antibody. Immunoprecipitates were incubated with recombinant IκBα in kinase buffer and subjected to Western immunoblotting using anti-phospho-IκBα (Ser-32/36) antibody. These results are representative of three independent experiments.

We next attempted to interfere with the persistent nature of the IL-1 signaling pathway to confirm that prolonged triggering of the pathway is responsible for sustained activation of NFκB. IL-1 receptor antagonist (IL-1ra) was added to the cells 1 h post-IL-1β treatment, thereby allowing initial activation of the IKK pathway. Levels of endogenous phospho- and total IκBα were again measured from whole cell extracts. As before, phosphorylation of IκBα on serines 32 and 36 was observed 8 h after stimulation with IL-1β (Fig. 6*A*, *lane 2*). However, when IL-1ra was added post-IL-1β treatment, phospho-IκBα was undetectable at 8 h (Fig. 6*A*, *lane 3*). IL-1ra alone had no effect on endogenous levels of the protein (Fig. 6*A*, *lane 5*). Thus, IL-1ra terminated the long term phosphorylation of IκBα. This has important functional consequences because similar treatment of cells with IL-1ra 1 h post-IL-1β stimula-

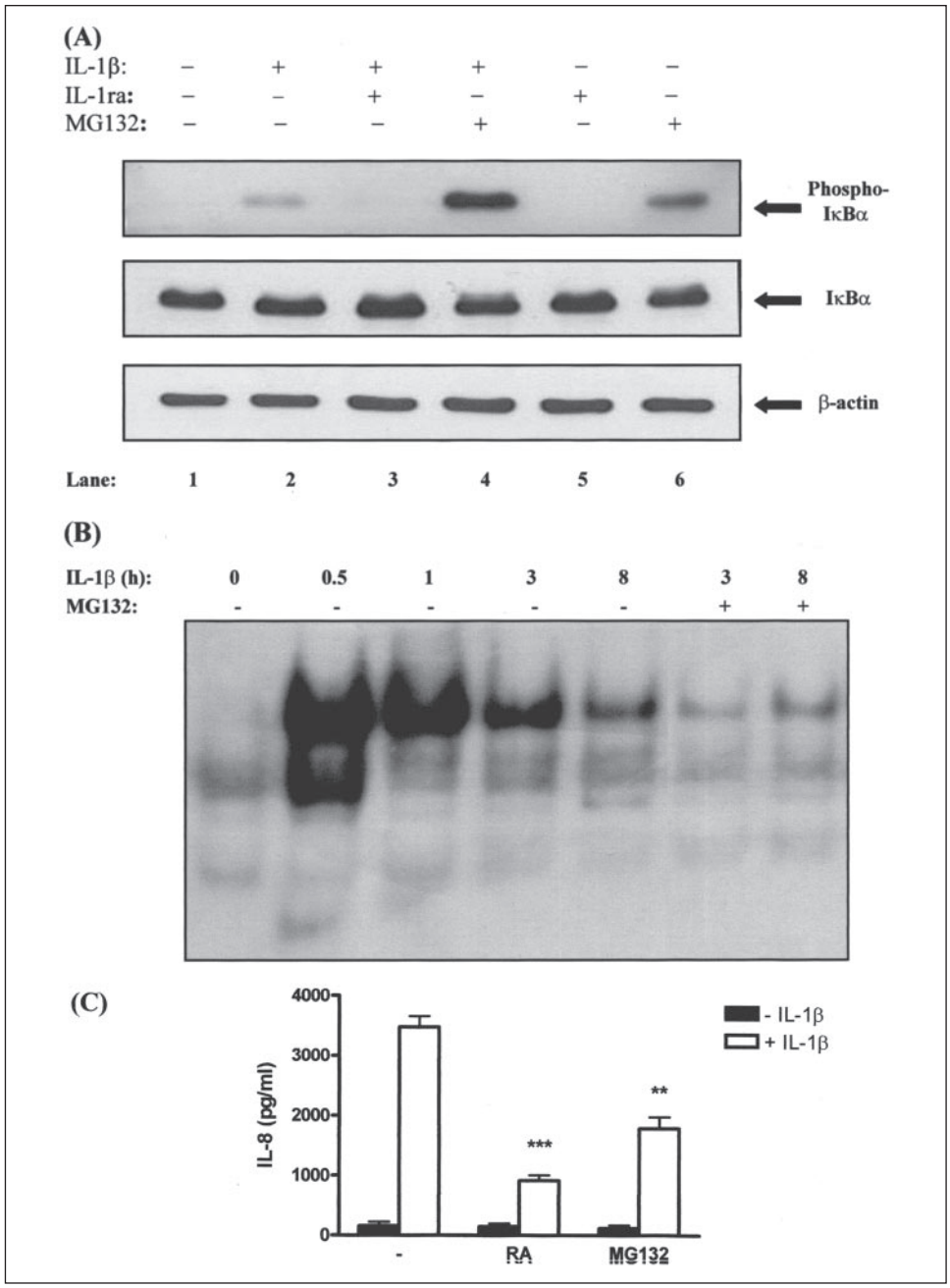


FIGURE 6. **IL-1 signaling inhibitors, post-IL-1β stimulation, attenuate the persistent activation of NFκB in 1321N1 astrocytomas.** 1321N1 astrocytomas were stimulated with or without IL-1β (10 ng/ml) for 8 h. 1 h post IL-1β addition, cells were treated with or without IL-1ra (200 ng/ml) or MG132 (10 μM). *A,* 1321N1 astrocytomas were grown to confluency in 6-well plates; whole cell extracts were generated, and levels of phospho-IκBα (Ser-32/36), total IκBα, and β-actin were determined by Western immunoblotting. The results shown are each representative of three independent experiments. *B,* 1321N1 astrocytomas were grown to confluency in 25-cm$^2$ flasks. Nuclear extracts (10 μg of protein) were assessed for the presence of NFκB DNA binding activity by EMSA. The results shown are each representative of three independent experiments. *C,* 1321N1 astrocytomas were grown to confluency in 96-well plates, and the medium was removed and assayed for IL-8 levels by sandwich enzyme-linked immunosorbent assay. Results are expressed as concentration of IL-8 in pg/ml. Data are presented as the mean ± S.E. of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$ *versus* sample treated with IL-1β alone (unpaired *t* test).

tion causes strong inhibition of IL-1β induction of the NFκB-regulated chemokine, IL-8 (Fig. 6*C*). These data strongly suggest that persistent triggering of the IL-1 receptor by IL-1β causes continuous activation of IKK, leading to phosphorylation and degradation of IκBα, and allowing for sustained activation of NFκB and chronic induction of NFκB-responsive genes. This model is further supported by the use of MG132, an inhibitor of the 26 S proteasome. The addition of MG132 1 h post-IL-1β stimulation caused considerable accumulation of phospho-IκBα at 8 h (Fig. 6*A*, compare *lanes 2* and *4*), strongly confirming continuous activation of IKK and phosphorylation of IκBα in response to persistent triggering of the pathway by IL-1β. Prolonged IL-1 signaling and IκB degradation are required to maintain chronic NFκB activation, as proteasome inhibition post-IL-1β stimulation was found to reduce the level of NFκB DNA binding activity (Fig. 6*B*). Furthermore, like IL-1ra, inhibiting this persistent signaling with MG132 strongly attenuates the long term induction of IL-8 by IL-1β (Fig. 6*C*). These findings demonstrate that the continuous binding of IL-1β to its astrocyte receptor leads to persistent phosphorylation and degradation of IκBα, resulting in sustained activation of NFκB.

*IL-1β Causes Sustained Activation of NFκB in a Promoter-specific Manner in Astrocytes*—The previous EMSA and confocal studies demonstrated that IL-1β causes peak activation of NFκB at 1 h, and although nuclear localization and DNA binding decreases over time, it is still sustained at 24 h. We finally addressed if this temporal regulation was the same for all NFκB-responsive promoters. The EMSA procedure provides information on the DNA binding activity of the global population of NFκB, but it does not examine the presence of NFκB on individual promoters and ignores the impact of chromatin structure on

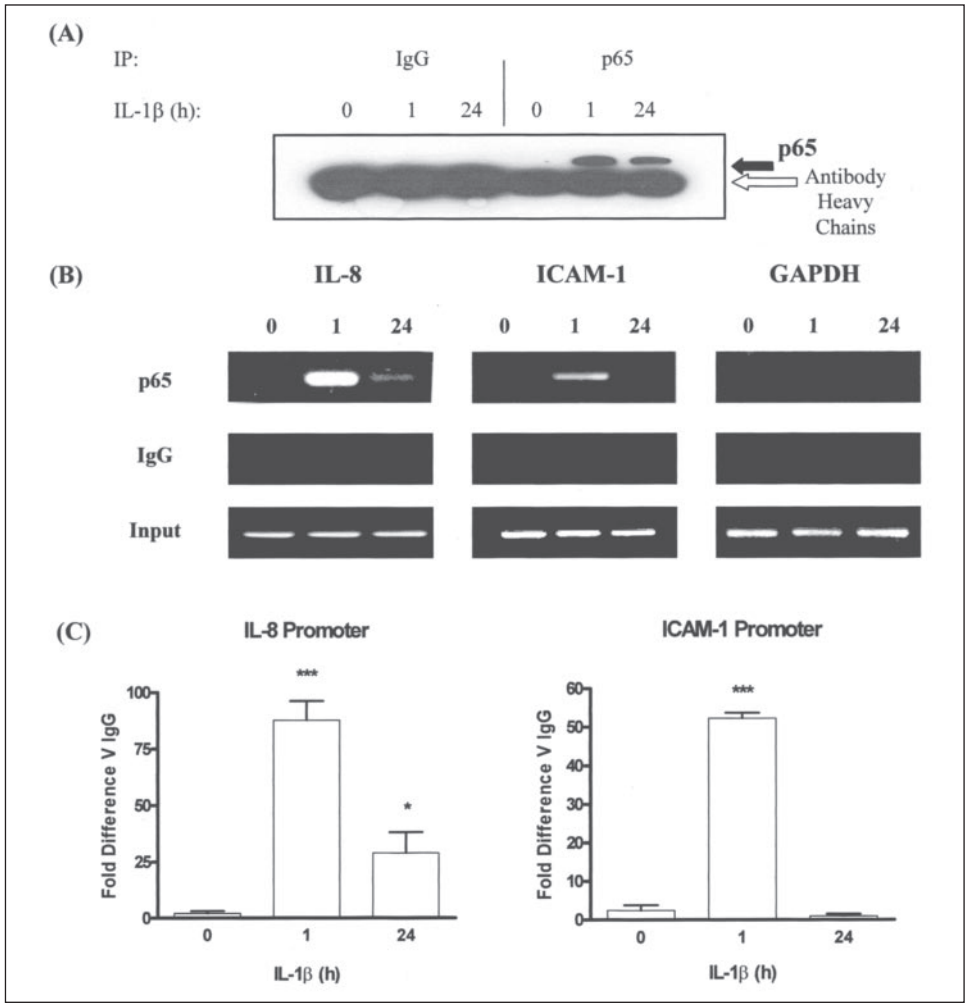


FIGURE 7. **IL-1β causes differential temporal profiles of p65 binding to κB-responsive promoters in 1321N1 astrocytomas.** 1321N1 astrocytomas were treated with or without IL-1β (10 ng/ml) for the indicated time periods. Nuclear lysates were generated and immunoprecipitated (*IP*) with anti-p65 or control IgG antibodies. *A*, following SDS-PAGE, immunoprecipitates were subjected to Western immunoblotting with anti-p65 antibody. Immunoprecipitated DNA was analyzed for IL-8, ICAM-1, and GAPDH promoters by PCR and gel electrophoresis (*B*) and for IL-8 and ICAM-1 promoters by quantitative real time PCR (*C*). Data from real time PCR are expressed as fold differences between DNA enrichment in the anti-p65 ChIP sample relative to IgG ChIP sample and are displayed as the means ± S.E. from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$ versus control at 0 h (unpaired *t* test).

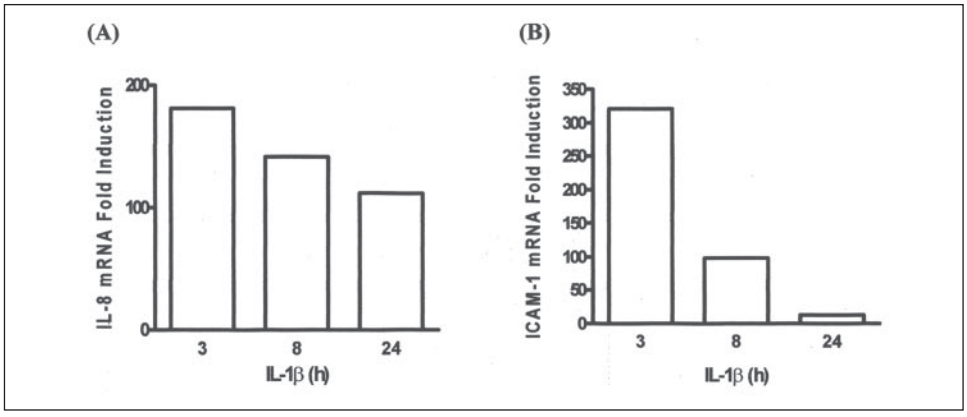


FIGURE 8. **IL-1β induces sustained production of IL-8 mRNA, but transient induction of ICAM-1 mRNA, in 1321N1 astrocytomas.** 1321N1 astrocytomas were treated with or without IL-1β (10 ng/ml) for the indicated time periods. RNA was isolated and converted into cDNA. Samples were assayed by quantitative real time PCR for levels of IL-8 (*A*) and ICAM-1 (*B*) mRNA using the ABI PRISM 7900 HT thermal cycler. Data are expressed as mRNA induced by IL-1β treatment normalized to a time-matched control and are displayed as means from two independent experiments.

DNA-binding ability. Chromatin immunoprecipitation studies were therefore performed to monitor the presence of p65 at the *IL-8* and *ICAM-1* promoters in 1321N1 astrocytomas previously stimulated with or without IL-1β for 1 or 24 h. SDS-PAGE and Western immunoblotting of anti-p65 and control IgG immunoprecipitates from nuclear extracts demonstrated the presence of nuclear p65 both 1 and 24 h after IL-1β treatment (Fig. 7*A*). Analysis of immunoprecipitated DNA by PCR indicated that p65 was present at both the *IL-8* and *ICAM-1* promoters 1 h post-stimulation with IL-1β (Fig. 7*B*). However, 24 h after stimulation, it was detected only at the *IL-8* promoter. This was confirmed by quantitative real time PCR analysis of the immunoprecipitated DNA, where a statistically significant level of p65 binding was recorded at the *IL-8*, but not *ICAM-1*, promoter at the later time point (Fig. 7*C*). *GAPDH* promoter was not detected in p65-immunoprecipitates, demonstrating the specificity of IL-8 and ICAM-1 promoter enrichment (Fig. 7*B*). Thus the sustained activation of NFκB by IL-1β in astrocytes is promoter-specific. Finally, we addressed the functional consequence of this promoter-specific effect by quantitating levels of mRNA encoding IL-8 and ICAM-1 by real time PCR and assessing how their induction by IL-1β varied over time. IL-1β caused an ~175-fold induction of IL-8 mRNA in 1321N1 cells at 3 h, and this was maintained above 100-fold at 24 h (Fig. 8*A*). ICAM-1 mRNA was induced over

300-fold by 3 h of stimulation with IL-1β. However, in contrast to IL-8, the levels of ICAM-1 mRNA decreased almost to control levels by 24 h (Fig. 8*B*). It is highly likely that the sustained expression of IL-8 mRNA is because of the prolonged presence of p65 on the IL-8 promoter, whereas transient association of p65 with the ICAM-1 promoter closely correlates with short lived induction of ICAM-1 mRNA.

## DISCUSSION

Although leukocyte extravasation is a necessary response to pathogenic invasion, chronic or aberrant inflammation underlies numerous disease states. Increased expression of molecules mediating inflammation is seen in various disorders of the CNS. Studies on MS and on EAE have demonstrated the positive correlation of pro-inflammatory cytokine expression with disease progression (15, 22). ICAM-1 expression is up-regulated in MS and EAE, where it is expressed on astrocytes and microglia in addition to endothelial cells (35, 36). Efficient suppression of EAE has been achieved by treatment with anti-ICAM-1 monoclonal antibody (37). Furthermore, studies on disease progression and remission in EAE animals indicate that the T-lymphocyte entry into and subsequent departure from the CNS is coincident with increased and decreased expression of pro-inflammatory cytokines and ICAM-1 (36, 38). IL-8 is also expressed in EAE animals during disease onset (38), but as a chemoattractant acting predominantly on neutrophils, it appears to have a more important role in conditions such as bacterial meningoencephalitis and cerebral reperfusion/ischemia injury where there is a significant neutrophilic contribution to the CNS lesion (39–41). In terms of controlling the cerebral recruitment of leukocytes and the ensuing neuropathogenesis, an increased understanding of the mechanisms regulating the expression of adhesion molecules, like ICAM-1, and chemokines, like IL-8, in resident brain cells will be of great import.

Regulation of gene expression commonly occurs at the level of transcription. The inducible transcription factor NFκB plays a key role in controlling the transcription of many immune and inflammatory response genes including adhesion molecules and chemokines (2). IL-1β has been shown previously to activate NFκB in glial cells, leading to the expression of IL-8 and ICAM-1 (23, 24). The rapid inducibility of the NFκB pathway is undoubtedly beneficial for immune response to infection or injury. However, long term activation of the transcription factor has been observed in chronic inflammatory disease states including rheumatoid arthritis, asthma, and MS (14, 16). Given the central role of NFκB in the pathogenesis and maintenance of these chronic inflammatory states, the importance of examining the mechanisms behind its sustained activation is clear. Some such mechanisms have been elucidated. Agents causing long term activation of NFκB are known to cause degradation of IκBβ in addition to IκBα (13). In pre-B-cells stimulated with LPS or IL-1β, IκBβ is degraded and resynthesized in a hypophosphorylated form, which binds NFκB without inhibiting nuclear translocation or DNA binding. Within this complex, NFκB is refractory to inhibition by resynthesized IκBα (26). The same mechanism is responsible for the continuous activation of NFκB in early mature B-cells (42).

This group has shown previously that IL-1β stimulation of glial cells causes long term, persistent activation of NFκB (25). As IκBβ remained absent over the course of sustained NFκB activity, the question arose as to how IκBα failed to terminate this activity upon resynthesis. Here we show that IκBα is able to enter the nucleus upon resynthesis, as evidenced by its accumulation in that compartment after IL-1β treatment under conditions where nuclear export is inhibited. Co-immunoprecipitation studies revealed that resynthesized IκBα is able to interact with p65, although indirect immunofluorescence indicated that the majority of p65-containing NFκB dimers are returned to the cytoplasm 3 h post-IL-1β stimulation. The apparent discrepancy between the ability of IκBα to mediate some degree of post-induction repression and the persistent presence of NFκB DNA binding activity were resolved by assessing the temporal profile of the IL-1 signaling cascade. IκBα, phosphorylated on serines 32 and 36, was detected up to 24 h following initial IL-1β stimulation. Furthermore, *in vitro* kinase assays demonstrated that IKKβ, the enzyme responsible for the inducible phosphorylation, was still active at this late time point. As NFκB-bound IκBα constitutes a better substrate for IKK than free IκBα (43), the phosphorylated form of the protein detected most likely represents IκBα that has been resynthesized and has returned NFκB to the cytoplasm before becoming subject to further degradation.

That the persistent activation of NFκB observed in these glial cells was dependent on continuous IL-1β signaling was verified using IL-1 receptor antagonist. When applied post-IL-1β stimulation to allow initial NFκB activation, IL-1ra inhibited the IKK-mediated phosphorylation of IκBα. It also attenuated the IL-1β-induced production of IL-8, a process dependent on NFκB transcriptional activity. The latter effect was also achieved through inhibition of the 26 S proteasome using MG132. The signaling and proteasome dependence of sustained NFκB activity indicates that there is persistent IKK activation and breakdown of IκBα following IL-1β stimulation. IκBβ remains absent at the protein level despite the continued presence of the mRNA transcript (25). It is possible that IκBβ protein is subject to immediate degradation upon resynthesis. Unlike IκBα, IκBβ expression is not induced by NFκB activation (13). Therefore, the low level of basal production in these cells may not be sufficient to overcome the IL-1β-mediated protein breakdown.

The *in vivo* recruitment of NFκB to two immunologically relevant, κB-responsive promoters, IL-8 and ICAM-1, was analyzed by chromatin immunoprecipitation studies. The Rel subunit chosen for detection for ChIP studies, as for indirect immunofluorescence, was p65 because of its transactivating ability (44). Furthermore, the predominant forms of NFκB activated in these cells are p65-p50 heterodimers and p65 homodimers (25). Finally, the variant κB sites in both the IL-8 and ICAM-1 promoters are known to recruit p65 homodimers following NFκB activation (18, 19). ChIP studies revealed that p65 is found at both promoters 1 h following IL-1β stimulation, but only at the IL-8 promoter after 24 h IL-1β. This confirms the finding of other groups that the overall NFκB-DNA binding activity as assessed by EMSA does not necessarily reflect the *in vivo* binding of NFκB to specific promoters (45). The transcriptional activation mediated by NFκB at these promoters was examined by quantitative real time PCR analysis of IL-8 and ICAM-1 mRNA levels. In line with ChIP data, divergence in the IL-1β-mediated induction of *ICAM-1* and *IL-8* gene expression was observed. Although ICAM-1 mRNA had returned almost to control levels 24 h after IL-1β treatment, IL-8 expression was still up-regulated.

A large degree of similarity exists between the IL-8 and ICAM-1 promoters. The form of NFκB that binds preferentially to the κB sites of both promoters is the p65 homodimer, and NFκB binding is essential for IL-1β-mediated gene induction. In both cases, binding occurs to a variant κB site that lacks the run of three or four consecutive guanine residues required for p50 binding at the 5′ end (18, 19). The sequence of the IL-8 κB site is in fact identical to the ICAM-1 κB site except for an A to T transition in the center of the decameric sequence. The nucleotides immediately flanking the κB site in the ICAM-1 promoter appear to play an important role in NFκB binding and transactivation, with the minimal ICAM-1-κB site incapable of supporting tumor necrosis factor-α-mediated induction when placed upstream of a reporter gene (46). However, a similar situation does not seem to exist in the IL-8 promoter

(47). Both promoters also contain adjacent C/EBP-binding sites in their 5′-flanking region, adjacent to the κB sites. IL-1β-mediated transcriptional activation is regulated by cooperative effects between C/EBP and NFκB. In the *IL-8* promoter C/EBP and NFκB binding occurs cooperatively, with resulting synergistic activation of *IL-8* gene expression (17). However, there are conflicting reports on the outcome of this interaction on the *ICAM-1* promoter, with one study demonstrating that C/EBPβ attenuated the transactivating effects of p65 on ICAM-1 expression in primary astrocytes (48). Thus, although the Rel binding characteristics and identity of cis-acting elements in the *IL-8* and *ICAM-1* promoters are similar, differences exist that could contribute to the persistence of p65 at one but not the other. Selective binding of NFκB to a subset of target promoters has been demonstrated previously in macrophages (45). In this case, NFκB was shown to bind to certain promoters immediately upon entering the nucleus following activation. Recruitment occurred later at other target sites, where stimulus-dependent alterations of promoter acetylation status may have been required. Promoter accessibility is therefore another level of control of NFκB activity. Diverse affinity of NFκB for the κB sites, interaction with other trans-acting factors, and modified promoter accessibility could account for the sustained presence of p65 at the *IL-8* but not *ICAM-1* promoter. It is also possible that newly synthesized IκBα is capable of removing NFκB from the *ICAM-1* promoter, whereas the IL-8 promoter-bound transcription factor remains refractory to the post-induction repression. The persistent presence of NFκB on a subset of target promoters following IL-1β synthesis has been proposed previously to be due to the presence of hypophosphorylated IκBβ in a complex with DNA-bound NFκB (45). However, IκBβ does not reappear at the protein level in IL-1β-stimulated astrocytes, ruling out this mechanism.

In summary, we have shown that persistent IL-1β signaling in an astrocytic cell system leads to sustained NFκB activity despite the ability of newly synthesized IκBα to remove NFκB from the nucleus. Over the course of this long term NFκB activation, there is an alteration of the profile of κB-responsive promoters at which the transcription factor persists, leading to differential expression of target genes. This significantly advances our molecular appreciation of the mechanisms underlying chronic NFκB activation in glial cells and provides important clues for informing new therapeutic strategies in the treatment of neuropathological conditions.

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Bryan D. Griffin and Paul N. Moynagh

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