

Loss of I κ B- β Is Associated with Prolonged NF- κ B Activity in Human Glial Cells*

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Nuclear factor- κ B (NF- κ B) is an inducible transcription factor central in the regulation of expression of a wide variety of genes and synthesis of several proteins involved in the generation of the immune response and inflammatory processes. In resting cells, NF- κ B is maintained in an inactive state through cytoplasmic retention by I κ B inhibitors. Stimulation of cells with a wide variety of inducers results in proteolytic degradation of these I κ B proteins, leading to activation of NF- κ B. The present study shows that interleukin-1 (IL-1) causes persistent activation of NF- κ B in glial cells. Stimulation with IL-1 also causes rapid but transient degradation of I κ B- α and I κ B- ϵ . However, NF- κ B remains active even after these I κ B isoforms have returned to control levels. In contrast, the I κ B- β isoform fails to reappear following its initial degradation by IL-1, coincident with sustained activation of NF- κ B. In addition, *in vivo* overexpression of the various I κ B isoforms revealed that I κ B- β is the only isoform that has the ability to inhibit IL-1-induced NF- κ B-driven transcription. The findings also suggest that the inability of I κ B- α and I κ B- ϵ to modulate NF- κ B activity is due to their modification *in vivo*. These findings indicate that I κ B- β is the key regulator of the activity of NF- κ B in human glial cells.

Nuclear factor κ B (NF- κ B)¹ is a transcription factor that is employed in the inducible expression of a wide variety of cellular genes, in particular those encoding immunoreceptors, cytokines, and viral proteins (1, 2). NF- κ B exists in the cytosol of resting cells as a homo- or heterodimer of proteins of the Rel family of transcription factors. Each member of this family contains a conserved N-terminal region called the Rel-homology domain, which contains the DNA-binding domain, the dimerization domain, and the nuclear localization signal (3). To date, five Rel-related NF- κ B proteins have been identified in mammalian cells: p65 (Rel A), Rel B, the proto-oncogene *c-rel*, p50/p105, and p52/p100. The transcriptional activity of the Rel proteins is tightly regulated by their association with members of the inhibitory molecule family, I κ B (4). Several I κ B isoforms have been identified, but the most extensively characterized

isoforms are I κ B- α , I κ B- β , and I κ B- ϵ . This family of proteins share conserved motifs referred to as ankyrin repeats, which are required for their association with the Rel homology region of the NF- κ B proteins. This association has two functions. First, I κ B sequesters NF- κ B in the cytosol by masking its nuclear localization signal through direct protein-protein interactions, and second, I κ B can prevent NF- κ B from binding to DNA by masking its DNA-binding domain (5). Exposure of cells to certain stimuli, *e.g.* the cytokines, IL-1 and tumor necrosis factor or bacterial and viral products such as LPS, double-stranded RNA, and the Tax protein from the human T-cell leukemia virus 1, causes phosphorylation of I κ B on two specific N-terminal serines: Ser-32 and Ser-36 on I κ B- α (6, 7), Ser-19 and Ser-23 on I κ B- β (8), and Ser-18 and Ser-22 on I κ B- ϵ (9). Such signal-induced phosphorylation is believed to be carried out by the newly discovered I κ B kinases (10–14) and represents a signal for ubiquitin conjugation of I κ B, followed by degradation via the 26 S proteasome. This allows translocation of active NF- κ B to the nucleus, where it binds to its consensus sequences within the promoter regions of genes, thus activating transcription (15–17). In addition to the signal-induced phosphorylation of the I κ Bs, it has also been shown that sites of constitutive phosphorylation exist within the C-terminal PEST (proline-, glutamic acid-, serine- and threonine-rich) domains of I κ B- α and - β , which affect intrinsic protein stability (18–21). Casein kinase II has been shown to be responsible for this phosphorylation, which occurs preferentially on Ser-293 in I κ B- α (20) and Ser-313 and Ser-315 in I κ B- β (21). This casein kinase II-mediated phosphorylation is required for efficient turnover of free I κ B- α and I κ B- β but is not involved in signal-induced I κ B phosphorylation. The mechanism of degradation following this basal phosphorylation has yet to be characterized.

Following stimulation, the nature of NF- κ B activation may be transient or persistent, depending entirely on the cellular stimulus. Tumor necrosis factor and phorbol 12-myristate 13-acetate cause rapid but transient activation of NF- κ B (22). Typically, activity peaks within 30 min, followed by a return to basal activity within 4–6 h. In contrast, stimulation with IL-1, LPS, or the human T-cell lymphotropic virus 1 Tax protein tends to result in long term persistent activation of NF- κ B, which has been shown to last for up to 48 h (8, 22). Interestingly, these studies reported that all stimuli of NF- κ B activation cause degradation of I κ B- α , whereas I κ B- β is only degraded by those inducers that cause long term persistent activation of NF- κ B. Preliminary studies on the recently discovered I κ B- ϵ indicate that this isoform may also be degraded by all inducers of NF- κ B (9). Hence, it has been suggested that differential patterns of degradation of the I κ B isoforms represent an important mechanism in the regulation of NF- κ B activation.

Previous work has shown that NF- κ B activation is sustained in glial cells in response to stimulation with IL-1 (23, 24). However, the mechanistic basis for this persistent activation of

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¹ The abbreviations used are: NF- κ B, nuclear factor κ B; IL-1, interleukin-1; LPS, lipopolysaccharide; PEST, proline-, glutamic acid-, serine-, threonine-rich; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; bp, base pair(s); DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus.

NF- κ B was not resolved. This is of clinical relevance, because the sustained activation of NF- κ B in brain cells may lead to prolonged induction of leukocyte adhesion molecules and chemokines that will facilitate cerebral recruitment of leukocytes, culminating in the generation of neuropathological states. The purpose of this study was to examine the relative importance of the I κ B isoforms (α , β , and ϵ) in regulating the temporal activation of NF- κ B in glial cells. We show that prolonged disappearance of I κ B- β in response to IL-1 underlies concurrent sustained activation of NF- κ B, and we provide direct evidence for the first time that NF- κ B in glial cells is subject to selective inactivation by the β form of the I κ B members.

EXPERIMENTAL PROCEDURES

Materials—The human astrocytoma cell line 1321N1 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). DMEM, fetal calf serum, penicillin-streptomycin, and trypsin were from Life Technologies, Inc. (Paisley, UK). Human rIL-1 β was purchased from R&D Systems Europe (Oxon, UK). T4 polynucleotide kinase, the 22-bp oligonucleotide containing the NF- κ B consensus sequence (*undrilled*) (5'-AGTTGAGGGGACTTCCAGGC-3') were supplied by Promega (Madison, WI). [γ -³²P]ATP, [α -³²P]dATP, and nylon transfer membranes were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Rabbit polyclonal antibodies against I κ B- α and I κ B- β were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Affinity-purified rabbit polyclonal antiserum against I κ B- ϵ was a gift from Nancy Rice (National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD). Sheep anti-rabbit IgG peroxidase conjugate and the Enhanced Chemiluminescence detection system were purchased from Roche Molecular Biochemicals UK Ltd. (East Sussex, UK). The pCMV4 vectors containing full-length cDNA encoding I κ B- α and I κ B- β were generously provided by Dean Ballard (Vanderbilt University, Nashville, TN). The pcDNA3-I κ B- ϵ plasmid was generously provided by Simon T. Whiteside (Pasteur Institute, Paris, France). The NF- κ B-luciferase reporter construct (consisting of five tandem κ B sites cloned into the polylinker of pGL3 Basic vector (Promega), the anti-p50, p65, and *c-rel* antisera and the polymerase chain reaction primers for β -actin (exon 3 forward: 5'-CGTAACTGGCATCGTG-3' and exon 4 reverse: 5'-GTTTCGTGGATGCCACAG-3') were generously provided by Luke O'Neill (Trinity College, Dublin, Ireland). The C-terminal histidine-tagged recombinant forms of I κ B- α , I κ B- β , and I κ B- ϵ were gifts from Jorg Eder (Novartis Pharma AG, Switzerland) and have been described previously (25).

Cell Culture—The human astrocytoma cell line, 1321N1, was cultured in DMEM supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, and 10% (v/v) of fetal calf serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. All cells were passaged using 1% (w/v) of trypsin in DMEM. IL-1 β stimulation was performed on cells in serum-containing medium at 37 °C for all experiments.

Preparation of Subcellular Fractions—1321N1 astrocytoma were seeded into 25-cm² flasks (5 ml; 2 \times 10⁵ cells/ml) and allowed to adhere for 72 h before stimulation. Cells were exposed to IL-1 β for various times. Stimulation was terminated by removal of medium followed by washing of the adherent cells with 3 ml of ice-cold PBS. Washed cells were scraped into 1 ml of hypotonic buffer (10 mM HEPES-NaOH buffer, pH 7.9, containing 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF). Cells were pelleted in hypotonic buffer by centrifugation at 21,000 \times g for 10 min and then lysed for 10 min on ice in hypotonic buffer (35 μ l) containing 0.1% (v/v) of Nonidet P-40. Lysates were centrifuged at 21,000 \times 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 μ l), containing 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (w/v) glycerol, and 0.5 mM phenylmethylsulfonyl fluoride and incubated for 15 min on ice. Incubations were then centrifuged at 21,000 \times g for 10 min, and the supernatants were transferred to 10 mM HEPES/NaOH buffer, pH 7.9 (75 μ l), containing 50 mM KCl, 0.2 mM EDTA, 20% (w/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT. Such samples constituted nuclear extracts. Protein concentrations of the cytosolic and nuclear extracts were determined by the method of Bradford (26). All of the above steps were carried out at 4 °C.

Electrophoretic Mobility Shift Assay—Nuclear extracts (4–10 μ g of protein) were incubated with 20,000 dpm of a 22-bp oligonucleotide containing the NF- κ B consensus sequence, which previously had been labeled with [γ -³²P]ATP (10 mCi/mmol) by T4 polynucleotide kinase

(27). 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 μ g of poly(dI-dC), and 1 mg/ml nuclease-free BSA. In the supershift analysis, polyclonal antisera (1 μ l) against the NF- κ B subunits p65, p50, p52, and *c-rel* were added to the extracts and incubated for 30 min on ice prior to incubation with labeled oligonucleotide. I κ B- α , I κ B- β , and I κ B- ϵ were examined for their modulation of the DNA binding activity of NF- κ B by incubating the extracts in the absence and presence of recombinant forms of I κ B (1 μ g) for 30 min at room temperature prior to incubation with labeled oligonucleotide. All incubations were subjected to electrophoresis on 4% non-denaturing polyacrylamide gels that were subsequently dried and autoradiographed.

Western Immunoblotting—Cytosolic extracts (20 μ g of protein) were boiled in an equal volume of 2 \times sample buffer for 5 min prior to loading. 2 \times sample buffer consisted of 0.125 M Tris-HCl buffer, pH 6.8, containing 20% (w/v) glycerol, 4% (w/v) SDS, 1.4 M 2- β -mercaptoethanol, and 0.0025% (w/v) bromophenol blue. The extracts and biotinylated molecular mass markers (14–100 kDa) were separated by SDS-PAGE using a 12% (w/v) resolving gel. The proteins were transferred electrophoretically to nitrocellulose and blocked by overnight incubation at room temperature in 20 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl (TBS) and 5% (w/v) powdered milk (Marvel). The blots were washed three times (10 min each) in TBS containing 0.05% (v/v) Tween 20 (TTBS) and then incubated for 2 h with primary polyclonal antibody against I κ B- α or I κ B- β (1 μ g/ml) or antiserum against I κ B- ϵ (1:2000) diluted in TTBS containing 1% (w/v) powdered milk (Marvel). The blots were subsequently washed in TTBS before incubation for 45 min with horseradish-peroxidase-conjugated sheep anti-rabbit IgG (1:1000 dilution) and horseradish-peroxidase-conjugated streptavidin (1:5000 dilution). The blots were washed, and immunoreactive bands were detected using the Enhanced Chemiluminescence detection system from Roche Molecular Biochemicals (Germany) according to the instructions of the manufacturer.

Northern Blot Analysis—Total RNA was extracted from cells by guanidinium thiocyanate and isolated as described previously (28). Total RNA (12 μ g per sample) was separated by 1% (w/v) formaldehyde-agarose gel electrophoresis and transferred overnight onto nylon membranes by capillary action. The RNA was cross-linked to the membranes by exposing to 120,000 μ J/cm² for 30 s within the Stratagene Stratalinker UV cross-linker. The membranes were incubated at 42 °C for 15 min in a solution containing 50% (v/v) formamide, 5 \times SSC, 5 \times Denhardt's solution, 1% (w/v) SDS, and 100 μ g/ml salmon sperm DNA. Radiolabeling of I κ B- α , I κ B- β , I κ B- ϵ , and human β -actin cDNA probes was achieved by nick translation with [α -³²P]dATP using *Escherichia coli* DNA polymerase I and DNase I (28). The probes were added to the hybridization solution at a concentration of 10 ng/ml, and hybridization was carried out overnight at 42 °C with rotation. The membranes were washed twice with 2 \times SSC containing 0.1% (w/v) SDS and twice with 0.2 \times SSC containing 0.1% (w/v) SDS at room temperature (low stringency). Two further washes were carried out using 0.2 \times SSC/0.1% (w/v) SDS at 42 °C (medium stringency), followed by two final washes with 0.1 \times SSC/0.1% (w/v) SDS at 68 °C (high stringency). High stringency washes were carried out on the I κ B- α , I κ B- β , and β -actin blots. I κ B- ϵ was detected under medium stringency conditions. The blots were then rinsed in 2 \times SSC, covered in UV-transparent wrap, and autoradiographed at -70 °C for 72 h.

Transient Transfections—The pCMV4-I κ B- α , pCMV4-I κ B- β , and pcDNA3-I κ B- ϵ constructs were described previously (8, 9). For control transfections, the empty pCMV4 vector was used. Human 1321N1 astrocytoma were seeded at 1 \times 10⁵ cells/ml and allowed to grow until confluent. Cells were trypsinized, and 1 \times 10⁷ cells were transfected by electroporation (using the Invitrogen Electroporator II) with 3 μ g of NF- κ B luciferase construct and 6 μ g of either pCMV4, pCMV4-I κ B- α , pCMV4-I κ B- β , or pcDNA3-I κ B- ϵ . As an internal control for transfection efficiency, 2 μ g of pHook- β -gal plasmid was co-transfected for all experiments. Aliquots (3 ml) of cells were then seeded into 6-well plates (4 \times 10⁵ cells/well), and the cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 48 h. Cells were then treated with or without IL-1 β (10 ng/ml), and cellular extracts were prepared 12 h later using reporter lysis buffer (Promega). Extracts were centrifuged at 21,000 \times g for 10 min, and the supernatants were analyzed for luciferase and β -galactosidase activity using kits from Promega and Stratagene, respectively, according to the instructions of the manufacturers.

RESULTS

IL-1 Causes Rapid and Persistent Activation of NF- κ B in Glial Cells—Nuclear extracts were prepared from unstimulated and IL-1 β -treated human 1321N1 astrocytoma and as-

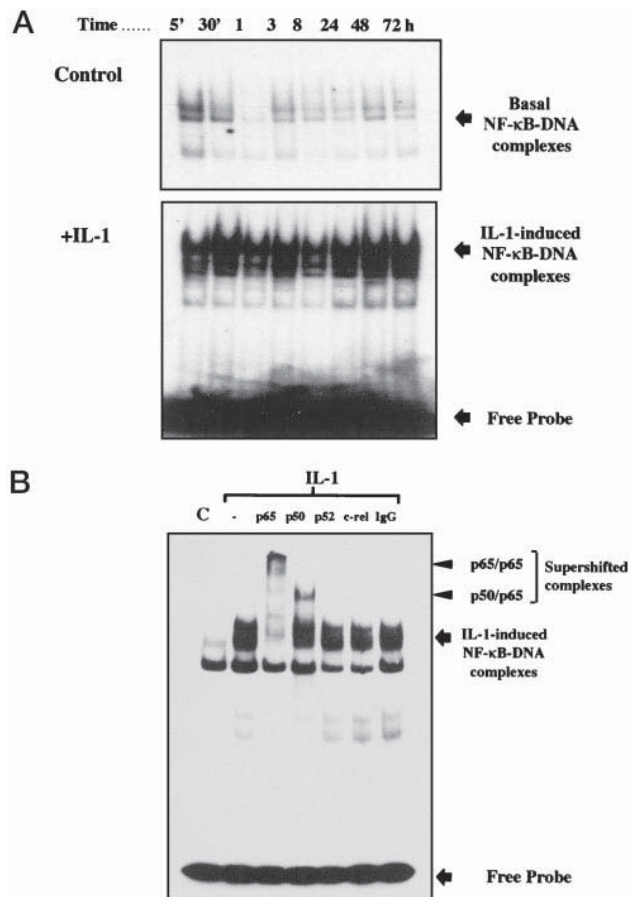


FIG. 1. Rapid and persistent activation of NF- κ B in 1321N1 astrocytoma cells in response to IL-1. 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. **A**, cells were treated in the absence (*upper panel*) and presence (*lower panel*) of IL-1 β (10 ng/ml) for the indicated time periods. Control cells were treated with vehicle (PBS/0.1% (w/v) BSA). Nuclear extracts (4 μ g of protein) were assayed for NF- κ B-DNA binding activity by EMSA as described under "Experimental Procedures." **B**, 1321N1 cells were incubated in the absence or presence of IL-1 β (10 ng/ml) for 1 h, after which nuclear extracts were prepared and assessed for NF- κ B binding activity in the absence and presence of polyclonal antisera against the NF- κ B subunits p50, p65, p52, and *c-rel*. Non-immune IgG was used as a control. The *arrows* indicate the mobility of unbound oligonucleotide (*Free Probe*), the specific NF- κ B-DNA complexes activated by IL-1, and basal NF- κ B-DNA complexes. The *arrowheads* indicate the mobilities of the supershifted complexes. These results are representative of three independent experiments.

sayed for NF- κ B activity by EMSA (Fig. 1). Trace levels of basal NF- κ B activation were apparent in the form of faint DNA-protein complexes in extracts from unstimulated cells. Rapid and sustained activation of NF- κ B was observed in response to stimulation with IL-1 β and was represented as the appearance of a NF- κ B-DNA complex of slow electrophoretic mobility. NF- κ B activity was detectable after 5-min stimulation and remained for at least 72 h. Unbound radioactive probe migrated freely through the gel and appeared as a dark band at the bottom of the gel. The subunit composition of the NF- κ B complexes induced by IL-1 was investigated by incubating nuclear extracts from IL-1-stimulated cells with antisera against p50, p65, p52, or *c-rel* and observing the effects on the electrophoretic mobility of the NF- κ B-DNA complexes (Fig. 1B). IL-1-induced complexes were unaffected by pretreatment with anti-*c-rel*, p52, and the non-immune IgG isotype control. In contrast, anti-p65 caused a reduction in the levels of the IL-1-induced complexes with the concomitant appearance of two supershifted complexes of slow electrophoretic mobility. The anti-

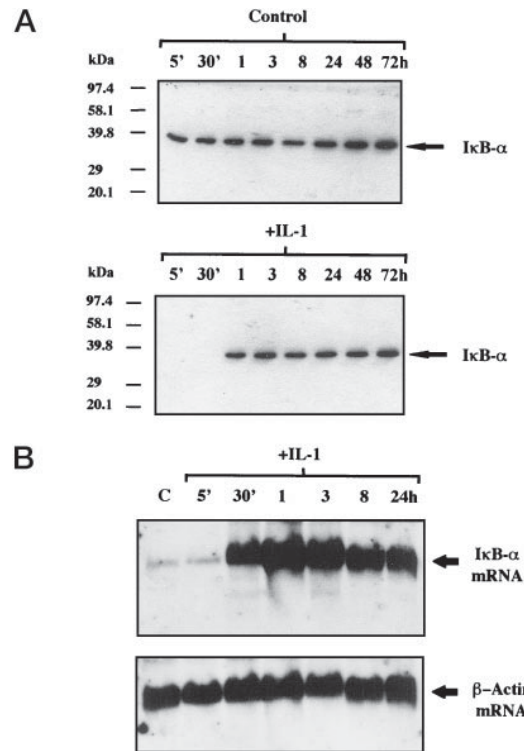


FIG. 2. Rapid degradation and subsequent resynthesis of I κ B- α in response to IL-1. 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. Cells were then treated with IL-1 β (10 ng/ml) for the indicated time periods. Control cells (*C*) were treated with vehicle (PBS/0.1% (w/v) BSA). **A**, cytosolic extracts (20 μ g of protein) were subjected to SDS-PAGE and probed by Western immunoblotting with anti-human I κ B- α antibody. Proteins were visualized by enhanced chemiluminescence. The electrophoretic mobility of molecular mass markers are indicated. **B**, total RNA extracts were prepared and 12 μ g of RNA subjected to 1% (w/v) agarose electrophoresis, blotted onto nylon membrane, and probed with a radiolabeled probe complementary to I κ B- α mRNA (*upper panel*). The nylon was then stripped and probed for β -actin mRNA (*lower panel*). These results are representative of three independent experiments.

p50 antibody also caused a reduction in the levels of the IL-1-induced complexes, albeit not to the same extent as the anti-p65 antibody, with the associated appearance of a single supershifted complex. The latter shows a similar mobility to the faster moving supershifted complex using anti-p65, suggesting that it consists of a p50-p65 heterodimeric complex. The supershifted complex of slowest electrophoretic mobility, using the anti-p65 antibody, is not formed by the other antibody, thus suggesting that this complex represents a p65-p65 homodimer. This would indicate that the IL-1-induced NF- κ B complexes consist primarily of p50-p65 and p65-p65 dimers. Because the levels of NF- κ B are controlled by the I κ B isoforms, it was decided to probe the effects of IL-1 in relation to the levels of the three I κ B isoforms (α , β , and ϵ) in an attempt to explain the underlying mechanisms to sustained activation of NF- κ B in these cells.

IL-1 Causes Rapid Degradation but Subsequent Resynthesis of I κ B- α —Cytosolic extracts were prepared from unstimulated and IL-1 β -treated human 1321N1 astrocytoma and assessed for I κ B- α protein levels by Western blot analysis (Fig. 2A). High levels of I κ B- α were detectable in cytosolic extracts from unstimulated cells. I κ B- α was rapidly degraded upon stimulation with IL-1 and was undetectable in the cytosol after only 5 min of stimulation. This degradation was transient because the level of I κ B- α returned to basal values after 1 h and remained at this level for up to 72 h. To characterize the mechanism of the transient degradation, the levels of mRNA encoding I κ B- α were measured after IL-1 treatment.

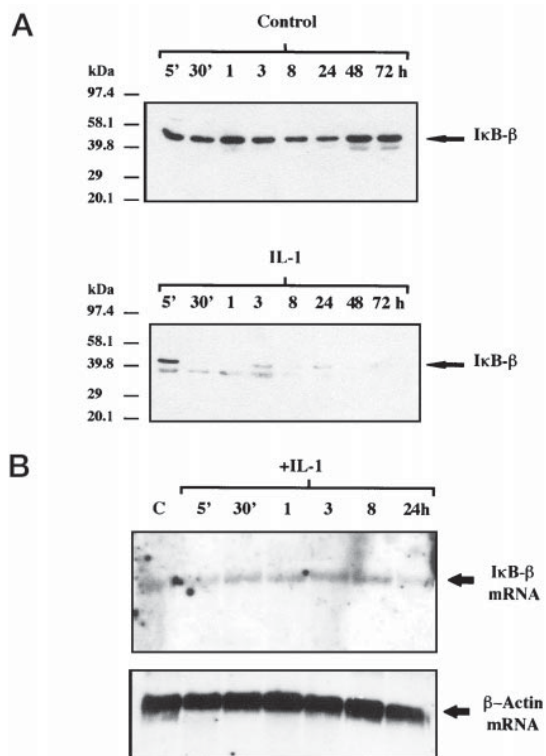


FIG. 3. IL-1 causes sustained degradation and fails to resynthesize I κ B- β . 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. Cells were then treated with IL-1 β (10 ng/ml) for the indicated time periods. Control cells (C) were treated with vehicle (PBS/0.1% (w/v) BSA). **A**, cytosolic extracts (20 μ g of protein) were subjected to SDS-PAGE and probed by Western immunoblotting with anti-human I κ B- β antibody. Proteins were visualized by enhanced chemiluminescence. The electrophoretic mobility of molecular mass markers are indicated. **B**, total RNA extracts were prepared and 12 μ g of RNA subjected to 1% (w/v) agarose electrophoresis, blotted onto nylon membrane, and probed with a radiolabeled probe complementary to I κ B- β mRNA (upper panel). The nylon was then stripped and probed for β -actin mRNA (lower panel). These results are representative of three independent experiments.

Total RNA extracts were prepared from unstimulated and IL-1 β -treated human 1321N1 astrocytoma and assessed for I κ B- α mRNA levels by Northern blot analysis (Fig. 2B). A very low level of I κ B- α mRNA expression was detectable in extracts from unstimulated cells. The expression of I κ B- α mRNA was rapidly up-regulated within 30 min of IL-1 treatment and reached peak levels by 1 h. This up-regulation was sustained because levels remained elevated for at least 24 h. These findings are consistent with the rapid reappearance of the I κ B- α protein in the cytosol of these cells, as detected by Western immunoblotting. The same blot was then reprobed for the housekeeping gene, β -actin. As expected, IL-1 had no effect on the expression of β -actin mRNA. The transient disappearance of I κ B- α did not correlate with the sustained activation of NF- κ B, suggesting that the other forms of I κ B may be more important in regulating NF- κ B. The regulation of I κ B- β by IL-1 was next examined.

IL-1 Causes Sustained Degradation and Fails to Resynthesize I κ B- β —Cytosolic extracts were prepared from unstimulated and IL-1 β -treated cells and assessed for I κ B- β protein levels by Western immunoblotting (Fig. 3A). High levels of I κ B- β were detectable in cytosolic extracts from unstimulated cells. I κ B- β was degraded more slowly than I κ B- α upon stimulation with IL-1; high levels of I κ B- β remained detectable in the cytosol after 5 min of stimulation. However, by 30 min of stimulation, I κ B- β was undetectable. In contrast to I κ B- α ,

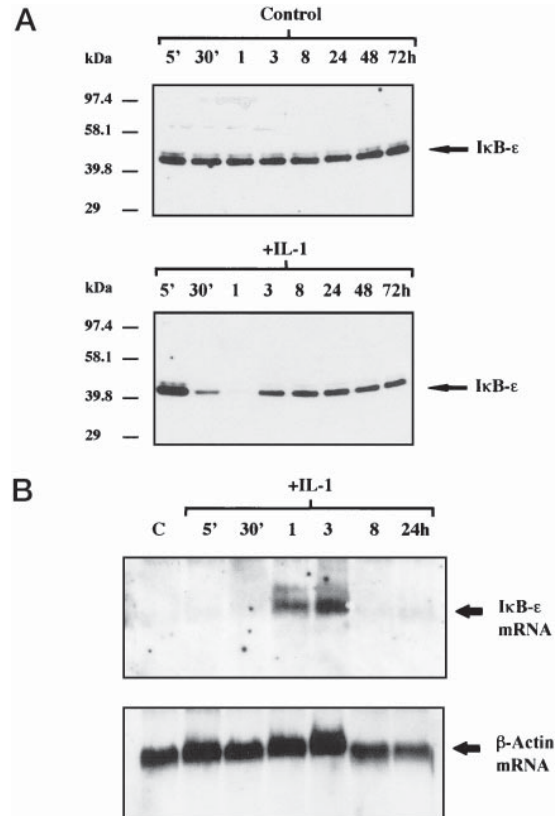


FIG. 4. Degradation and subsequent resynthesis of I κ B- ϵ in response to IL-1. 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. Cells were then treated with IL-1 β (10 ng/ml) for the indicated time periods. Control cells (C) were treated with vehicle (PBS/0.1% (w/v) BSA). **A**, cytosolic extracts (20 μ g of protein) were subjected to SDS-PAGE and probed by Western immunoblotting with anti-human I κ B- ϵ antibody. Proteins were visualized by enhanced chemiluminescence. The electrophoretic mobility of molecular mass markers are indicated. **B**, total RNA extracts were prepared and 12 μ g of RNA subjected to 1% (w/v) agarose electrophoresis, blotted onto nylon membrane, and probed with a radiolabeled probe complementary to I κ B- ϵ mRNA (upper panel). The nylon was then stripped and probed for β -actin mRNA (lower panel). These results are representative of three independent experiments.

I κ B- β degradation was sustained, because the levels remained undetectable over most of the time points for up to 72 h. The mechanism of regulation of I κ B- β was next assessed by characterizing the regulation of mRNA levels of I κ B- β by IL-1.

Total RNA extracts were prepared from unstimulated and IL-1 β -treated cells and assessed for I κ B- β mRNA levels by Northern blot analysis (Fig. 3B). A low level of I κ B- β mRNA expression was detectable in extracts from unstimulated cells. In contrast to I κ B- α mRNA expression, the expression of I κ B- β mRNA did not change from basal levels upon stimulation with IL-1. However the prolonged disappearance of I κ B- β protein in response to IL-1 indicates that translation of I κ B- β may be blocked or that IL-1 causes its continuous degradation. The sustained disappearance of I κ B- β correlates well with prolonged activation of NF- κ B, suggesting a crucial role for I κ B- β in regulating NF- κ B in glial cells. However, the relative role of I κ B- ϵ remained to be determined.

IL-1 Causes Degradation but Subsequent Resynthesis of I κ B- ϵ —Cytosolic extracts were prepared from unstimulated and IL-1 β -treated cells and assessed for I κ B- ϵ protein levels by Western blot analysis (Fig. 4A). High levels of I κ B- ϵ were detectable in cytosolic extracts from unstimulated cells. I κ B- ϵ was degraded more slowly than I κ B- α in response to IL-1; I κ B- ϵ remained detectable in the cytosol after 5- and 30-min stimu-

lation. However, similar to I κ B- α , I κ B- ϵ degradation was transient. The amount of I κ B- ϵ returned to basal levels after 3 h of stimulation and remained at this level up to 72 h. Again, the basis of this resynthesis was assessed at the level of mRNA.

Total RNA extracts were prepared from unstimulated and IL-1 β -treated cells and assessed for I κ B- ϵ mRNA levels by Northern blot analysis (Fig. 4B). A very low level of I κ B- ϵ mRNA expression was detectable in extracts from unstimulated cells. The expression of I κ B- ϵ mRNA was up-regulated upon stimulation with IL-1 and reached peak levels by 3 h. These findings are consistent with the rapid reappearance of the I κ B- ϵ protein in the cytosol of these cells, as detected by Western blot. These findings suggest circumstantially that the I κ B- β isoform is the crucial form in controlling the activity status of NF- κ B in glial cells. However, the reappearance of I κ B- α and - ϵ with no coincident reduction in NF- κ B levels questions the role, if any, of these isoforms in controlling the activity of NF- κ B in glial cells. To examine directly the relative contributions of each of the I κ B isoforms in controlling NF- κ B, genes encoding the three forms were cotransfected with a NF- κ B-regulated reporter gene and examined for their inhibitory potential on the induction of the reporter gene.

In Vivo Overexpression of the I κ B Isoforms—1321N1 astrocytoma were transfected with the various expression vectors for the I κ B isoforms, and the resulting cell lysates were analyzed for expression of the respective proteins by Western blot analysis (Fig. 5A). Lane 1 corresponds to cells transfected with empty pCMV4 vector, and no I κ B proteins were detectable in these extracts. It must be noted that these extracts contained considerably less protein than those in Figs. 2A, 3A, and 4A, in which 20 μ g of protein had been loaded per lane compared with 0.2 μ g in this case. This explains why endogenous levels of the I κ B isoforms were undetectable in these blots. Lanes 2, 3, and 4, corresponding to cells transfected with expression vectors encoding I κ B- α , I κ B- β , and I κ B- ϵ , respectively, show high levels of the respective I κ B isoforms. This experiment confirms that the transfected plasmids are being expressed efficiently *in vivo*.

Overexpression of I κ B- β Inhibits Transactivation by NF- κ B—1321N1 astrocytoma were co-transfected with the plasmids containing cDNAs encoding the various I κ B proteins and a κ B-dependent luciferase reporter gene and were subsequently stimulated in the absence and presence of IL-1. Cells lysates were then prepared and analyzed for luciferase activity (Fig. 5B). In the presence of empty pCMV4 vector, IL-1 strongly induced transcription of the reporter gene (an 11-fold induction over control). IL-1 induced similar levels of luciferase expression in the presence of co-transfected I κ B- α or I κ B- ϵ . Therefore, expression of I κ B- α or I κ B- ϵ had no effect on κ B-dependent transcription induced by IL-1. In contrast, cotransfection with I κ B- β resulted in significant inhibition of IL-1-induced transcription of the luciferase reporter construct. This experiment demonstrates that I κ B- β is the only I κ B isoform capable of inhibiting the transactivation activity of NF- κ B in 1321N1 astrocytoma.

Recombinant I κ B Isoforms Inhibit the *in Vitro* DNA Binding Activity of NF- κ B—The inability of overexpressed I κ B- α and I κ B- ϵ to inhibit the transactivation of NF- κ B coupled to the earlier findings showing that the activation of NF- κ B was sustained even in the presence of newly synthesized I κ B- α and I κ B- ϵ prompted an investigation into their lack of inhibitory potential. The modification of the NF- κ B complexes or I κ B- α and I κ B- ϵ were plausible alternative proposals for the lack of inhibition. To resolve these alternative mechanisms the ability of recombinant forms of the three I κ B proteins were assessed for their *in vitro* ability to inhibit the DNA binding activity of the sustained complexes, which were activated by IL-1 in

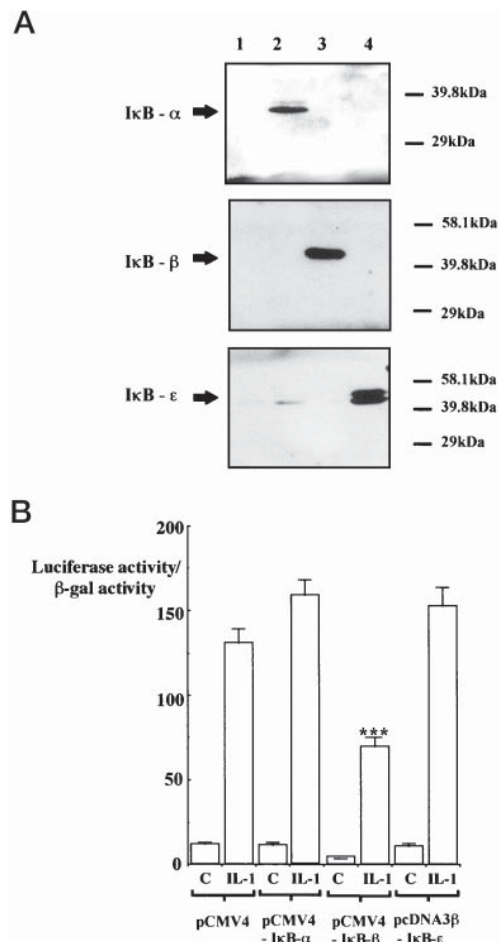


FIG. 5. Overexpression of I κ B- β inhibits transactivation by NF- κ B. 1321N1 astrocytoma (1×10^7 cells) were transfected with 3 μ g of NF- κ B-luciferase reporter construct and 2 μ g of a CMV-driven β -gal reporter construct. In addition to these constructs, the cells were transfected with 6 μ g of one of the following: empty pCMV4 vector (pCMV4), pCMV4 containing I κ B- α (pCMV4-I κ B- α), pCMV4 containing I κ B- β (pCMV4-I κ B- β), or pCMV4 containing I κ B- ϵ (pCMV4-I κ B- ϵ). The cells were allowed to recover for 48 h before stimulation with or without (C) IL-1 β (10 ng/ml) for a further 12 h. A, cell lysates were analyzed for the expression of the I κ B proteins by Western immunoblotting. Lysates (0.2 μ g of protein) were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with anti-human I κ B- α (upper panel), anti-mouse I κ B- β (middle panel), and anti-human I κ B- ϵ (lower panel) polyclonal antibodies. The figure shows extracts from cells transfected with: empty vector pCMV4 (lane 1), pCMV4-I κ B- α (lane 2), pCMV4-I κ B- β (lane 3), and pCMV4-I κ B- ϵ (lane 4). The electrophoretic mobility of molecular mass markers is also shown. B, cell lysates were analyzed for luciferase and β -galactosidase activity. The results are expressed as luciferase activity/ β -gal activity and represent the mean \pm S.E. ($n = 4$) for a representative experiment. A repeat of this experiment gave a similar finding. Statistical analysis was carried out using an unpaired Student *t* test (***) denotes a *p* value of <0.0001 .

1321N1 astrocytoma. Thus nuclear extracts were generated from cells that had been stimulated with IL-1 for 48 h. The extracts were examined for NF- κ B activity by EMSA in the absence and presence of C-terminal histidine-tagged recombinant forms of I κ B- α , I κ B- β , and I κ B- ϵ (25). IL-1 caused the typical activation of two NF- κ B complexes of slow electrophoretic mobility. Intriguingly, I κ B- α and I κ B- ϵ blocked the DNA binding activity of both complexes, whereas I κ B- β only inhibited the complex of slowest mobility. This strongly opposes the *in vivo* inhibitory effects of the overexpressed I κ B isoforms where the opposite inhibitory profile was observed. The ability of I κ B- α and I κ B- ϵ to inhibit the DNA binding of the NF- κ B complexes contrasts with their lack of effect when over-

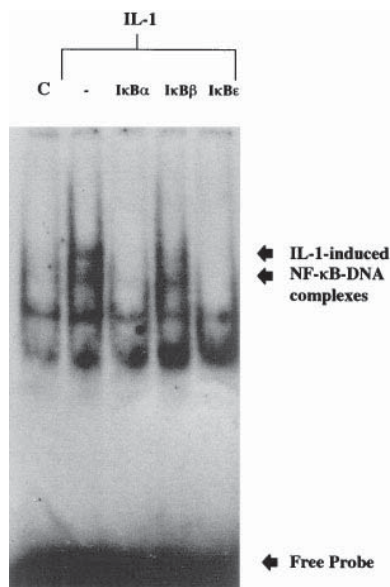


FIG. 6. I κ B- α and I κ B- ϵ but not I κ B- β inhibit the DNA binding activity of sustained NF- κ B complexes. 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. Cells were treated in the absence (C) and presence of IL-1 β (10 ng/ml) for 48 h, after which nuclear extracts were prepared and assessed for NF- κ B binding activity in the absence and presence of C-terminal histidine-tagged recombinant forms of I κ B- α , I κ B- β , or I κ B- ϵ (1 μ g). The arrows indicate the mobility of unbound oligonucleotide (Free Probe) and NF- κ B-DNA complexes activated by IL-1. These results are representative of three independent experiments.

expressed *in vivo*. This strongly suggests that, in the *in vivo* environment, I κ B- α and I κ B- ϵ proteins are modified in some manner that abolishes their inhibitory potential.

DISCUSSION

The regulation of NF- κ B activation by the I κ B isoforms has recently become an area of intensive research in many cell types. The present study probes the molecular nature of this control in glial cells. This is of much importance, because the activation of NF- κ B in brain cells will lead to the induction of pro-inflammatory genes resulting in cerebral inflammation. Indeed, one of the authors has previously reported that IL-1 activates NF- κ B and induces sustained expression of leukocyte adhesion molecules in glial cells (23, 24). This may underlie leukocyte accumulation, which is a prologue to neuropathology. The temporal regulation of NF- κ B by IL-1 in glial cells is addressed in the present study, and the relative importance of the various I κ B isoforms in regulating NF- κ B activity is also evaluated.

IL-1 β caused rapid and sustained activation of NF- κ B in human 1321N1 astrocytoma. This confirms previous studies in this laboratory, which have shown IL-1 β to activate NF- κ B for up to 24 h in rat and human glial cells (23, 24). It is also consistent with studies in other cells types, such as B- and T-cells, in which sustained activation of NF- κ B by IL-1 was also observed (8, 22). This persistent activation of NF- κ B prompted an investigation of the mechanisms underlying the regulation of NF- κ B in these cells concentrating specifically on the role of the different I κ B isoforms.

The effect of IL-1 on the intracellular levels of I κ B was initially assessed. I κ B- α was observed to be rapidly and completely degraded upon stimulation with IL-1. However, degradation was only transient, because control levels of the protein were detectable within 1 h of IL-1 stimulation. This rapid reappearance of I κ B- α is due to increased transcription of the gene encoding I κ B- α , because Northern analysis showed sig-

nificant increases in the levels of I κ B- α mRNA with a time profile corresponding closely to the reappearance of the protein. In addition, transcriptional up-regulation continued for up to 24 h thus ensuring that high I κ B- α levels were maintained in the cell. These results are consistent with previous studies, which indicate that I κ B- α regulates NF- κ B through a novel autoregulatory feedback loop (29–34). Although this model explains the activation of NF- κ B by agents that cause transient activation, it fails to explain the persistent activation of NF- κ B, observed in response to IL-1 stimulation in the present study. It appears that I κ B- α is unable to shut down the NF- κ B response in this case, because NF- κ B remains fully active even after cellular I κ B- α levels have returned to normal. It was apparent from this observation that I κ B- α was not the sole regulator of NF- κ B activation in response to IL-1, which led to examination of the second I κ B isoform, I κ B- β .

I κ B- β shares many common properties with I κ B- α . Both isoforms interact with the same spectrum of Rel proteins with equal affinity, resulting in the inhibition of both nuclear localization and DNA binding ability of NF- κ B (5). Upon stimulation, both proteins become phosphorylated on similarly located N-terminal serines and are subsequently degraded by the ubiquitin/proteasome pathway (35). The present study demonstrated that IL-1 induced I κ B- β degradation in glial cells, accompanied by persistent long term activation of NF- κ B. Degradation of I κ B- β occurred with slightly slower kinetics than I κ B- α , which is in agreement with previous observations in B- and T-cells (8, 22). Once phosphorylated, I κ B is quickly ubiquitinated and degraded. Thus, the efficiency of phosphorylation dictates how rapidly I κ B degradation occurs. I κ B- β has been shown to be a less-efficient substrate for the I κ B kinase subunits when compared with I κ B- α , therefore, it has been proposed that the slower rate of degradation of I κ B- β , is due to a slower rate of phosphorylation (10, 11).

Unlike I κ B- α , the degradation of I κ B- β was not followed by its resynthesis. As already outlined, the rapid reappearance of the I κ B- α protein is due to NF- κ B-directed up-regulation of the I κ B- α gene. Although the *cis*-acting elements that regulate the I κ B- β gene remain undefined, recent reports suggest that this transcriptional unit is not under NF- κ B control (8, 22). Indeed, this was confirmed in the present study with the observation that I κ B- β mRNA transcription was unaffected by stimulation with IL-1. These findings establish a strong correlation between the chronic down-regulation of I κ B- β protein expression and the failure of IL-1 to induce I κ B- β gene transcription. The slow but persistent degradation of I κ B- β observed in response to IL-1 stimulation in these cells suggests that I κ B- β may not be utilized for regulating rapid responses but may respond to persistent signals that yield a more permanent change.

The third isoform of I κ B to be examined in this study was the recently discovered, I κ B- ϵ (9, 36). I κ B- ϵ is similar in structure to I κ B- α and I κ B- β , containing multiple ankyrin repeats and two conserved serine residues, which are necessary for degradation of the protein by the ubiquitin/proteasome pathway. However, in this study I κ B- ϵ displayed its own unique pattern of degradation in response to IL-1 stimulation. I κ B- ϵ was degraded in 1321N1 astrocytoma with relatively slow kinetics, a characteristic shared with I κ B- β . But like I κ B- α , I κ B- ϵ was rapidly resynthesized, returning to control levels by 3 h after IL-1 stimulation. This was explained by findings from Northern analysis, which showed strong up-regulation of I κ B- ϵ mRNA transcription upon stimulation with IL-1. The short period of time between degradation and resynthesis indicates that, like I κ B- α , I κ B- ϵ is tightly regulated in these cells. Whiteside *et al.* (9) reported similar kinetics for the degradation of I κ B- ϵ in Jurkat cells in response to LPS. This group proposed

that I κ B- ϵ could control a third type of NF- κ B response: Those that are slow but still transient in nature.

The kinetics of degradation and resynthesis of the various I κ B isoforms observed in this study suggests that the inability of NF- κ B to up-regulate transcription of the I κ B- β gene transcription is the key to the persistent activation of NF- κ B in glial cells. It appears that the resulting long term down-regulation of I κ B- β protein synthesis allows NF- κ B released from NF- κ B-I κ B- β complexes to remain fully active for long periods of time. However, this can only be achieved if the NF- κ B released from I κ B- β is resistant to the newly synthesized I κ B- α and I κ B- ϵ . In B-cells, such resistance is achieved by association of NF- κ B with a modified form of I κ B- β (37). The initial degradation of I κ B- β in B-cells in response to stimulation with LPS is followed by resynthesis of the protein in a hypophosphorylated form. This newly synthesized I κ B- β associates with free NF- κ B, preventing it from interacting with I κ B- α . Unlike basally phosphorylated I κ B- β , unphosphorylated I κ B- β is unable to mask the nuclear localization signal or DNA-binding domains of NF- κ B. Therefore, the NF- κ B bound to unphosphorylated I κ B- β can still enter the nucleus and remains transcriptionally active. Due to its association with hypophosphorylated I κ B- β , NF- κ B is resistant to inhibition by I κ B- α , allowing it to remain persistently active. However, the present study demonstrates that I κ B- β is degraded and is not resynthesized even in the hypophosphorylated form in glial cells, suggesting a novel regulatory mechanism. Interestingly, the inability of the recombinant form of I κ B- β to inhibit *in vitro* the DNA binding activity of one of the IL-1-activated NF- κ B complexes is likely due to its unphosphorylated state. This is further discussed below.

Previous studies had also suggested that when NF- κ B is released from I κ B- β , it may become modified, conferring resistance to inhibition by I κ B- α and I κ B- ϵ (22). To determine whether activated NF- κ B is modified in such a way as to affect its interactions with the I κ B proteins in glial cells, the present study went on to investigate the effects of overexpression of the I κ B isoforms on IL-1-induced NF- κ B-driven transcription. High levels of the various I κ B isoforms were detected in lysates from transfected cells. Interestingly, the overexpression of I κ B- α or I κ B- ϵ had no effect on the strong induction of the NF- κ B-luciferase gene observed upon stimulation with IL-1. In contrast, I κ B- β significantly inhibited this induction. This implies that the p50-p65 subunits driving the expression of the reporter gene are only susceptible to inhibition by I κ B- β . The fact that I κ B- β expression is chronically down-regulated following IL-1 stimulation explains why NF- κ B remains active for long periods of time. The mechanism underlying the inability of overexpressed I κ B- α or I κ B- ϵ to modulate the transactivation potential of NF- κ B was finally explored. Such lack of inhibition could be explained by modification of the NF- κ B complexes to states that would make them refractory to inhibition by the two I κ B isoforms. Alternatively, the I κ B proteins may be subject to their own modification, which may reduce their inhibitory potential. This was directly addressed by probing the *in vitro* inhibitory effects of recombinant forms of the I κ B proteins on the DNA binding activity of the sustained NF- κ B complexes. The recombinant forms represent the unmodified state and would allow for resolution of the mechanism. The strong *in vitro* inhibitory effects of the recombinant I κ B- α and I κ B- ϵ contrast with their lack of influence on the *in vivo* expression of a NF- κ B-regulated reporter gene. This strongly suggests that I κ B- α and I κ B- ϵ proteins are modified in 1321N1 astrocytoma to states that are ineffectual in modulating NF- κ B. I κ B- β was less effective in inhibiting the *in vitro* DNA binding activity of the sustained NF- κ B complexes. However, this is likely due to its hypophosphorylated state, whereas its capacity to strongly

reduce the induction of the NF- κ B-regulated reporter gene is presumably due to basal phosphorylation of I κ B- β in the cells. The inhibition of the NF- κ B-regulated reporter gene by I κ B- β is incomplete suggesting additional regulatory mechanisms that are independent of I κ B- α , I κ B- β , and I κ B- ϵ .

In summary, it has been demonstrated that IL-1 causes long term, persistent activation of NF- κ B in glial cells. Although the breakdown of I κ B- α and I κ B- ϵ may be involved in the initial transient phases of activation, the prolonged degradation of I κ B- β may be the key step in mediating persistent NF- κ B activation in these cells. Knowledge of this system may be exploited in the development of novel therapeutic agents, and the above findings immediately suggest I κ B- β as a suitable target for an anti-inflammatory regime in glial cells.

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Loss of I κ B- β Is Associated with Prolonged NF- κ B Activity in Human Glial Cells
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