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Loss of IκB-β Is Associated with Prolonged NF-κB Activity in Human Glial Cells*

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

Nuclear factor κB (NF-κB)[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-κB 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-κB 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, IκB (4). Several IκB isoforms have been identified, but the most extensively characterized isoforms are IκB-α, IκB-β, and IκB-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-κB proteins. This association has two functions. First, IκB sequesters NF-κB in the cytosol by masking its nuclear localization signal through direct protein-protein interactions, and second, IκB can prevent NF-κB 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 IκB on two specific N-terminal serines: Ser-32 and Ser-36 on IκB-α (6, 7), Ser-19 and Ser-23 on IκB-β (8), and Ser-18 and Ser-22 on IκB-ε (9). Such signal-induced phosphorylation is believed to be carried out by the newly discovered IκB kinases (10–14) and represents a signal for ubiquitin conjugation of IκB, followed by degradation via the 26 S proteasome. This allows translocation of active NF-κB 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 IκBs, 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 IκB-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 IκB-α (20) and Ser-313 and Ser-315 in IκB-β (21). This casein kinase II-mediated phosphorylation is required for efficient turnover of free IκB-α and IκB-β but is not involved in signal-induced IκB phosphorylation. The mechanism of degradation following this basal phosphorylation has yet to be characterized.

Following stimulation, the nature of NF-κB 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-κB (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 lymphotrophic virus 1 Tax protein tends to result in long term persistent activation of NF-κB, which has been shown to last for up to 48 h (8, 22). Interestingly, these studies reported that all stimuli of NF-κB activation cause degradation of IκB-α, whereas IκB-β is only degraded by those inducers that cause long term persistent activation of NF-κB. Preliminary studies on the recently discovered IκB-ε indicate that this isoform may also be degraded by all inducers of NF-κB (9). Hence, it has been suggested that differential patterns of degradation of the IκB isoforms represent an important mechanism in the regulation of NF-κB activation.

Previous work has shown that NF-κB 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-κB, nuclear factor κB; 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.

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NF-κB was not resolved. This is of clinical relevance, because the sustained activation of NF-κB 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 IκB isoforms (α, β, and ϵ) in regulating the temporal activation of NF-κB in glial cells. We show that prolonged disappearance of IκB-β in response to IL-1 underlies concurrent sustained activation of NF-κB, and we provide direct evidence for the first time that NF-κB in glial cells is subject to selective inactivation by the β form of the IκB 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-1β was purchased from R&D Systems Europe (Oxon, UK). T4 polynucleotide kinase, the 22-bp oligonucleotide containing the NF-κB consensus sequence (*underlined*) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were supplied by Promega (Madison, WI). [γ-^{32}P]ATP, [α-^{32}P]dATP, and nylon transfer membranes were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Rabbit polyclonal antibodies against IκB-α and IκB-β were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Affinity-purified rabbit polyclonal antiserum against IκB-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 IκB-α and IκB-β were generously provided by Dean Ballard (Vanderbilt University, Nashville, TN). The pcDNA3-IκB-ϵ plasmid was generously provided by Simon T. Whiteside (Pasteur Institute, Paris, France). The NF-κB-luciferase reporter construct (consisting of five tandem κB 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′-CGTAACACTGGCATCGTG-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 IκB-α, IκB-β, and IκB-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 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 DMEM. IL-1β 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-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. 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 21,000 × *g* for 10 min and 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 21,000 × *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 phenylmethylsulfonyl fluoride and incubated for 15 min on ice. Incubations were then centrifuged at 21,000 × *g* for 10 min, and the supernatants were transferred to 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 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 μg of protein) were incubated with 20,000 dpm of a 22-bp oligonucleotide containing the NF-κB consensus sequence, which previously had been labeled with [γ-^{32}P]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 μg of poly(dI-dC), and 1 mg/ml nuclease-free BSA. In the supershift analysis, polyclonal antisera (1 μl) against the NF-κB 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. IκB-α, IκB-β, and IκB-ϵ were examined for their modulation of the DNA binding activity of NF-κB by incubating the extracts in the absence and presence of recombinant forms of IκB (1 μg) 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 μg of protein) were boiled in an equal volume of 2× sample buffer for 5 min prior to loading. 2× 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) bromphenol 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 IκB-α or IκB-β (1 μg/ml) or antiserum against IκB-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 μg 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 μJ/cm^2 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× SSC, 5× Denhardt's solution, 1% (w/v) SDS, and 100 μg/ml salmon sperm DNA. Radiolabeling of IκB-α, IκB-β, IκB-ϵ, and human β-actin cDNA probes was achieved by nick translation with [α-^{32}P]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× SSC containing 0.1% (w/v) SDS and twice with 0.2× SSC containing 0.1% (w/v) SDS at room temperature (low stringency). Two further washes were carried out using 0.2× SSC/0.1% (w/v) SDS at 42 °C (medium stringency), followed by two final washes with 0.1× SSC/0.1% (w/v) SDS at 68 °C (high stringency). High stringency washes were carried out on the IκB-α, IκB-β, and β-actin blots. IκB-ϵ was detected under medium stringency conditions. The blots were then rinsed in 2× SSC, covered in UV-transparent wrap, and autoradiographed at −70 °C for 72 h.

Transient Transfections—The pCMV4-IκB-α, pCMV4-IκB-β, and pcDNA3β-IκB-ϵ constructs were described previously (8, 9). For control transfections, the empty pCMV4 vector was used. Human 1321N1 astrocytoma were seeded at 1×10^5 cells/ml and allowed to grow until confluent. Cells were trypsinized, and 1×10^7 cells were transfected by electroporation (using the Invitrogen Electroporator II) with 3 μg of NF-κB luciferase construct and 6 μg of either pCMV4, pCMV4-IκB-α, pCMV4-IκB-β, or pcDNA3β-IκB-ϵ. As an internal control for transfection efficiency, 2 μg of pHook-β-gal plasmid was co-transfected for all experiments. Aliquots (3 ml) of cells were then seeded into 6-well plates (4×10^5 cells/well), and the cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. Cells were then treated with or without IL-1β (10 ng/ml), and cellular extracts were prepared 12 h later using reporter lysis buffer (Promega). Extracts were centrifuged at 21,000 × *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-κB in Glial Cells—Nuclear extracts were prepared from unstimulated and IL-1β-treated human 1321N1 astrocytoma and as-

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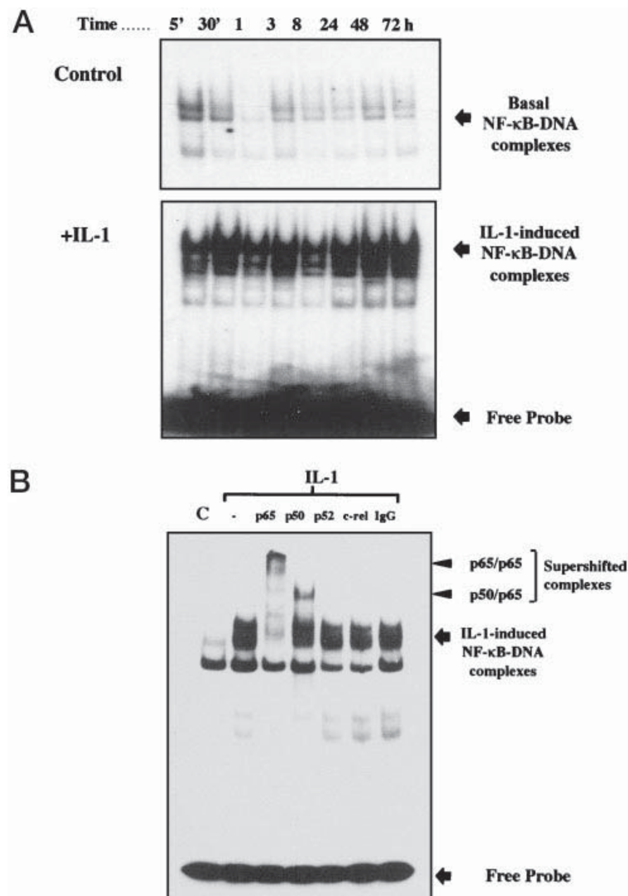

FIG. 1. **Rapid and persistent activation of NF-κB in 1321N1 astrocytoma cells in response to IL-1.** 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. *A*, cells were treated in the absence (*upper panel*) and presence (*lower panel*) of IL-1β (10 ng/ml) for the indicated time periods. Control cells were treated with vehicle (PBS/0.1% (w/v) BSA). Nuclear extracts (4 μg of protein) were assayed for NF-κB·DNA binding activity by EMSA as described under "Experimental Procedures." *B*, 1321N1 cells were incubated in the absence or presence of IL-1β (10 ng/ml) for 1 h, after which nuclear extracts were prepared and assessed for NF-κB binding activity in the absence and presence of polyclonal antisera against the NF-κB 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-κB·DNA complexes activated by IL-1, and basal NF-κB·DNA complexes. The *arrowheads* indicate the mobilities of the supershifted complexes. These results are representative of three independent experiments.

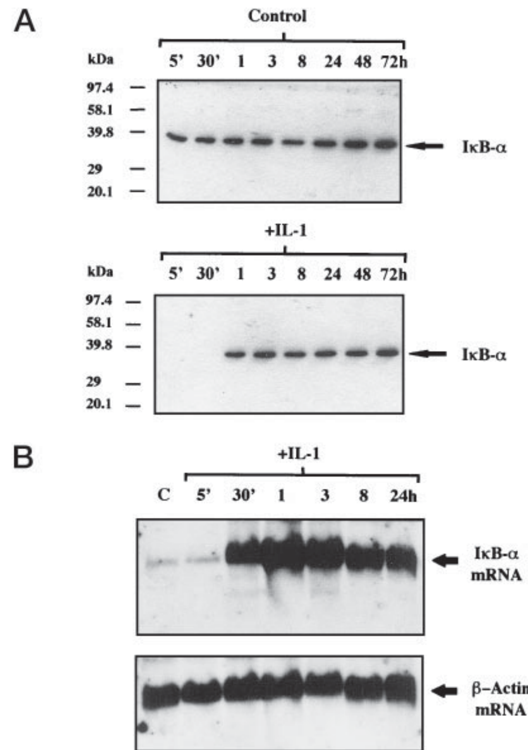

FIG. 2. **Rapid degradation and subsequent resynthesis of IκB-α in response to IL-1.** 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. Cells were then treated with IL-1β (10 ng/ml) for the indicated time periods. Control cells (*C*) were treated with vehicle (PBS/0.1% (w/v) BSA). *A*, cytosolic extracts (20 μg of protein) were subjected to SDS-PAGE and probed by Western immunoblotting with anti-human IκB-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 μg of RNA subjected to 1% (w/v) agarose electrophoresis, blotted onto nylon membrane, and probed with a radiolabeled probe complementary to IκB-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.

sayed for NF-κB activity by EMSA (Fig. 1). Trace levels of basal NF-κB activation were apparent in the form of faint DNA·protein complexes in extracts from unstimulated cells. Rapid and sustained activation of NF-κB was observed in response to stimulation with IL-1β and was represented as the appearance of a NF-κB·DNA complex of slow electrophoretic mobility. NF-κB 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-κB 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-κB·DNA complexes (Fig. 1*B*). 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-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-κB complexes consist primarily of p50·p65 and p65·p65 dimers. Because the levels of NF-κB are controlled by the IκB isoforms, it was decided to probe the effects of IL-1 in relation to the levels of the three IκB isoforms (α, β, and ε) in an attempt to explain the underlying mechanisms to sustained activation of NF-κB in these cells.

IL-1 Causes Rapid Degradation but Subsequent Resynthesis of IκB-α—Cytosolic extracts were prepared from unstimulated and IL-1β-treated human 1321N1 astrocytoma and assessed for IκB-α protein levels by Western blot analysis (Fig. 2*A*). High levels of IκB-α were detectable in cytosolic extracts from unstimulated cells. IκB-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 IκB-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 IκB-α were measured after IL-1 treatment.

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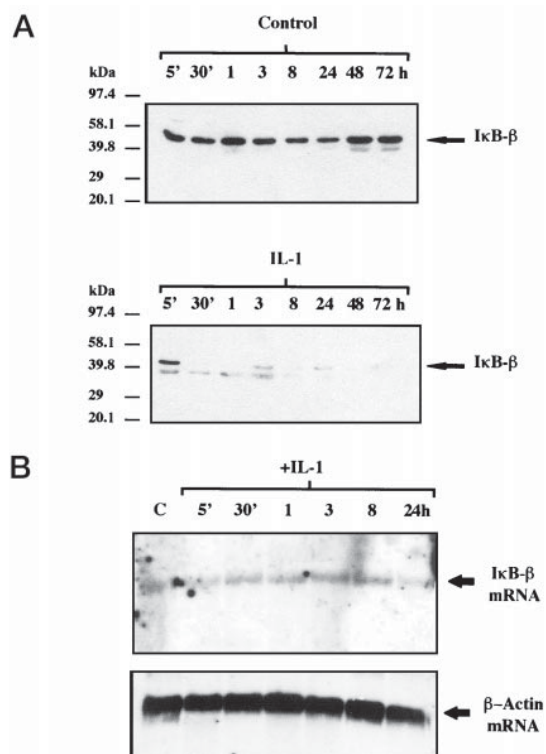

FIG. 3. **IL-1 causes sustained degradation and fails to resynthesize IκB-β.** 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. Cells were then treated with IL-1β (10 ng/ml) for the indicated time periods. Control cells (*C*) were treated with vehicle (PBS/0.1% (w/v) BSA). *A*, cytosolic extracts (20 μg of protein) were subjected to SDS-PAGE and probed by Western immunoblotting with anti-human IκB-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 μg of RNA subjected to 1% (w/v) agarose electrophoresis, blotted onto nylon membrane, and probed with a radiolabeled probe complementary to IκB-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.

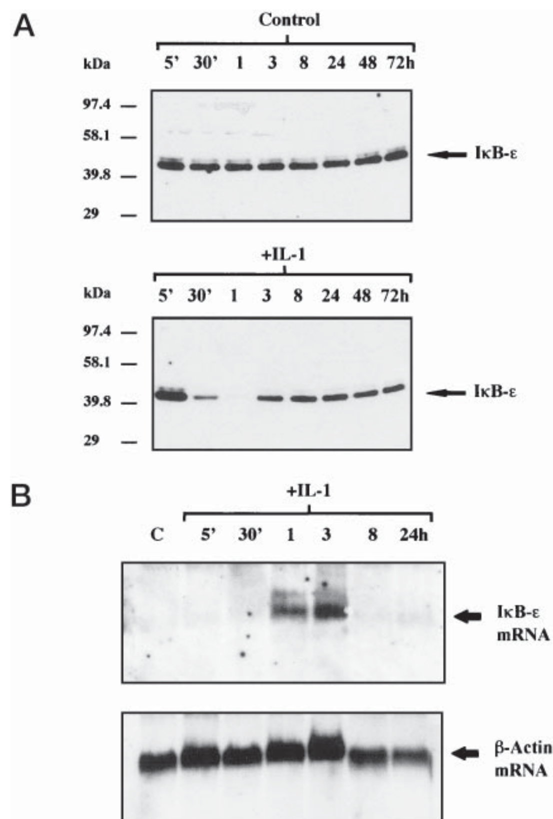

FIG. 4. **Degradation and subsequent resynthesis of IκB-ε in response to IL-1.** 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. Cells were then treated with IL-1β (10 ng/ml) for the indicated time periods. Control cells (*C*) were treated with vehicle (PBS/0.1% (w/v) BSA). *A*, cytosolic extracts (20 μg of protein) were subjected to SDS-PAGE and probed by Western immunoblotting with anti-human IκB-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 μg of RNA subjected to 1% (w/v) agarose electrophoresis, blotted onto nylon membrane, and probed with a radiolabeled probe complementary to IκB-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.

Total RNA extracts were prepared from unstimulated and IL-1β-treated human 1321N1 astrocytoma and assessed for IκB-α mRNA levels by Northern blot analysis (Fig. 2*B*). A very low level of IκB-α mRNA expression was detectable in extracts from unstimulated cells. The expression of IκB-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 IκB-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 IκB-α did not correlate with the sustained activation of NF-κB, suggesting that the other forms of IκB may be more important in regulating NF-κB. The regulation of IκB-β by IL-1 was next examined.

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

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

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

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lation. However, similar to IκB-α, IκB-ε degradation was transient. The amount of IκB-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-1β-treated cells and assessed for IκB-ε mRNA levels by Northern blot analysis (Fig. 4*B*). A very low level of IκB-ε mRNA expression was detectable in extracts from unstimulated cells. The expression of IκB-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 IκB-ε protein in the cytosol of these cells, as detected by Western blot. These findings suggest circumstantially that the IκB-β isoform is the crucial form in controlling the activity status of NF-κB in glial cells. However, the reappearance of IκB-α and -ε with no coincident reduction in NF-κB levels questions the role, if any, of these isoforms in controlling the activity of NF-κB in glial cells. To examine directly the relative contributions of each of the IκB isoforms in controlling NF-κB, genes encoding the three forms were cotransfected with a NF-κB-regulated reporter gene and examined for their inhibitory potential on the induction of the reporter gene.

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

Overexpression of IκB-β Inhibits Transactivation by NF-κB—1321N1 astrocytoma were co-transfected with the plasmids containing cDNAs encoding the various IκB proteins and a κB-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. 5*B*). 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 IκB-α or IκB-ε. Therefore, expression of IκB-α or IκB-ε had no effect on κB-dependent transcription induced by IL-1. In contrast, cotransfection with IκB-β resulted in significant inhibition of IL-1-induced transcription of the luciferase reporter construct. This experiment demonstrates that IκB-β is the only IκB isoform capable of inhibiting the transactivation activity of NF-κB in 1321N1 astrocytoma.

Recombinant IκB Isoforms Inhibit the in Vitro DNA Binding Activity of NF-κB—The inability of overexpressed IκB-α and IκB-ε to inhibit the transactivation of NF-κB coupled to the earlier findings showing that the activation of NF-κB was sustained even in the presence of newly synthesized IκB-α and IκB-ε prompted an investigation into their lack of inhibitory potential. The modification of the NF-κB complexes or IκB-α and IκB-ε were plausible alternative proposals for the lack of inhibition. To resolve these alternative mechanisms the ability of recombinant forms of the three IκB 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

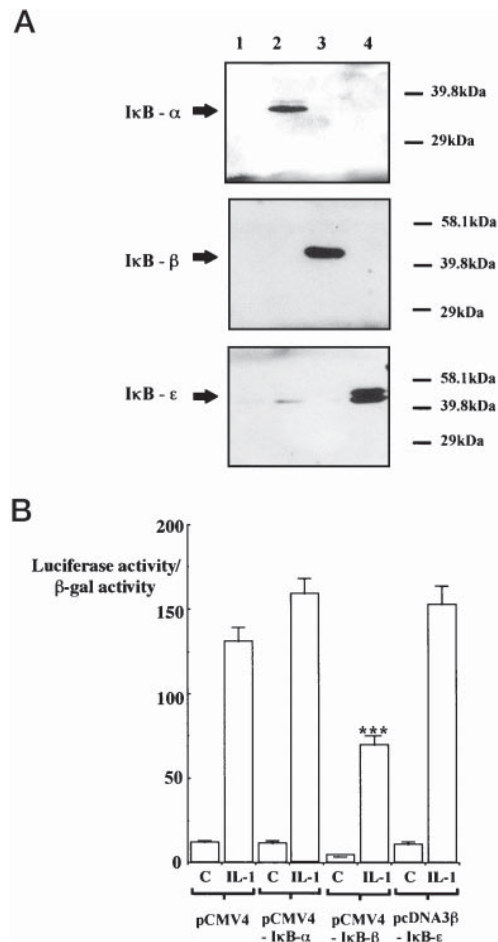

FIG. 5. **Overexpression of IκB-β inhibits transactivation by NF-κB.** 1321N1 astrocytoma (1×10^7 cells) were transfected with 3 μg of NF-κB-luciferase reporter construct and 2 μg of a CMV-driven β-gal reporter construct. In addition to these constructs, the cells were transfected with 6 μg of one of the following: empty pCMV4 vector (*pCMV4*), pCMV4 containing IκB-α (*pCMV4-IκB-α*), pCMV4 containing IκB-β (*pCMV4-IκB-β*), or pcDNA3β containing IκB-ε (*pcDNA3β-IκB-ε*). The cells were allowed to recover for 48 h before stimulation with or without (*C*) IL-1β (10 ng/ml) for a further 12 h. *A*, cell lysates were analyzed for the expression of the IκB proteins by Western immunoblotting. Lysates (0.2 μg of protein) were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed with anti-human IκB-α (*upper panel*), anti-mouse IκB-β (*middle panel*), and anti-human IκB-ε (*lower panel*) polyclonal antibodies. The figure shows extracts from cells transfected with: empty vector pCMV4 (*lane 1*), pCMV4-IκB-α (*lane 2*), pCMV4-IκB-β (*lane 3*), and pcDNA3β-IκB-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 ± 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-κB activity by EMSA in the absence and presence of C-terminal histidine-tagged recombinant forms of IκB-α, IκB-β, and IκB-ε (25). IL-1 caused the typical activation of two NF-κB complexes of slow electrophoretic mobility. Intriguingly, IκB-α and IκB-ε blocked the DNA binding activity of both complexes, whereas IκB-β only inhibited the complex of slowest mobility. This strongly opposes the *in vivo* inhibitory effects of the overexpressed IκB isoforms where the opposite inhibitory profile was observed. The ability of IκB-α and IκB-ε to inhibit the DNA binding of the NF-κB complexes contrasts with their lack of effect when over-

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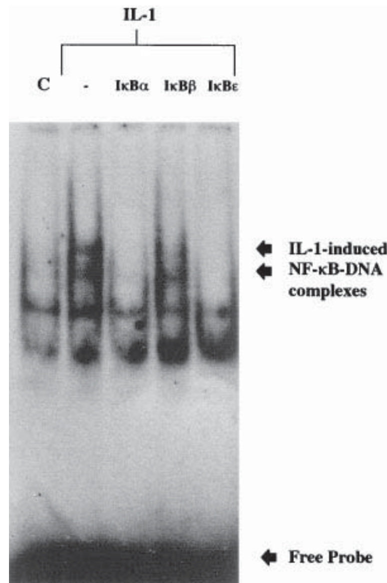

FIG. 6. **IκB-α and IκB-ε but not IκB-β inhibit the DNA binding activity of sustained NF-κB complexes.** 1321N1 astrocytoma were seeded at 2×10^5 cells/ml and grown to confluency. Cells were treated in the absence (*C*) and presence of IL-1β (10 ng/ml) for 48 h, after which nuclear extracts were prepared and assessed for NF-κB binding activity in the absence and presence of C-terminal histidine-tagged recombinant forms of IκB-α, IκB-β, or IκB-ε (1 μg). The *arrows* indicate the mobility of unbound oligonucleotide (*Free Probe*) and NF-κB·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, IκB-α and IκB-ε proteins are modified in some manner that abolishes their inhibitory potential.

DISCUSSION

The regulation of NF-κB activation by the IκB 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-κB 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-κB 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-κB by IL-1 in glial cells is addressed in the present study, and the relative importance of the various IκB isoforms in regulating NF-κB activity is also evaluated.

IL-1β caused rapid and sustained activation of NF-κB in human 1321N1 astrocytoma. This confirms previous studies in this laboratory, which have shown IL-1β to activate NF-κB 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-κB by IL-1 was also observed (8, 22). This persistent activation of NF-κB prompted an investigation of the mechanisms underlying the regulation of NF-κB in these cells concentrating specifically on the role of the different IκB isoforms.

The effect of IL-1 on the intracellular levels of IκB was initially assessed. IκB-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 IκB-α is due to increased transcription of the gene encoding IκB-α, because Northern analysis showed significant increases in the levels of IκB-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 IκB-α levels were maintained in the cell. These results are consistent with previous studies, which indicate that IκB-α regulates NF-κB through a novel autoregulatory feedback loop (29–34). Although this model explains the activation of NF-κB by agents that cause transient activation, it fails to explain the persistent activation of NF-κB, observed in response to IL-1 stimulation in the present study. It appears that IκB-α is unable to shut down the NF-κB response in this case, because NF-κB remains fully active even after cellular IκB-α levels have returned to normal. It was apparent from this observation that IκB-α was not the sole regulator of NF-κB activation in response to IL-1, which led to examination of the second IκB isoform, IκB-β.

IκB-β shares many common properties with IκB-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-κB (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 IκB-β degradation in glial cells, accompanied by persistent long term activation of NF-κB. Degradation of IκB-β occurred with slightly slower kinetics than IκB-α, which is in agreement with previous observations in B- and T-cells (8, 22). Once phosphorylated, IκB is quickly ubiquitinated and degraded. Thus, the efficiency of phosphorylation dictates how rapidly IκB degradation occurs. IκB-β has been shown to be a less-efficient substrate for the IκB kinase subunits when compared with IκB-α, therefore, it has been proposed that the slower rate of degradation of IκB-β, is due to a slower rate of phosphorylation (10, 11).

Unlike IκB-α, the degradation of IκB-β was not followed by its resynthesis. As already outlined, the rapid reappearance of the IκB-α protein is due to NF-κB-directed up-regulation of the IκB-α gene. Although the *cis*-acting elements that regulate the IκB-β gene remain undefined, recent reports suggest that this transcriptional unit is not under NF-κB control (8, 22). Indeed, this was confirmed in the present study with the observation that IκB-β mRNA transcription was unaffected by stimulation with IL-1. These findings establish a strong correlation between the chronic down-regulation of IκB-β protein expression and the failure of IL-1 to induce IκB-β gene transcription. The slow but persistent degradation of IκB-β observed in response to IL-1 stimulation in these cells suggests that IκB-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 IκB to be examined in this study was the recently discovered, IκB-ε (9, 36). IκB-ε is similar in structure to IκB-α and IκB-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 IκB-ε displayed its own unique pattern of degradation in response to IL-1 stimulation. IκB-ε was degraded in 1321N1 astrocytoma with relatively slow kinetics, a characteristic shared with IκB-β. But like IκB-α, IκB-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 IκB-ε mRNA transcription upon stimulation with IL-1. The short period of time between degradation and resynthesis indicates that, like IκB-α, IκB-ε is tightly regulated in these cells. Whiteside *et al.* (9) reported similar kinetics for the degradation of IκB-ε in Jurkat cells in response to LPS. This group proposed

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

The kinetics of degradation and resynthesis of the various IκB isoforms observed in this study suggests that the inability of NF-κB to up-regulate transcription of the IκB-β gene transcription is the key to the persistent activation of NF-κB in glial cells. It appears that the resulting long term down-regulation of IκB-β protein synthesis allows NF-κB released from NF-κB·IκB-β complexes to remain fully active for long periods of time. However, this can only be achieved if the NF-κB released from IκB-β is resistant to the newly synthesized IκB-α and IκB-ε. In B-cells, such resistance is achieved by association of NF-κB with a modified form of IκB-β (37). The initial degradation of IκB-β in B-cells in response to stimulation with LPS is followed by resynthesis of the protein in a hypophosphorylated form. This newly synthesized IκB-β associates with free NF-κB, preventing it from interacting with IκB-α. Unlike basally phosphorylated IκB-β, unphosphorylated IκB-β is unable to mask the nuclear localization signal or DNA-binding domains of NF-κB. Therefore, the NF-κB bound to unphosphorylated IκB-β can still enter the nucleus and remains transcriptionally active. Due to its association with hypophosphorylated IκB-β, NF-κB is resistant to inhibition by IκB-α, allowing it to remain persistently active. However, the present study demonstrates that IκB-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 IκB-β to inhibit *in vitro* the DNA binding activity of one of the IL-1-activated NF-κB complexes is likely due to its unphosphorylated state. This is further discussed below.

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

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

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Loss of IκB-β Is Associated with Prolonged NF-κB Activity in Human Glial Cells
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