RESEARCH COMMUNICATION

Interleukin-1 activates transcription factor NFκB in glial cells

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Recombinant human interleukin-1 (IL-1) α and β were found to activate a latent cytosolic form of the transcription factor NFκB in rat C6 glioma. IL-1β was 10 times more potent than IL-1 α for this activity and both were inhibited by the IL-1 receptor antagonist. The activation was detectable from 20 min and remained sustained for up to 24 h. The electrophoretic mobility of the activated complex was shown to be different from that of the corresponding complexes in another IL-1-responsive cell line, the murine thymoma line EL4.NOB-1. C6 cells, when transiently transfected with five NFκB consensus sequence repeats linked to the reporter gene chloramphenicol acetyltransferase (CAT), demonstrated increased CAT activity in response to IL-1β treatment. The activation of NFκB in glial cells may thus represent an early step in IL-1 signalling in brain and is likely to have consequences for IL-1-induced gene expression in these cells.

INTRODUCTION

Interleukin 1 (IL-1) is a cytokine with a range of biological activities which suggest that it is a key mediator of inflammation [1]. Several of these activities are due to the effects of IL-1 in the brain. In addition to regulation of the febrile process, intracerebroventricular administration of IL-1 induces acute-phase-glycoprotein synthesis, slow-wave sleep, loss of appetite and sickness syndrome [2]. Such physiological effects of IL-1 suggest that it can regulate brain-cell function. However, the molecular mechanism by which IL-1 can activate cells of neuronal and glial origin remains to be elucidated.

IL-1 is a potent stimulator of the transcription factor NFκB in other cells and serves to regulate a set of genes encoding immunoreceptors, cytokines and viral proteins [3,4]. NFκB contacts its decameric DNA recognition sequence as a heterodimer consisting of two DNA-binding subunits of 50 kDa (p50) and 65 kDa (p65) [5]. This heterodimer is constitutively present and active in the nucleus only in a restricted subset of cells such as B cells [6], certain T-cell lines [7] and monocytes [8]. NFκB is also found in other cell types, but in an inactive form in the cytosol, where it is associated with an inhibitory protein, IκB, which prevents its translocation to the nucleus [9,10]. Activation of cells by various agents (including IL-1) leads to the dissociation of IκB, releasing an active heterodimer, which is translocated into the nucleus [4]. Cell-free activation of latent NFκB has also been achieved by treating cytosolic fractions with sodium deoxycholate, thus allowing detection of active NFκB [9].

It was envisaged that some of the central effects of IL-1 may be mediated by activation of brain-cell transcription factors, possibly NFκB. The present work thus assessed the ability of IL-1 to activate NFκB in brain cells using the rat C6 glioma cell line as a model cell. The novel finding of NFκB and its activation by IL-1 in C6 cells are described and compared with an immune cell represented by the murine thymoma EL4.NOB-1 cell line. The ability of IL-1 to increase the expression of a reporter gene under the control of an NFκB-containing promoter region demonstrates the functional significance of IL-1-activated NFκB in C6 cells. The results may suggest a basis for the various effects mediated by IL-1 in different cell types and may act as a first step in dissecting the signalling pathway of IL-1 in brain.

MATERIALS AND METHODS

Materials

The rat glioma C6 and murine thymoma EL4.NOB-1 lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.). RPMI 1640, Dulbecco's modified Eagle's medium (DMEM) and foetal-calf serum (FCS) were from Greiner (Frickenhausen, Germany). Trypsin was from GIBCO (Grand Island, NY, U.S.A.). Recombinant human IL-1α and 1β were gifts from Dr. Jeremy Saklatvala (Strangeways Research Laboratory, Cambridge, U.K.). Recombinant human IL-1 receptor antagonist (IL-1RA) was given by Dr. Robert Thompson (Synergen Inc., Boulder, CO, U.S.A.). T4 polynucleotide kinase, the 22 bp oligonucleotide containing the NFκB consensus sequence (underlined) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and the 22 bp oligonucleotide containing the consensus sequence (underlined) for the immunoglobulin enhancer octanucleotide factor-1 (OCT1) (5′-TGTCGAATGCAAATCACTAGAA-3′) were from Promega (Madison, WI, U.S.A.). [γ-^{32}P]ATP (10 mCi/mmol), [^{14}C]chloramphenicol (54 mCi/mmol) and poly(dI-dC) were from Amersham International (Amersham, Bucks., U.K.). Nonidet P40, nuclease-free BSA, sodium deoxycholate and DEAE-dextran were from Sigma (Poole, Dorset, U.K.). The pCAT Promoter plasmid in which five NFκB sequence motifs had been cloned into the promoter region of the chloramphenicol acetyltransferase (CAT) gene was a gift from Dr. Timothy Bird (Immunex Research and Development Corporation, Seattle, WA, U.S.A.).

Cell culture

The rat glioma C6 cell line was cultured in DMEM containing 10% FCS, and the murine thymoma EL4.NOB-1 cell line was cultured in RPMI 1640 containing 10% FCS. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. C6 glioma cells were passaged using 0.25% trypsin in DMEM. Both

Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FCS, foetal-calf serum; HIV-1, human immunodeficiency virus type 1; IL-1, interleukin-1; IL-1RA, interleukin-1 receptor antagonist; OCT1, octanucleotide factor-1; PMSF, phenylmethanesulphonyl fluoride.

cell types were stimulated during their exponential growth phase in serum-containing medium at 37 °C for all experiments.

Preparation of subcellular fractions [11]

C6 and EL4.NOB-1 cells [$(0.5–1) \times 10^6$ cells/ml] were stimulated with various concentrations of IL-1α and 1β respectively for different time periods. In some experiments cells were pretreated with IL-RA for 1 h before IL-1 α/β treatment. Stimulation was terminated by the addition of 5 vol. of ice-cold PBS, followed by centrifugation of the cells at 1500 *g* for 5 min. It was necessary to scrape off the C6 cells prior to centrifugation.

Pelleted cells [$(1.5–5.0) \times 10^6$ cells] were washed in 1 ml of hypo-osmotic buffer [10 mM Hepes/NaOH buffer, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethanesulphonyl fluoride (PMSF)] by resuspension and centrifugation at 14000 *g* for 10 min. Cells were then lysed for 10 min on ice in hypo-osmotic buffer (20 ml) containing 0.1 % (v/v) Nonidet P40. Lysates were centrifuged at 14000 *g* for 10 min. Cytosolic extracts were prepared from the supernatants by adding 300 mM Hepes/NaOH buffer, pH 7.9 (18 μl), containing 1.4 M KCl and 30 mM $MgCl_2$, followed by 176 μl of water, centrifuging at 100000 *g* for 1 h and dialysing the resulting supernatants overnight against 10 mM Hepes/NaOH buffer, pH 7.9 (20 ml), containing 50 mM KCl, 0.2 mM EDTA, 20 % (w/v) glycerol, 0.5 mM PMSF and 0.5 mM DTT. The dialysis residue constituted the cytosolic extract. The pellets from the lysis step were resuspended in 20 mM Hepes/NaOH buffer, pH 7.9 (15 ml), 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. Incubation mixtures were then centrifuged at 14000 *g* for 10 min and the supernatants removed into the above dialysis buffer (75 ml). Nuclear extracts were thus obtained. Protein concentrations of cytosolic and nuclear extracts were determined [12] and the extracts assayed immediately for NFκB activity or stored at −70 °C until further use. All of the steps in the above procedure were performed at 4 °C unless otherwise stated.

Electrophoretic-mobility-shift assay

Nuclear and cytosolic extracts (4 μg of protein) were incubated with 10000 c.p.m. of a 22 bp oligonucleotide containing the NFκB consensus sequence which had been previously labelled with [γ-^{32}P]ATP (10 mCi/mmol) by T4 polynucleotide kinase [13]. Incubations were performed for 30 min at room temperature in the presence of 2 μg of poly(dI-dC) and 100 mM Tris/HCl buffer, pH 7.5, containing 1 M NaCl, 10 mM EDTA, 50 mM DTT, 40 % (w/v) glycerol and 1 mg/ml nuclease-free BSA. In some experiments, unlabelled oligonucleotides containing either the NFκB or the OCT1 consensus sequence (1.75 pmol each) were added to the extracts before incubating with labelled oligonucleotide. Cell-free activation of latent NFκB activity required pretreatment of extracts (4 μg of protein) with 0.8 % (w/v) sodium deoxycholate for 10 min on ice before incubation as above with the labelled oligonucleotide probe. The latter incubation mixtures also included 1.1 % (w/v) Nonidet P40. All incubation mixtures were subjected to electrophoresis on native 4 % (w/v) polyacrylamide gels which were subsequently dried and autoradiographed.

DNA transfection

C6 cells (1×10^6 cells) were plated into 60 mm dishes in 5 ml of DMEM containing 10 % (v/v) FCS and allowed to adhere for 20 h. The medium was removed, and the adherent cells washed with 5 ml of cold (4 °C) DMEM. The pCAT Promoter plasmid with an insert of five NFκB repeats (1 μg of DNA) was then added to the washed cells in 1 ml of DMEM containing 0.2 mg/ml DEAE-dextran and incubated for 1 h at 37 °C. The cells were subsequently shocked for 30 s by addition of 10 % (v/v) glycerol in DMEM (1 ml) and the shock was terminated with cold DMEM (5 ml). The medium was removed and the cells washed twice with 5 ml of cold DMEM before addition of 5 ml of DMEM supplemented with 10 % (v/v) FCS. The cells were incubated for 20 h at 37 °C and treated for a further 24 h in the absence or presence of IL-1β (10 ng/ml) before harvesting of cells for CAT activity determinations. In some experiments, cells were pretreated with IL-1RA (5 μg/ml) for 1 h before IL-1β treatment.

CAT assays

Medium was removed from cells, which were then washed twice with 5 ml of PBS. The cells were scraped into PBS (1 ml) and pelleted by centrifugation at 14000 *g* for 5 min. Pellets were suspended in 0.25 M Tris/HCl buffer, pH 8 (100 μl) and subjected to three cycles of freezing in liquid N_2 and thawing at 37 °C. Cell extracts were separated from debris by centrifugation as described above and their protein concentrations determined [12]. Extracts (100 μg of protein) were incubated for 2 h at 37 °C in 0.25 M Tris/HCl buffer, pH 8 (61 μl) containing 1 mM acetyl-CoA and 0.2 μCi of [^{14}C]chloramphenicol (54 mCi/mmol). Reactions were stopped by addition of 350 μl of ethyl acetate, vortex-mixed for 30 s and the phases separated by centrifugation at 14000 *g* for 1 min. The upper phase was removed, dried under vacuum and suspended in ethyl acetate (12 μl). Acetylated and non-acetylated products were resolved by silica-gel t.l.c. in chloroform/methanol (19:1; v:v). The separated products were revealed by autoradiography and their respective regions on the tlc plate isolated before liquid-scintillation counting.

RESULTS

Nuclear extracts were prepared from unstimulated and IL-1β-treated C6 glioma and assayed for NFκB activity. Trace levels of constitutive NFκB were apparent in the form of two faint DNA–protein complexes in extracts from untreated cells (Figure 1). Rapid and sustained activation of substantial amounts of NFκB activity was apparent in response to IL-1β. IL-1β-induced

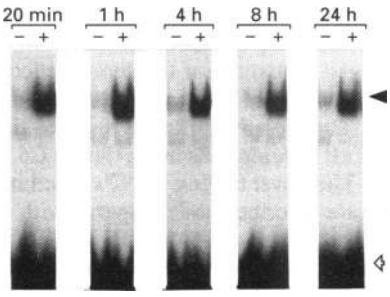

Figure 1 Time course of induction of NFκB by IL-1β in C6 glioma

Cultures of C6 cells (4×10^5 cells/ml) were incubated in the absence (−) or presence (+) of IL-1β (10 ng/ml) for the indicated times, after which nuclear extracts were prepared and assayed for NFκB binding activity as described in the Materials and methods section. The filled arrowhead (◀) indicates IL-1β-induced DNA–protein complexes and the open arrowhead (◁) indicates unbound DNA fragments.

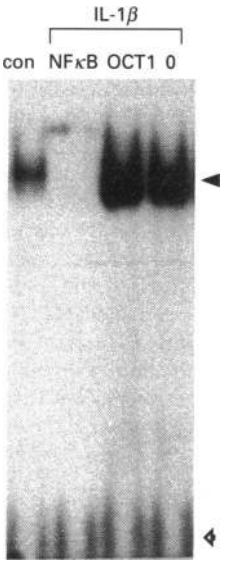

Figure 2 Specific inhibition of DNA–protein complex formation by NFκB sequence motif

C6 cells (4×10^5/ml) were stimulated with IL-1β (10 ng/ml) for 1 h, and nuclear extracts were prepared and assayed for DNA–protein complex-formation in the absence (0) or presence of unlabelled oligonucleotides (1.75 pmol) containing the NFκB or the OCT1 consensus sequences as described in the Materials and methods section. Nuclear extracts from unstimulated cells (con) were also assayed in the absence of these oligonucleotides. The filled arrowhead (◀) indicates IL-1β-induced DNA–protein complexes and the open arrowhead (◁) indicates unbound DNA fragments.

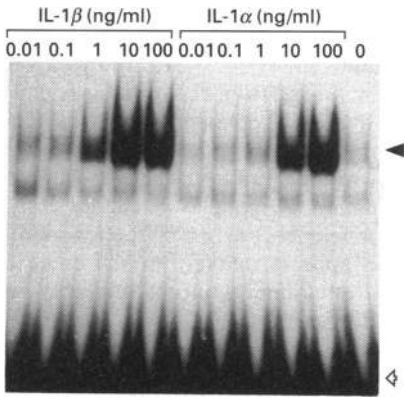

Figure 3 Dose–response relationship of IL-1α and IL-1β induction in C6 glioma cells

Cultures of C6 cells (4×10^5 cells/ml) were incubated with various concentrations of IL-1α or IL-1β for 1 h and the nuclear extracts subsequently assessed for NFκB induction as described in the Materials and methods section. The filled arrowhead (◀) indicates IL-1-induced DNA–protein complexes and the open arrowhead (◁) indicates unbound DNA fragments.

activity was detectable at 20 min in the form of a single DNA–protein complex and remained for at least 24 h. The DNA–protein interaction was inhibited by addition of unlabelled oligonucleotide, containing the NFκB binding site, to nuclear extracts before incubation with labelled probe (Figure 2). An unlabelled oligonucleotide containing the OCT1 sequence motif

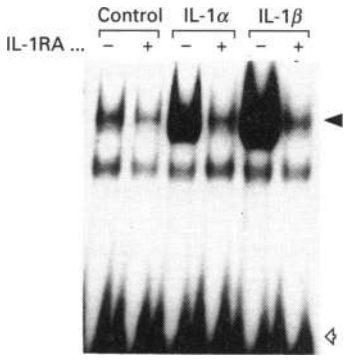

Figure 4 Inhibition of IL-1-induced NFκB in C6 glioma by IL-1RA

C6 cells (4×10^5 cells/ml) were incubated in the absence (−) or presence (+) of IL-1RA (5 μg/ml) for 1 h at 37 °C before stimulation with IL-1α (10 ng/ml) or IL-1β (10 ng/ml) for 1 h. Nuclear extracts were subsequently prepared and analysed for NFκB activity as described in the Materials and methods section. Control samples represent nuclear extracts from unstimulated cells. The filled arrowhead (◀) indicates IL-1 induced DNA–protein complexes and the open arrowhead (◁) indicates unbound DNA fragments.

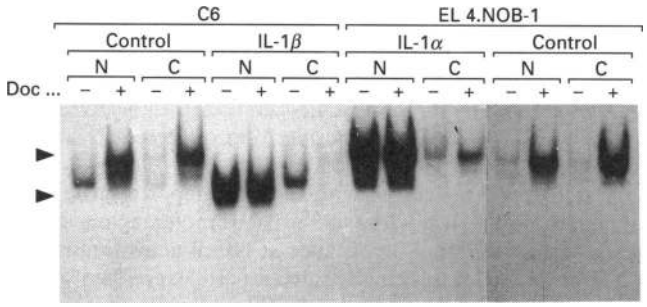

Figure 5 Effect of IL-1 stimulation on the subcellular distribution of NFκB in C6 glioma and EL4.NOB-1 thymoma

Cultures of C6 (4×10^5 cells/ml) and EL4.NOB-1 (1×10^6 cells/ml) were stimulated with IL-1β (10 ng/ml) and IL-1α (10 ng/ml) for 1 h at 37 °C. Control cells were left unstimulated. Nuclear (N) and cytosolic (C) extracts were prepared and left untreated (−) or subjected to deoxycholate (Doc) treatment (+) before examination for NFκB activity as described in the Materials and methods section. The arrowheads (►) indicate induced DNA–protein complexes. The free DNA probe is not shown.

failed to exhibit any such inhibitory effect. The two forms (α and β) of human recombinant IL-1 were assessed for their relative abilities to stimulate NFκB activity (Figure 3). IL-1β was approx. 10 times more effective than IL-1α. The effect of IL-1RA on the induction of NFκB by both forms of IL-1 in C6 glioma was next studied (Figure 4). Pretreatment of cells with IL-1RA abolished the ability of both IL-1α and 1β to activate NFκB. IL-1RA alone did not induce NFκB activity at the concentration used.

Cytosolic and nuclear extracts were examined for NFκB activity after cellular or cell-free activation in order to characterize the state and location of NFκB in stimulated and unstimulated cells (Figure 5). Such studies were performed on both C6 glioma and the murine thymoma EL4.NOB-1 for comparison purposes. Both cell types, when unstimulated, demonstrated little or no NFκB activity in either the nuclear or cytosolic extracts. However, addition of sodium deoxycholate to these samples revealed latent activity in all cases. Upon IL-1 stimulation of cells, NFκB activity was strongly increased in the nuclear extracts of both cells, even in the absence of deoxycholate. The cytosolic extracts from stimulated cells showed only traces

Table 1 Effect of IL-1β on CAT expression in C6 glioma cells

C6 cells (1×10^6) were transfected with a pCAT Promoter plasmid with an insert of five NFκB repeats (1 μg of DNA) in its promoter region. Transfected cells were incubated in the absence or presence of IL-1RA (5 μg/ml) for 1 h at 37 °C before stimulation without (Control) and with IL-1β (10 ng/ml) for 24 h. Cell extracts (100 μg of protein) were subsequently prepared and assayed for CAT activity as described in the Materials and methods section. CAT activity is expressed as percentage of chloramphenicol recovered as an acetylated form. Each value represents the mean ± S.E.M. for triplicate determinations.

Conditions	Acetylation (%)
Control	1.69 ± 0.28
IL-1	6.38 ± 0.69
IL-1 + IL-1RA	1.63 ± 0.26
IL-1RA	1.43 ± 0.13

of activity, which was reduced in C6 cells and increased only slightly in EL4.NOB-1 cells in response to deoxycholate treatment. Indeed, both cell types appear to be significantly depleted of latent cytosolic NFκB activity upon cellular activation of IL-1. The electrophoretic mobility of the DNA–protein complex induced by IL-1β in C6 cells differs from that of the complexes induced by IL-1α in EL4.NOB-1 cells. The major complex induced by IL-1α in EL4.NOB-1 cells displayed a similar mobility to the complexes resulting from cell-free activation of the two cell types by deoxycholate.

C6 cells were transfected with the CAT reporter gene which contained five NFκB-binding sites in its promoter region, in order to assess the functional significance of NFκB activation in these cells. Transfected cells demonstrated a basal expression of CAT activity which was increased 3.8-fold upon IL-1β treatment (Table 1). Pre-treatment of transfected cells with IL-1RA abolished the efficacy of IL-1β. IL-1RA alone did not affect basal CAT activity.

DISCUSSION

It is well known that IL-1 induces the transcription factor NFκB in many cell types [3,4], but up until now, such induction has not been studied in any brain cells. The present work describes the novel characterization of IL-1-induced activation of NFκB in a glial-cell line. NFκB activity was defined as that component which formed a complex with a short radiolabelled oligonucleotide containing the recognition 5′-GGGACTTTCC-3′. The failure of a similarly sized oligonucleotide, lacking such a sequence (e.g. an oligonucleotide containing the OCT1 recognition sequence) to inhibit the formation of the protein–DNA complex demonstrated the specificity of the interaction.

IL-1β was approx. 10 times more potent than the α-form with respect to activation of NFκB in C6 cells. This is consistent with previous studies which reported human recombinant IL-1β to be more effective than the α-form in eliciting cellular and systemic responses using rat model systems [14,15]. The greater potency of IL-1β has been proposed to reflect its higher affinity for IL-1 receptors in the rat. The IL-1 activation of NFκB in the present study appears to be receptor-mediated, since the stimulatory effects of the two forms of IL-1 are inhibited by human IL-1RA. It is likely that the type I receptor is responsible, as has been shown in T- and B-cells [16].

Cell-free activation studies reveal that unstimulated C6 cells contain latent forms of NFκB in both the cytosol and the nucleus. However, latent activity in the latter fraction is probably due to cytosolic contaminants, as determined by lactate dehydrogenase measurements (results not shown). Cellular stimulation by IL-1β appears to cause activation of the latent cytosolic form, which subsequently translocates into the nucleus. This is based on the observation that the cytosols from IL-1-stimulated cells contain only trace amounts of activated NFκB and little or no latent activity, whereas the nuclear extracts demonstrate substantial activity, even in the absence of deoxycholate. Interestingly, deoxycholate tended to decrease slightly the amounts of already-activated NFκB in IL-1-stimulated C6 cells. The nature of the susceptibility of preactivated NFκB to deoxycholate is not understood, but suggests that the active form of NFκB manifested from cell-free activation by deoxycholate is not the same as that resulting from cellular activation by IL-1. Indeed, further evidence is provided for this proposal in the different electrophoretic mobilities of the induced complexes resulting from the two forms of activation. Such mobility differences may represent distinct subunit compositions. Thus, on a speculative note, the lower-mobility complex apparent upon deoxycholate treatment may represent a p50–p65 heterodimer, whilst the higher-mobility complex, induced by IL-1β, may constitute a p50–p50 homodimer, which has been observed previously [17]. However, previous work [4] would suggest that the concentration of poly(dI-dC) used in the present study would tend to preclude detection of the homodimer–oligonucleotide complex by the band-shift assay, and thus novel, as yet uncharacterized, forms of NFκB may be present.

Unstimulated EL4.NOB-1 cells, like C6 cells, appear to contain latent forms of NFκB in both the nucleus and the cytosol. However, latency in the nuclear fraction was probably due to cytosolic contamination (as in the case of C6 cells). Cellular stimulation of EL4.NOB-1 cells by IL-1α apparently activates the latent cytosolic form, which subsequently translocates to the nucleus. However, unlike C6, the active form of NFκB resulting from cell-free activation by deoxycholate appears to be the same as the major form arising from IL-1 cellular stimulation, at least with respect to their electrophoretic mobilities. This may reflect distinct forms or differential processing of NFκB in the two cell types, expecially with respect to the active forms revealed by IL-1 cellular stimulation. Although one cannot exclude the possibility that such differences are merely species-specific differences, such a scenario appears unlikely in the context of previous reports demonstrating high sequence similarity between NFκB subunits from different species [17,18]. C6 and EL4.NOB-1 cells also display differential regulation of IL-1-induced NFκB activity. The present study demonstrates the rapid and sustained IL-1 activation of NFκB in C6 cells, which contrasts with previous reports describing transient activation in EL4.NOB-1 [16,19] cells. If C6 and EL4.NOB-1 cells are valid models for assessing the effects of IL-1 in the central nervous system and the immune system respectively, the different NFκB species and their distinct regulation by IL-1 in the two cell types may form a basis for differential IL-1 signalling in the brain and the immune system.

C6 cells, when transfected with a CAT reporter gene whose expression is regulated by NFκB-binding sites in its promoter region, showed basal levels of CAT activity even in the absence of IL-1β. Such expression of CAT is probably driven by constitutive levels of NFκB. The IL-1β-induced CAT expression confirms the functional nature of the NFκB activated by IL-1β in C6 cells, at least with respect to its ability to increase the expression of a reporter gene. However, more generally one would predict that NFκB may regulate the expression of those genes in glial cells which contain binding sites for this tran-

scription factor as part of their promoter regions. The inhibitory nature of IL-1RA on the ability of IL-1β to induce CAT expression implies a receptor involvement which was predicted on the basis of IL-1RA antagonism of the activation of NFκB.

The activation of NFκB by IL-1 may be of substantial physiological importance in brain, especially with respect to immunomodulation and inflammation, since most of the target genes for NFκB so far described are those encoding cytokines and their receptors and cell-adhesion molecules [20]. Indeed, whilst the present study was in progress, a report was published describing the likely significance of NFκB in mediating IL-1-induced IL-6 gene expression in astrocytes [21]. However, NFκB may also be involved in pathological conditions in the brain, most notably expression of human immunodeficiency virus type 1 (HIV-1). An NFκB-binding site exists in the enhancer region of HIV-1 [22], and it may be that latent provirus in glia exploits the activation of NFκB for increased viral expression. Indeed, the progression of AIDS (acquired immunodeficiency syndrome) correlates well with the presence of NFκB-activating agents in patients [23]. Thus manipulation of the activation of NFκB in brain may serve an important role in controlling IL-1-induced physiological and pathological conditions. The present study may provide the foundation for a molecular understanding of such processes and suggest ways of regulating them differentially in distinct environments such as the central nervous and the immune systems.

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