Transient Nuclear Factor κB (NF-κB) Activation Stimulated by Interleukin-1β May Be Partly Dependent on Proteasome Activity, but Not Phosphorylation and Ubiquitination of the IκBa Molecule, in C6 Glioma Cells

REGULATION OF NF-κB LINKED TO CHEMOKINE PRODUCTION*

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Takashi Uehara‡, Junko Matsuno‡, Masayuki Kaneko‡, Tadashi Nishiya‡, Masahiro Fujimuro§, Hideyoshi Yokosawa§, and Yasuyuki Nomura¶

From the Departments of ‡Pharmacology and §Biochemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

We previously reported that several stresses can induce cytokine-induced neutrophil chemoattractant expression in a nuclear factor κB (NF-κB)-dependent manner. In this study, we attempted to elucidate how NF-κB is regulated. We report that CINC2, the expression of multiple immediate-early genes involved in immune, acute-phase, and inflammatory responses (1). NF-κB is a heterodimeric protein of the Rel family of transcription factors. In mammalian cells, the factors include p65 (RelA), c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). NF-κB proteins are constitutively present in cells and are retained in the cytoplasm associated with the inhibitory protein IκB (2, 3). Activated NF-κB complexes, typically composed of p50 and p65, are translocated to the nucleus in response to several cytokines (TNF-α, IL-1β, and IL-2), bacterial endotoxin, and stresses (UV, H2O2). (1, 4–6). The activation of NF-κB appears to require the phosphorylation and degradation of the IκB proteins, thereby allowing the rapid translocation of NF-κB from the cytoplasm to the nucleus (4, 7–9). In particular, it has been shown that the phosphorylation of Ser-32 and Ser-36 and the ubiquitination at Lys-21 and Lys-22 are essential for targeting IκB for signal-induced degradation by the ubiquitin/proteasome system (10).

The ubiquitin-dependent degradation of regulatory short-lived proteins plays an important role in cellular processes, including the cell cycle, immune system functions, inflammatory responses, and tissue differentiation. A key element in the regulation process is E3, a member of the ubiquitin-substrate ligase family of enzymes. After binding of the substrate through a specific structural motif, E3 transfers activated ubiquitin moieties from a ubiquitin-conjugating enzyme, E2, to a Lys residue in the target protein to generate a polyubiquitin chain. The tagged substrate is proteolyzed by a 26 S proteasome complex with the release of free and reutilizable ubiquitin (11–13). The activation of the enzymatic component of the ubiquitin system can render the substrates susceptible to conjugation and subsequent degradation. The proteasome is considered to be a crucial component in the ubiquitin-dependent proteolytic system (14). In this system, the proteasome functions in an ATP-dependent manner as a 26 S complex, which is assembled from a 20 S complex and other several regulatory subunits in the presence of ATP (15–17). These proteins are involved in physiological homeostasis processes such as the cell cycle, DNA replication, and stress response. However, regulation of these activities is unclear.

In this study, we attempted to elucidate how NF-κB activation in response to IL-1β is regulated. We report that CINC production through NF-κB induced by IL-1β is sensitive to proteasome inhibitors. The phosphorylation of Ser-32 and the degradation of IκB occurred rapidly, followed by IκBα protein resynthesis. Interestingly, we found that the resynthesized IκBα protein was already phosphorylated (Ser-32), suggesting...
that upstream kinases are still activated during this period. Possible Mechanism of Transient NF-κB Activation by IL-1β

EXPERIMENTAL PROCEDURES

Materials—C6 glioma cells were obtained from the American Type Culture Collection. Restriction endonucleases, dNTP mixture, and RNase inhibitor were purchased from Takara (Kyoto, Japan). Oligonucleotides were purchased from TaKaRa Biotechnology (Osaka, Japan). Anti-IκBα and anti-phospho-specific IκBα (Ser-32) antibodies were from Santa Cruz Biotechnology and New England Biolabs, Inc., respectively. Suc-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide, benzyl-oxycarbonyl-leucyl-leucinal (calpain inhibitor), and E-64 d (inhibitor of thiol protease) were from Peptide Inc. (Osaka, Japan). All other reagents were purchased from Sigma.

Cell Culture—C6 glioma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator containing 5% CO2.

RNA Isolation and PCR Analysis—Total RNA was prepared from 1.2 × 106 cells using guanidine/cesium chloride as described previously (18, 19). PCR was performed on total RNA extracted from cultures. Total RNA (2 μg) was incubated at 37 °C for 60 min with a mixture of 100 units of reverse transcriptase, 1× first strand buffer, 10 mM DTT, a 0.5 mM concentration of each dNTP, and 50 units of RNase inhibitor to a final volume of 20 μl. The reaction mixture was then incubated for 10 min at 70 °C to inactive the reverse transcriptase. An aliquot (2 μl) of reverse transcriptase product was mixed with 1 milliunit of DNA polymerase and a 200 nM concentration of each of the sense and antisense primers for 1 h at room temperature. The PCR products were then amplified by 20 cycles of PCR as described (19). The number of cycles that produced a linear relationship between the amount of input RNA and the resulting PCR products was used for each primer pair. The PCR products were then separated by electrophoresis in 1× TAE buffer and visualized by ethidium bromide and photographed. The primers used are as follows: rat CINC-1, 5′-AAA CCC ATC-3′ (positions 578–598; downstream) and 5′-TTA TAA CGT CAG ACG CTG GCC TCC-3′ (positions 578–598; upstream); rat IκBα, 5′-GGG ACC CTT CAT CTT TTG-3′ (positions 298–318; downstream); rat 18S, 5′-GAG ACC CTT CAT CTT TTG-3′ (positions 513–532; upstream); and rat GAPDH, 5′-AAA ACC ACC ACC ACC TTC TTG CAG-3′ (positions 236–258; upstream) and 5′-AGG GCC CAT CCA CAG TTC TCT-3′ (positions 578–598; downstream). ELISA—ELISA for rat IL-8 (CINC/cgro) antigen expressed in the culture supernatant was performed using a kit from Amersham International. The limit of detection was <0.08 ng/ml.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared using previously described methods (6, 19). Briefly, 5 × 106 cells were harvested; washed once with 2 ml of ice-cold phosphate-buffered saline and resuspended in 400 μl of buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 20 μl/ml aprotinin. After incubation for 15 min on ice, Nonidet P-40 was added to a final concentration of 0.6%, and the mixture was vortexed vigorously for 10 s. The nuclei were precipitated; resuspended in 50 μl of buffer containing 20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 20 μl/ml aprotinin; and vortexed vigorously for 15 min at 4 °C. The lysate was centrifuged at 15,000 rpm for 20 min at 4 °C; and the supernatants containing the nuclear proteins were transferred into new vials. The protein concentration of each extract was measured using a Bio-Rad protein assay kit.

EMSA’s were performed by incubating 7.5 μg of nuclear extracts with 2 μg of poly(dI-dC) in binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg/ml bovine serum albumin, 0.05% Nonidet P-40, and 5% glycerol) in a 20-μl final volume for 30 min at 4 °C. Then, an end-labeled double-stranded oligonucleotide probe (50,000 cpm/0.3 ng) was added, and the reaction mixture was incubated for 15 min at room temperature. For the supershift assay with specific antibodies against NF-κB, nuclear extracts were preincubated with 1 μg of each antibody for 2 h at 4 °C before the addition of the end-labeled double-stranded oligonucleotide probe. Samples were separated by 5% native polyacrylamide gel electrophoresis in low ionic strength buffer (0.25× TBE/0.5% Triton X-100).

Oligonucleotides—The double-stranded oligonucleotides used as competitors in the EMSA are as follows: blunt-ended competitors and NF-κB-binding site (5′-AGT TGA GGC GAC TTC CTT AGG C-3′; the core recognition sequence of this oligonucleotide is underlined).

Western Blot Analysis—Cells (5 × 106) were washed twice with ice-cold phosphate-buffered saline, and then 200 μl of lysis buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM DTT, and 1% Nonidet P-40) was added. In particular, for the detection of ubiquitinated IκBα proteins, the cells were lysed with lysis buffer containing 0.1% SDS and 5 mM N-ethylmaleimide (20). The total lysates were centrifuged at 15,000 rpm for 30 min at 4 °C, and the supernatants were removed as crude cytosolic fractions. The cytosol was boiled with SDS sample buffer for 5 min. Equal amounts of each sample were subjected to 12% SDS-polyacrylamide gel electrophoresis at 100 V for 1 h at 4 °C, followed by transfer to a nitrocellulose filter. The filters were then blocked with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 containing 5% Cytochrome c suspension for 1 h at room temperature. Anti-IκBα and anti-phospho-specific IκBα antibodies were used as primary antibodies, and horseradish peroxidase-labeled rabbit Ig was used as the secondary antibody. The antibody-reactive bands were revealed by chemiluminescence (ECL Western detection kit).

Metabolic Labeling and Immunoprecipitation—Cells (1 × 106) in 10-cm plates were washed twice with prewarmed (37 °C) methionine- and cysteine-free Dulbecco’s modified Eagle’s medium. The cells were then labeled 15 min before and 30 min after IL-1β stimulation in 10 ml of methionine- and cysteine-free Dulbecco’s modified Eagle’s medium containing 0.2 mM Ci/ml Tran35S-label (ICN Biomedicals, Irvine, CA). Pulse-labeled cells were chased for various times for up to 240 min in complete medium containing 2.5 mM methionine and cysteine, removed at the end of each time point, the cells were solubilized in lysis buffer, and the supernatants were centrifuged at 100,000 g for 20 min. The radioactivity in the supernatants was determined by liquid scintillation counting.

Antibody Reactivity—Antibody reactivity was determined by Western blot analysis using anti-IκBα antibody and analyzed on 12% SDS-polyacrylamide gel. Dried gels on Whatman No. 3MM paper were exposed on an imaging plate and visualized on a Fuji BAS 2000 apparatus.

Assay for Chymotrypsin-like Activity in Cell Lysates—Cells (1 × 106) were stimulated with 5 ng/ml IL-1β for the indicated time periods (0–60 min) in complete medium containing 2.5 mM methionine and cysteine-buffered saline, scraped into 200 μl of lysis buffer (50 mM HEPES (pH 7.5), 2 mM ATP, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT), and homogenized by 20 strokes in a Dounce homogenizer on ice. The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C, and the resultant supernatants were centrifuged at 100,000 × g for 20 min or 5 h at 4 °C. Each supernatant was used for quantification of the chymotrypsin-like activity as described (21, 22). The activity was assayed at 37 °C for 15 min in 50 mM Tris-HCl (pH 7.8) containing 1 mM DTT, 2 mM ATP, 10 mM MgCl2, and 0.1 mM Suc-LLLY-4-methylcoumaryl-7-amide as a substrate. The reaction was stopped by adding 1 ml of 10% SDS. The amounts of released 4-methylcoumaryl-7-amide were measured with a spectrophotometer (Hitachi F-2000 fluorescence spectrophotometer) with excitation at 380 nm and emission at 460 nm.

Ability to Degrade Ubiquitinated IκBα in Cytosolic Fractions Stimulated by IL-1β—Cells (5 × 106) were stimulated with 5 ng/ml IL-1β for the indicated time periods (0, 15, and 60 min), and the supernatants were isolated as described above and used for the assay of ubiquitinated IκBα degradation. Each cytosolic fraction (20 μg) was incubated with the undegraded ubiquitinated IκBα-accumulated fraction (40 μg) (see Fig. 7A, lane 6) at 37 °C for 60 min. The reaction was terminated by adding SDS sample buffer. The samples were then boiled for 5 min and subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by transfer to a nitrocellulose filter. The degradation of ubiquitinated IκBα was detected by Western blot analysis using anti-phospho-specific IκBα antibody.

RESULTS

Induction of CINC mRNA and Protein in Response to IL-1β—Initially, we examined whether mRNA levels of CINC and GAPDH were affected by IL-1β. A 24-h exposure to IL-1β did not alter cell viability (data not shown). Furthermore, the housekeeping gene GAPDH mRNA was not altered at the
induction by 5 ng/ml IL-1β and continued to accumulate for up to 48 h (Fig. 1A). CINC mRNA was not detected after treatment with IL-1β. Kinetic analysis showed that IL-1β-induced CINC mRNA expression peaked at 30–60 min. We then used ELISA to investigate the levels of CINC protein after treatment with IL-1β. Treatment resulted in time-dependent induction of CINC proteins into the culture supernatant. CINC protein in the culture medium was detected by a specific ELISA. The data represent typical results from four independent experiments. CINC protein in the culture medium was detected by a specific ELISA. The data represent typical results from four independent experiments. CINC protein in the culture medium was detected by a specific ELISA. The data represent typical results from four independent experiments.

Inhibitory Effects of Proteasome Inhibitors and Herbimycin A on IL-1β-induced CINC mRNA and Protein Expression—To elucidate the involvement of proteasomes and herbimycin A-sensitive proteins (possibly tyrosine kinases) in IL-1β-induced CINC expression, we investigated the effects of several inhibitors. Both MG132 and PSI, which are cell-permeable protease inhibitors, attenuated CINC mRNA induction in a concentration-dependent manner (Fig. 2, A and B). Furthermore, CINC proteins were significantly suppressed by pretreatment with either inhibitor (Fig. 2D). Herbimycin A, a potent tyrosine kinase inhibitor, also attenuated CINC mRNA and protein expression (Fig. 2, C and D).

Induction and Identification of NF-κB Complexes in Response to IL-1β—We previously demonstrated that NF-κB activation in C6 glioma cells in response to stressors is involved in CINC expression (19). In this study, we examined whether IL-1β can also induce NF-κB activation. Nuclear extracts were prepared, and samples were subjected to EMSA using a DNA probe containing the NF-κB-binding element as described under “Experimental Procedures.” As shown in Fig. 3A, no binding of NF-κB to radiolabeled probes was detected in unstimulated cells. However, after treatment with IL-1β, there was significant transient binding, peaking at 15 min. Binding completely disappeared when the nuclear extracts were incubated with non-radiolabeled DNA (Fig. 3B). Supershift assays in EMSA using specific antibodies against NF-κB p50 and p65 revealed that IL-1β-stimulated NF-κB is a heterodimer (p50/p65) (Fig. 3C).

Since proteasome inhibitors and herbimycin A suppressed IL-1β-induced CINC mRNA and protein expression, we examined the effects of these inhibitors on NF-κB activity by IL-1β. Proteasome inhibitors and herbimycin A partially and completely attenuated NF-κB activation, respectively (Fig. 4).

IsBo Is Degraded and Thereafter Rapidly Resynthesized during IL-1β Treatment—NF-κB activation is mainly dependent on IκB degradation. Before degradation, IκB is serine-phosphorylated by IκB kinase and then ubiquitinated by ubiquitin ligase (23). We analyzed IκBα levels at the indicated sampling times by Western blotting using anti-IκBα antibody. The levels of IκBα were decreased after 5 min in response to IL-1β and then disappeared after 15 min. Surprisingly, IκBα was re-detected at 30 min and returned to basal levels at 45–60 min (Fig. 5A). Cycloheximide, a protein synthesis inhibitor, completely blocked the resynthesis of IκBα. Proteasome inhibitors and herbimycin A, which inhibited NF-κB activation by IL-1β, blocked the degradation of IκBα. Fig. 5B shows the mRNA levels of IκBα during IL-1β treatment. IκBα mRNA was detected 5 min after stimulation and peaked at 45–60 min.

The phosphorylation of IκBα at Ser-32 and Ser-36 stimulates conjugation with ubiquitin and subsequent proteasome-mediated degradation, resulting in the translocation of NF-κB. Therefore, the phosphorylation of IκBα at Ser-32 and Ser-36 is essential for the activation of NF-κB. We investigated whether IκBα is phosphorylated in response to IL-1β using antibody that specifically recognizes the phosphorylated Ser-32 on IκBα. No detectable bands were observed in the unstimulated state. Treatment with IL-1β stimulated the phosphorylation of IκBα at 5 min, and then all phosphorylated IκBα was degraded at 15 min. Interestingly, resynthesized IκBα was phosphorylated at Ser-32 (Fig. 5C). Although neither MG132 nor PSI influenced the phosphorylation of IκBα, herbimycin A completely inhibited IL-1β-induced serine phosphorylation.

Next, we performed a pulse-chase study to examine the degradation rates during IL-1β stimulation in C6 glioma cells. The cells were labeled 15 min before and 30 min after IL-1β stimulation in medium containing [35S]Met/Cys and chased in medium containing 2.5 mM Met and Cys for 60 and 240 min, respectively. As shown in Fig. 6A, nascent IκBα degraded rapidly and almost disappeared 15 min after IL-1β stimulation, with a time course similar to the results of Western blot analysis using anti-IκBα antibody (Fig. 5A). Thereafter, IκBα protein was not detected. However, as shown in Fig. 6B, the resynthesized IκBα protein was not degraded, as seen in the early step (0–15 min after stimulation). These results indicate that the degradation rates of IκBα in the early (0–15 min after stimulation) and late (45–240 min) steps are different.

IL-1β Transiently Enhances Chymotrypsin-like Activity, but Not Ubiquitination—We further examined how the activation of NF-κB is regulated. It is believed that the phosphorylation and multi-ubiquitination of IκB are critical for degradation by 26 S proteasomes. We first measured the multi-ubiquitination state of IκBα by Western blotting using anti-phospho-specific IκBα antibody because only phosphorylated IκBα molecules are ubiquitinated. We prepared cell extracts at different times after treatment of C6 cells with 5 ng/ml IL-1β in the presence or absence of MG132 and then analyzed the samples by Western blotting. The extracts were prepared in the presence of SDS (0.1%) and N-ethylmaleimide (5 mM) to inhibit isopeptidase.
activities, as described (20). As shown in Fig. 7A, a ladder of high molecular mass proteins appeared following stimulation with IL-1β. The molecular mass increments of this ladder were ~8.5 kDa, which is the size of ubiquitin. The ubiquitination of IkBα peaked at 5 min following stimulation and then decreased at 15 min. Resynthesized and phosphorylated IkBα was upward-shifted again by 30–60 min. We next examined the effect of MG132 on IL-1β-induced ladder formation. Treatment of C6 cells with MG132 alone (60 min) led to a slight accumulation of phosphorylated and ubiquitinated IkBα. Although MG132 inhibited the degradation of IkBα, but not its phosphorylation (Fig. 5C), phosphorylated and ubiquitinated IkBα proteins were clearly detected under these conditions (Fig. 7B). The ubiquitination of IkBα peaked at 15 min after stimulation and then decreased by 30–60 min, possibly because of residual activities of proteasomes and isopeptidases.

On the other hand, chymotrypsin-like (Suc-LLVYase) activity in the presence of ATP in the proteasome-containing fraction, which was prepared by centrifugation at 100,000 × g for 20 min, was transiently enhanced and peaked 15 min after IL-1β stimulation (Fig. 7C). In contrast, the activity in the cytosolic fraction prepared by centrifugation at 100,000 × g for 5 h was reduced to ~75% of the sample prepared by centrifugation at 100,000 × g for 20 min because the high molecular mass proteasomes (700–1000 kDa) are precipitated by the prolonged centrifugation.2 In addition, we measured the activity in the TNF-α-treated cytosolic fractions. TNF-α, which can induce NF-κB activation, also increased Suc-LLVYase activity in a similar manner to IL-1β-treated cells.3 Next, we investigated the effects of several inhibitors on IL-1β-enhanced proteasome activation. The cells were pretreated with MG132 or PSI for a specified time period; the cytosolic fractions were isolated; and the activity was measured. The proteasome inhibitors (MG132 and PSI) completely blocked the activity in a quiescent state, and enhancement of the activity was induced by IL-1β (data

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2 T. Uehara, M. Kaneko, and Y. Nomura, unpublished data.
3 T. Uehara and Y. Nomura, unpublished data.
FIG. 5. Resynthesis and phosphorylation of IκBα at Ser-32 in C6 glioma cells challenged with IL-1β. Cells were pretreated with 10 μM MG132, 20 μM PSI, 1 μg/ml herbimycin A (Herb A), or 0.1 μM cycloheximide (CHX) for 1, 2, 12, or 1 h, respectively, and then stimulated with 5 ng/ml IL-1β for the indicated time periods. Cytosolic extracts (20 μg) and total RNA from the cells were subjected to Western blot analysis and RT-PCR, respectively. A, detection of IκBα during IL-1β treatment by Western blot analysis using anti-IκBα antibody; B, detection of resynthesized IκBα mRNA by the RT-PCR method; C, detection of the phosphorylated state of IκBα by Western blot analysis using anti-phospho-specific (Ser-32) IκBα antibody. Total cell extracts from HeLa cells prepared with TNF-α (10 ng/ml, 5 min) treatment were used as positive controls (P. C.).

FIG. 6. Degradation state of IκBα during IL-1β treatment. Cells were metabolically labeled for 15 min with Tran35S-label during the time periods indicated by the closed bars and then chased for the indicated time periods. In B, the cells were stimulated by IL-1β for 30 min and then metabolically labeled for 15 min with exposure to a similar concentration of IL-1β. Thereafter, the cells were chased for up to 240 min. The reaction was stopped at each time indicated by the arrow. IκBα proteins were immunoprecipitated and analyzed on SDS-polyacrylamide gels as described under “Experimental Procedures.”

not shown). Treatment with E-64-d, a thiol proteinase inhibitor, did not affect the activation in response to IL-1β. In addition, herbimycin A (a potent tyrosine kinase inhibitor), benzyl-oxyarboxon-2-leucyl-leucinal (a calpain inhibitor), and RO 31-8220 (a nonspecific serine/threonine kinase inhibitor) did not alter enhancement by IL-1β.

To elucidate the causality between the enhancement of Suc-LLVYase activity and the degradation of phosphorylated and multi-ubiquitinated IκBα, we finally analyzed the ability to degrade ubiquitinated IκBα in IL-1β-stimulated cytosolic fractions. As shown in Fig. 8, only the cytosol stimulated by IL-1β for 15 min, but not for 0 or 60 min, could degrade ubiquitinated IκBα in the cytosol 60 min after IL-1β treatment. Treatment with 10 μM MG132 suppressed the degradation of IκBα (Fig. 8, lane 4).

DISCUSSION

The aim of this study was to investigate the mechanism of NF-κB regulation, especially via IκBα, in C6 glioma cells. We showed here that IL-1β stimulates NF-κB activation and subsequent chemokine (CINC) production. Activation was sensitive to proteasome inhibitors (MG132 and PSI) and herbimycin A, indicating that both the proteasome, which degrades IκBα, and a tyrosine kinase are involved in this pathway. Furthermore, IL-1β treatment resulted in the rapid resynthesis of IκBα. Surprisingly, we found that Ser-32 in resynthesized IκBα was already phosphorylated. Although the phosphorylation of this residue is important for ubiquitination and degradation, no significant translocation of NF-κB was observed. These results suggest that some proteins downstream from the serine phosphorylation of IκBα inhibit degradation signaling. Therefore, we further examined why the activation of NF-κB stimulated by IL-1β is transient.

Recent studies have provided some insights into the mechanisms leading to IκBα degradation (7, 20, 24–30). The signal-induced degradation of IκBα is dependent on the presence of an intact COOH-terminal PEST (Pro, Glu, Ser, Thr) region as well as the phosphorylation of Ser-32 and Ser-36. These phosphorylated residues probably target the molecule for ubiquitination at Lys-21 and Lys-22 (29, 30). In turn, this targets the molecule for degradation by 26 S proteasomes (20), which occurs when the inhibitor and NF-κB are dissociated. The IκBα molecule is resynthesized rapidly following degradation, partly because the promoter of the IκBα gene is positively regulated by NF-κB. We confirmed that IL-1β stimulates the phosphorylation and degradation of IκBα, and its de novo resynthesis (Fig. 5).

Surprisingly, we found that resynthesized IκBα is already phosphorylated at Ser-32, indicating that the receptor-mediated signal for phosphorylation is active in IL-1β-challenged C6 glioma cells for at least up to 60 min. Why does phosphorylated IκBα not cause further NF-κB translocation? To address this problem, we examined the activities of ubiquitin ligase and proteasomes. We first measured the state of ubiquitination of the IκBα molecule by Western blotting. The multi-ubiquitination of IκBα was detected by anti-phospho-specific IκBα antibody. Multi-ubiquitinated IκBα proteins were detected as upward-shifted proteins (20). Treatment with IL-1β stimulated ubiquitination (Fig. 7A). However, multi-ubiquitinated proteins disappeared 15 min after IL-1β challenge, and resynthesized IκBα was phosphorylated and ubiquitinated. In addition, pretreatment with MG132, a proteasome inhibitor, caused the accumulation of multi-ubiquitinated IκBα, which peaked 15 min after IL-1β treatment. These results suggest that multi-ubiquitination in response to IL-1β is present for at least up to 60 min after treatment. In addition, phosphorylated and resynthesized IκBα proteins and the accumulated phosphorylated IκBα protein in MG132-pretreated cytosolic fractions were barely dephosphorylated (Fig. 5C). These results suggest that
inactivated enzyme such as some phosphatases is not mainly involved, or the activity has a very low level in this system, although the modification of IκBα (serine phosphorylation) that is essential for NF-κB activation rapidly occurs.

We next examined Suc-LLVY cleaving activity in the cytosolic fraction. The chymotrypsin-like (Suc-LLVYase) activity in the proteasome-containing cytosolic fraction prepared by centrifugation at 100,000 × g for 20 min in response to IL-1β was transiently activated with a time course similar to that of NF-κB translocation (Fig. 7C). Since it is well known that LLVY is a substrate for chymotrypsin (which is slight in glial cells), calpain, and 20 S and 26 S proteasomes, the activity in the cytosolic fraction may be derived from these proteins. We first investigated the possibility that the chymotrypsin-like (LLVYase) activity is partly derived from proteasome activity. The activity in the cytosolic fraction prepared by centrifugation at 100,000 × g for 5 h was reduced markedly compared with that in the sample prepared by centrifugation at 100,000 × g for 20 min because the high molecular mass proteasomes (700–1000 kDa) are precipitated by prolonged centrifugation. The resulting activity is considered to be derived from another protein such as calpain, and there was no enhancement of the activity seen during IL-1β treatment. Moreover, the calpain inhibitor did not block IL-1β-stimulated IκBα degradation in this system, suggesting that at least the proteasome is partly activated by treatment with IL-1β. These results indicate that a transient increase in Suc-LLVY cleaving activity is partly derived from the proteasome activity. However, the time courses of Suc-LLVYase and NF-κB translocation are merely coincidental; we cannot completely rule out the possibility that the other proteinase is involved in Suc-LLVY cleavage and IκBα degradation.

Subsequently, we studied the mechanism of the transient Suc-LLVYase activation stimulated by IL-1β using several inhibitors: E-64-d (a thiol protease inhibitor), Ro 31-8220 (a nonspecific serine/threonine kinase inhibitor), and herbimycin A (a potent tyrosine kinase inhibitor). Pretreatment with these inhibitors did not affect IL-1β-stimulated Suc-LLVYase activation. There have been few reports on the transient activation of proteasomes in response to several cytokines that can stimulate NF-κB translocation. However, Kawahara et al. (21) reported that the 26 S proteasome is activated in prophase and metaphase during the mitotic cell cycle of synchronously dividing ascidium embryos. Furthermore, proteasomes are activated during in vivo Xenopus egg activation induced by treatment with the calcium ionophore A23187 (22). The 26 S proteasome consists of at least two subunits: one is a 700-kDa proteolytic core complex called the 20 S proteasome with 28 subunits, and the other is a 700–1000-kDa regulatory subunit complex made up of ~20 subunits (31–38). Although there are many reports that several proteasome subunits can be phosphorylated, there is little or no direct effect on proteasome activity from these modifications (39–42). Thus, it is believed that phosphorylation is involved in assembly, targeting, or turnover of proteasome subunits. In contrast, Kenneth et al. (43) demonstrated that the phosphorylation of PA28, referred to as the 11 S regulator, is required for stimulation of peptidase activity, although the relevant mechanism remains to be defined. Therefore, in our system, it may be possible that some modifications such as phosphorylation are involved in the modulation of Suc-LLVYase activity, possibly 26 S proteasome activity; however, we could not directly demonstrate how the 26 S proteasome is regulated.

It is recognized that the level of resynthesized IκBα proteins is very important for the down-regulation of NF-κB activity. We have found here that the resynthesized proteins are phosphorylated and multi-ubiquitinated. If the proteasome is involved in the degradation of IκBα as reported previously, it was clearly expected that resynthesized (phosphorylated and ubiquitinated) IκBα would also be degraded, and then the activation of NF-κB would occur again. However, these events did not occur. Alternatively, if ubiquitinated IκBα is degraded by other proteinase in this system, the rates of degradation in the early (0–15 min after stimulation) and late (45–240 min after stim-
involved in TNF-α two processes (proteasome activation and NF-

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ulation). Hence, we investigated the ability to degrade ubiquiti-

proteasome-containing cytosolic fractions were prepared from the cells stimulated with 5 ng/ml IL-1β. Each cytosolic fraction was incubated with the undegraded ubiquitinated IκBα-accumulated fraction at 37 °C for 60 min. The reaction was terminated by adding SDS sample buffer. The degradation of ubiquitinated IκBα was detected by Western blot analysis using anti-phospho-specific IκB antibody. p-IκBα, phosphorylated IκBα; [Ub]n-p-IκBα, multi-ubiquitinated p-IκBα.

![Fig. 8. Degradation of ubiquitinated IκBα in IL-1β-stimulated cytosolic fractions.](Image)

| (kDa) | 1 | 2 | 3 | 4 |
|-------|---|---|---|---|
| cytosol | - | - | - | + |
| 0 min after IL-1β stimulation | - | - | - | + |
| 15 min after IL-1β stimulation | - | - | - | + |
| 60 min after IL-1β stimulation | - | - | - | + |
| MG-132 | - | - | - | + |

Fig. 8. Degradation of ubiquitinated IκBα in IL-1β-stimulated cytosolic fractions. Cells were stimulated with 5 ng/ml IL-1β for 60 min, and then the cytosolic fraction containing undegraded ubiquitinated IκBα was prepared as described under “Experimental Procedures.” The proteasome-containing cytosolic fractions were prepared from the cells stimulated with 5 ng/ml IL-1β for the indicated time periods (0, 15, and 60 min). Each cytosolic fraction was incubated with the undegraded ubiquitinated IκBα-accumulated fraction at 37 °C for 60 min. The reaction was terminated by adding SDS sample buffer. The degradation of ubiquitinated IκBα was detected by Western blot analysis using anti-phospho-specific IκB antibody. p-IκBα, phosphorylated IκBα; [Ub]n-p-IκBα, multi-ubiquitinated p-IκBα.

We propose here a novel regulation system for NF-κB activation in glial cells, IL-1β induces the rapidly sequential phos-

phorylation, ubiquitination, and degradation of IκBα and the subsequent NF-κB translocation into the nuclei that peaks 15 min after IL-1β treatment. Receptor-mediated signals seem to be activated at least 60 min after stimulation. We demonstrated that the transient enhancement of Suc-LLVYase (possibly proteasome) activity has a time course similar to that of the translocation of NF-κB. It is obvious that the regulation of proteasome activity partly contributes to the transient NF-κB activation. Furthermore, the cytosolic fraction stimulated by IL-1β for 15 min, but not for 0 and 60 min, had an ability to degrade ubiquitinated IκBα. In view of these results, it is suggested that the transient increase in proteasome activity is partly involved in the NF-κB regulation. Therefore, delineating the precise mechanisms of proteasome activation in response to IL-1β is needed.

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