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A MEK Inhibitor, PD98059 Enhances IL-1-Induced NF-κB Activation by the Enhanced and Sustained Degradation of IκBα

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Interleukin-1 (IL-1) mediates numerous host responses through rapid activation of nuclear factor-κB (NF-κB), but signal pathways leading to the NF-κB activation appear to be complicated and multiplex. We propose a novel regulatory system for NF-κB activation by the extracellular signal-related kinase (ERK) pathway. In a human glioblastoma cell line, T98G, IL-1-induced NF-κB activation was significantly augmented by the pretreatment of a specific MEK inhibitor, PD98059. In contrast, ectopic expression of a constitutive activated form of Raf (v-Raf) reduced IL-1-induced NF-κB activation, and this inhibition was completely reversed by PD98059. Interestingly, PD98059 sustained IL-1-induced NF-κB DNA binding activity by an eletrophoretic mobility shift assay and also IκBα degradation, presumably by augmenting and sustaining the proteasome activation. Concomitantly, two NF-κB dependent genes, A20 and IκBα expression were prolonged with PD98059. These data suggested that MEK-ERK pathway exerts a regulatory effect on NF-κB activation, providing a novel insight on the role of MEK-ERK pathway. © 2001 Academic Press

Key Words: **interleukin-1; NF-κB; PD98059; MEK; ERK; IκBα; proteasome.**

IL-1 as a proinflammatory cytokine induces various cytokines as well as many inflammatory genes in response to the infection, activated lymphocyte products, microbial toxins, and other stimuli (1, 2). Most of the IL-1-inducible genes are regulated by the nuclear factors, NF-κB (3, 4). Recently, intracellular events mediated by IL-1RI leading to NF-κB activation are being extensively explored. That is, binding of IL-1 to the IL-1R type I (IL-1RI) allows the association with the IL-1R accessory protein (IL-1R AcP), followed by the recruitment of MyD88, an adaptor protein to the IL-1RI complex. This in turn leads to the recruitment of serine/threonine kinase, IL-1 receptor-associated kinase (IRAK) to the receptor complex. Then, IRAK interacts with tumor necrosis factor receptor-associated factor-6 (TRAF6), recruiting TGF-β-activating kinase (TAK)-1, NF-κB-inducing kinase (NIK), IκB-kinase (IKK) α/β and resulting in the phosphorylation and degradation of IκB, thus facilitating the translocation of NF-κB to the nucleus (5–8).

Mitogen-activated protein (MAP) kinase pathway which implies three major family, ERK, SAPK/JNK, p38MAPK is also known to be activated by IL-1. Particularly, MEK/ERK pathway in which Raf stimulates MAPKK (MEK1 and MEK2) plays important roles in these responses. However, the interaction of these kinases with NF-κB activation remains to be controversial (9–12). Here, we focused on the cross-talks of MAP kinase pathway on NF-κB activation, since we found that a specific MEK inhibitor, PD98059 potently augmented NF-κB activation by IL-1. PD98059 augmented TRAF6-induced NF-κB activation as well, PD98059 appeared to interact at downstream of TRAF6 during the IL-1 signal pathway. In addition, expression of v-Raf, a constitutively activated form of Raf, inhibited both IL-1 and TNF-α-induced NF-κB activation, proposing an idea that Raf-MEK-ERK pathway plays a negative role on NF-κB.

MATERIALS AND METHODS

Cells, reagents, and antibodies. A human glioblastoma cell line, T98G was obtained from the Japanese Cancer Research Resources Bank (JCRB, Kamiyoga, Tokyo, Japan), and was maintained in tissue culture dishes in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 5% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Human recombinant IL-1α and TNF-α were kindly provided by Dainippon Pharmaceutical Co. (Suitashi, Osaka, Japan). PD98059, a specific inhibitor of MEK was purchased from Alexis Biochemicals (San Diego, CA).

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MG132 was obtained from Calbiochem. Polyclonal anti-IκBα and polyclonal anti-IKKα Ab were purchased from Santa Cruz Biotechnology. Anti-phospho-specific IκBα (Ser-32) Ab was obtained from New England Biolabs, Inc. Peroxidase-conjugated swine anti-rabbit IgG was purchased from Dako Japan, Tokyo. For all experiments, unless otherwise indicated, cells were incubated with PD98059 (10 μM) for 30 min at 37°C prior to stimulation with IL-1α (10 ng/mL) or TNF-α (10 ng/mL) for 12 h. All of the results shown are repeated at least three times and represented as mean ± SD.

Transient transfection and reporter gene assay. T98G cells (5×10^5) were transfected with 1 μg of pNF-κB-Luc or pAP-1-Luc, 0.1 μg of pRL-TK (Promega), and/or 3 μg of pCMV5-FLAG-TRAF6, and/or 2 μg of pNLC-Δ Raf1 (v-Raf). After 36 h, cells were treated with PD98059 or DMSO for 30 min and stimulated with IL-1α or TNF-α for 12 h. Then, cells were harvested and the luciferase activities were measured by the Lumat LB9501 (Bertold Japan, Tokyo). The efficiency of transfection was normalized with sea pansy luciferase activities.

Electrophoretic mobility shift assay (EMSA) and Western blot analysis. EMSA and Western blot were performed essentially as described elsewhere (13). In brief, 10 μg of nuclear proteins prepared from T98G cells were incubated with [γ-^{32}P]-labeled double-strand oligonucleotide probe containing a NF-κB binding site (5′-GGGACTTTCCGGGACTTTCC-3′). The binding reaction was carried out at room temperature for 30 min in a total vol of 25 μL. Bound complexes were separated on 5% TGE gel by electrophoresis in TGE buffer, dried, and visualized by autoradiography. For Western blot analysis, cells were lysed in 200 μL of lysis buffer (13, 14), followed by the removal of cell debris by centrifugation at 15,000 rpm for 15 min. Cell lysates were fractionated on the 10% SDS–PAGE, transfered to nitrocellulose membranes. Immunoblot was performed with polyclonal anti-IκBα Ab (1:1000 dilution) and horseradish peroxidase-coupled goat anti-rabbit Ig (1:1000 dilution), and were visualized by using the enhanced chemiluminescence Western blotting detection system (Amersham).

Kinase assay. Cells (1×10^5) were lysed with lysis buffer and the cell lysates were clarified by centrifugation at 15,000 rpm for 15 min. They were then immunoprecipitated using 1 μg anti-IKKα and anti-IKKβ specific Abs. The IKK-Ab complex was precipitated with protein-A sepharose beads for 2 h at 4°C, washed three times with lysis buffer and then washed three times with kinase buffer (25 mM Hepes-NaOH pH 7.5, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM DTT, 20 mM *p*-nitrophosphate), which was incubated with 1 μg purified GST-IκBαΔC (1–180) as a substrate. Kinase reaction was run for 30 min at 30°C using 100 μM ATP and terminated by the addition of 5 × SDS–PAGE sample buffer and boiling for 5 min. GST-IκBαΔC phosphorylation (IκBα-Ser-32) was determined by Western blot analysis using the Anti-phospho-specific IκBα (Ser-32) Ab.

RNA isolation and PCR analysis. Total RNA separation and reverse transcriptase-PCR analysis were done according to the manufacturer's protocols (Takara Syuzou, Shiga, Japan) using oligo $(dT)_{20}$ primer and 1 μg total RNA for first strand cDNA synthesis. PCR was performed at an annealing temperature of 60°C and 20 amplification cycles. The PCR products were resolved and electrophoresed on a 1% agarose gel in Tris borate/EDTA. The primers used are as following: human A20, 5′-CGCTCAAGGAAACAGACACA-3′ (upstream) and 5′-CTTCAGGGTCACC AAGGGTA (downstream); human IκBα, 5′-GGGGGATCCATGTTCCAGGCG GCCGAG-3′ (upstream) and 5′-GGGGGATCCTCATAACGTCAGACGCTG-3′ (downstream); and human GAPDH, 5′-GTCAGTGGTGGACCTGACCT-3′ (upstream) and 5′-TGAGGAGGGGAGATTCAGTG-3′ (downstream).

Assay of chymotrypsin-like activity in cell lysates. Cells (5×10^5) were washed twice with ice-cold PBS, 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 supernatants were centrifuged at 100,000*g* for 20 min at 4°C. Each supernatant was used for the measurement of the chymotrypsin-like activity. The activity was assayed at 37°C for 15 min in 50 mM Tris (pH 7.8) containing 1 mM DTT, 2 mM ATP, 10 mM $MgCl_2$, and 0.1 mM Suc-LLVY-4-metylcoumaryl-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 fluorescence spectrofluorometer (Hitachi F-4000, Tokyo) with excitation at 380 nm and emission at 460 nm (15–17).

RESULTS

PD98059 enhances IL-1α-induced NF-κB but not AP-1 activation. To investigate the involvement of the ERK/MAP kinase pathway in NF-κB activation, PD98059, a specific MEK inhibitor was tested, since PD98059 inhibits tyrosine phosphorylation and activation of ERK/MAPK through the inhibition of the ERK kinase/MAPKK (MEK) activation (18). IL-1α induced significant NF-κB activation in a human glioblastoma cell line, T98G as described previously (13). Pretreatment of 1–10 μM PD98059 augmented the IL-1-induced NF-κB luciferase activity up to double, while PD98059 alone failed to induce NF-κB activation (Fig. 1A). While AP-1 is also involved in the IL-1-mediated gene activation (19), PD98059 pretreatment did not augment but rather inhibited IL-1-induced AP-1 activation in a dose-dependent manner (Fig. 1B). Thus, it is suggested that in the IL-1 signaling, MEK-ERK pathway downregulates IL-1-induced NF-κB activation, while it is essential for the AP-1 activation. In addition to IL-1, PD98059 pretreatment also augmented TNF-α-induced NF-κB activation significantly (Fig. 1C).

PD98059 affects at downstream of TRAF6. Since TRAF6 is an important molecule in the IL-1-signaling, we transfected pCMV5-FLAG-TRAF6, an expression vector for TRAF6, in T98G cells and the effect of PD98059 was tested. As shown in Fig. 1D, ectopic expression of TRAF6 induced NF-κB activation moderately, which was enhanced further by the treatment with PD98059. These findings suggested that PD98059 affects at downstream of TRAF6 and, therefore, that MEK-ERK pathway downregulates IL-1-induced NF-κB activation at the downstream of TRAF6.

v-Raf inhibits NF-κB activation induced by both IL-1 and TNF-α. Above data gave a notion that MEK-ERK pathway negatively regulates NF-κB activation. We then explored whether overexpression of v-Raf, a constitutive active mutant of Raf, inhibits NF-κB activation. As shown in Fig. 2A, ectopic expression of v-Raf effectively inhibited IL-1-induced NF-κB activation by about 50%. Similarly, v-Raf also inhibited TNF-α-induced NF-κB activation by about 40% (Fig. 2B). When cells were incubated with PD98059 prior to IL-1α stimulation, PD98059 reversed the v-Raf-mediated inhibition with marked NF-κB activation

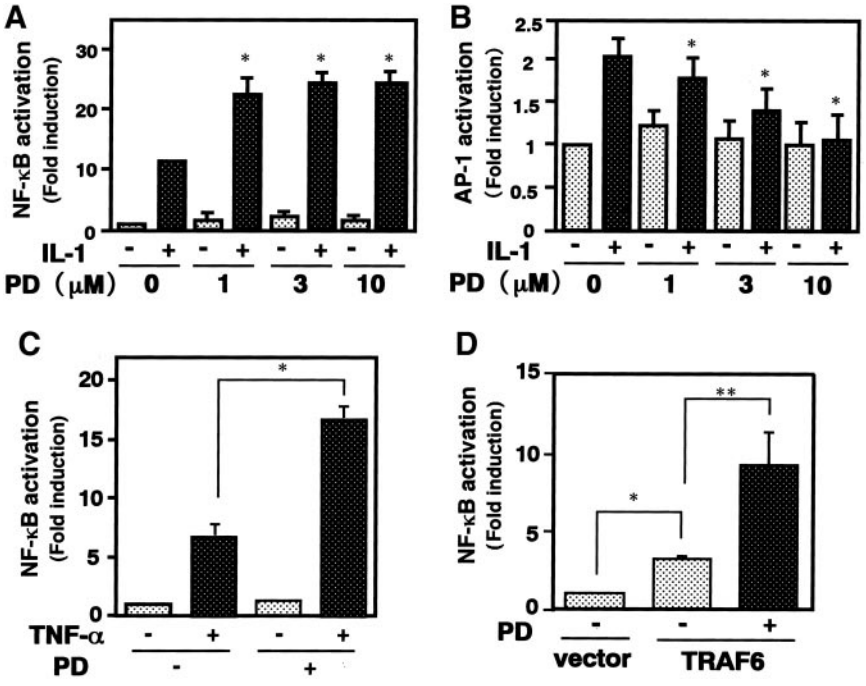

FIG. 1. Effect of PD98059 on IL-1-induced NF-κB and AP-1 activation. T98G cells were transfected with the NF-κB-dependent reporter plasmid (p NF-κB-Luc) (A, C), or the AP-1-dependent reporter plasmid (p-AP-1-Luc) (B). After transfection for 36 h, the cells were stimulated with IL-1α (10 ng/mL) (A, B) and TNFα (10 ng/mL) (C), for 12 h in the absence or presence of 1 to 10 μM PD98059. T98G cells were cotransfected with pNF-κB-Luc and a plasmid expressing wild type of TRAF6 (D) in the absence or presence of PD98059 (10 μM). After 12 h, as described under Materials and Methods, luciferase activity was measured. *$P < 0.005$ versus effect on IL-1α alone (A–C), *$P < 0.005$ versus vector control, and **$P < 0.005$ versus effect on TRAF6 value.

(Figs. 2A and 2B). These results supported the idea that ERK pathway downregulates NF-κB activation induced by IL-1 and TNF-α.

PD98059 prolongs NF-κB activation. To examine the precise role of PD98059 on the NF-κB activation, we investigated whether PD98059 affects the binding of nuclear extracts to the consensus κB oligonucleotide by an EMSA. As shown in Fig. 3A, IL-1 induced significant NF-κB binding activity, peaking at 0.5–1 h. This binding activity decreased to the basal level at 2 h after IL-1 stimulation (Fig. 3A). NF-κB band was confirmed by the competitive inhibition with non-radiolabeled oligomer DNA (Fig. 3B). Although the pretreatment with PD98059 had no substantial effect on the DNA binding of NF-κB by IL-1 until 1 h, it clearly prolonged the DNA binding activity until 2 h (Fig. 3A). NF-κB DNA binding activity in the presence of PD98059 continued until 4 h after IL-1 stimulation and decreased to the basal level at 6 h (data not shown). Thus, PD98059 sustains IL-1-induced NF-κB activation effectively, presumably by preventing MEK-ERK pathway, which affects negatively on NF-κB activation.

PD98059 augments IκB degradation. We focused further on the regulatory mechanism of NF-κB activation by PD98059. It is well established that activation of NF-κB requires the phosphorylation and degradation of the IκB proteins, thereby allowing the rapid translocation of NF-κB from cytoplasm to the nucleus (20–23). Therefore, kinetics of IκBα degradation was visualized by Western blotting using anti-IκBα Ab. Amounts of IκBα decreased after 15 min in response to IL-1 with complete disappearance at 30 min. However, IκBα reappeared at 60 min and returned basal level at 105 to 120 min (Fig. 3C). Presumably, pretreatment of PD98059 appeared to enhance the degradation of IκBα, and/or delay the reappearance of IκBα at 60 to 70 min, thus enabling to sustain NF-κB activation.

PD98059 has no enhancing effect on IL-1-stimulated IKK activity. It is presumed that the phosphorylation of IκB by IKKs is critical for the degradation by 26S proteasomes. The phosphorylation of IκBα at Ser-32 and Ser-36 is essential for the activation of NF-κB (22, 23). To explore the effects of PD98059 on the IKK activity, endogenous IKKs from IL-1-stimulated T98G cells were immunoprecipitated and determined the in vitro kinase assay using GST-IκB$\alpha\Delta$C (1–180) as a substrate. As shown in Fig. 4A, IL-1 induced a marked increase in IKKα activity at 15 min which continued until 90 min. Similarly, IKKβ activation was detected at 5 min, with a maximal activity at 30 min, followed by decline at 60

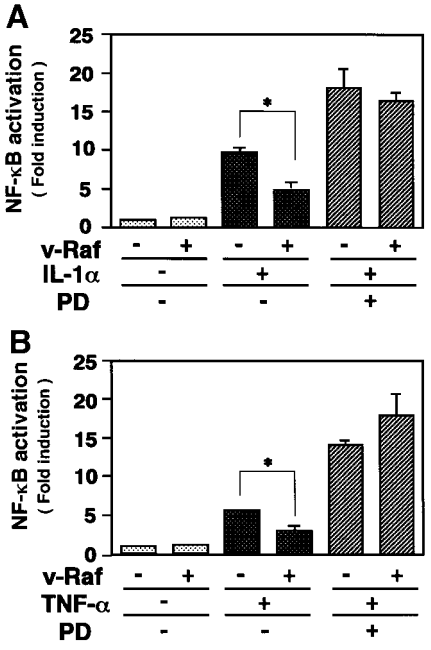

FIG. 2. Involvement of Raf-MEK-ERK pathway in regulating NF-κB activation by IL-1 or TNF-α. T98G cells were cotransfected with pNF-κB-Luc, an empty vector, or a plasmid expressing v-Raf. Thirty-six hours after transfection, cells were incubated with IL-1α (10 ng/mL) (A) or TNFα(10 ng/mL) (B) for 12 h in the absence or presence of 10 μM PD98059. $*P < 0.005$ versus effect on control value (Vector, IL-1) (A, B).

min. As shown in Fig. 3C, PD98059 had no significant enhancement of both IKKα and IKKβ activation by IL-1. Above results indicated that PD98059 augments degradation of IκBα without significant enhancement of IKK activity.

PD98059 augments proteasome activity. Activation of NF-κB requires the proteolysis of phosphorylated IκB by 26S proteasome complex (24–26). Therefore, we examined whether PD98059 affects on the proteasome activity. As shown in Fig. 5, chymotrypsin-like (Suc-LLVYase) activity in the presence of ATP in the proteasome-containing fraction was transiently enhanced with a peak at 45 min after IL-1 stimulation. The time course is similar to that of IκB degradation. It should be noted that PD98059 markedly augmented and sustained Suc-LLVYase activity induced by IL-1 (Fig. 5). These results were indicative that PD98059 increases IL-1-induced NF-κB activation by enhancing proteasome activity.

PD98059 sustains IL-1-stimulated A20 and IκBα expression. The zinc finger protein A20 is encoded by an immediate early response gene, whose expression is induced by inflammatory stimuli including IL-1 or TNF-α.

A20 gene induction (27, 28) as well as IκBα induction (29, 30) are known to be mediated through the activation of NF-κB. Therefore, the effect of PD98059 on A20 and IκBα expression was explored using RT-PCR analysis. Kinetics analysis demonstrated that IL-1 induced A20 mRNA expression with a peak at 1 to 3 h, followed by a rapid decline. Pretreatment with PD98059 sustained A20 expression until 12 h (Fig. 6A). IL-1 also induced IκBα mRNA expression, peaking at 1 h followed by a rapid decline, while its expression was significantly prolonged by the pretreatment with PD98059 until 12 h (Fig. 6B). Taken together, PD98059 augments the expression of some of NF-κB-dependent genes through the enhancement of NF-κB activation.

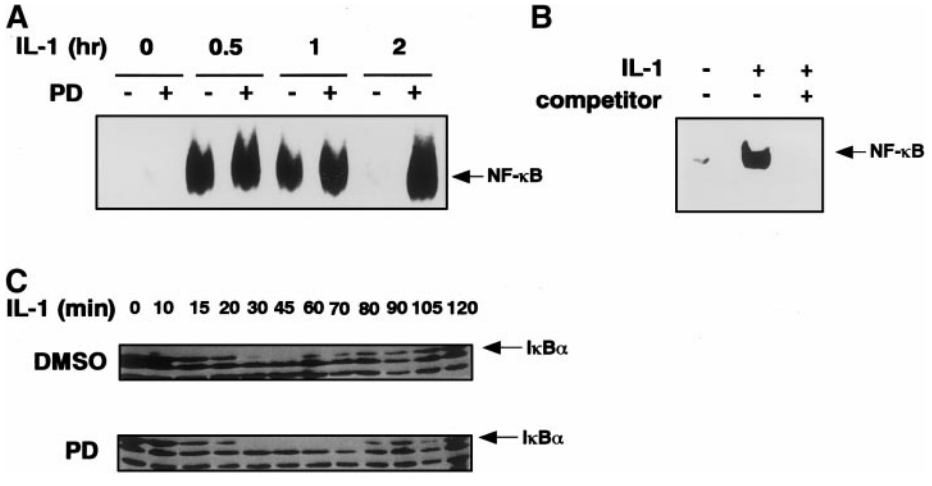

FIG. 3. PD98059 sustained IL-1-induced NF-κB activation by the enhancement of IκBα degradation. Nuclear extracts were prepared from T98G cells, pretreatment with 10 μM PD98059 (PD) for 30 min prior to IL-1α stimulation (10 ng/mL) for indicated periods. EMSAs were carried out as described under Material and Methods, and the binding of NF-κB in the nuclear extracts to ^{32}P-NF-κB oligonucleotide is shown. The position of the NF-κB-DNA complex is indicated with an arrow (A). Unlabeled competitor oligonucleotide (NF-κB consensus sequence) was used at a 100-fold excess (B). T98G cells were incubated with 10 μM PD98059 (PD) or DMSO for 30 min prior to stimulation with IL-1α for indicated times, and immunoblotted with an anti-IκBα Ab. IκBα is a most upper band in this Western blot (C).

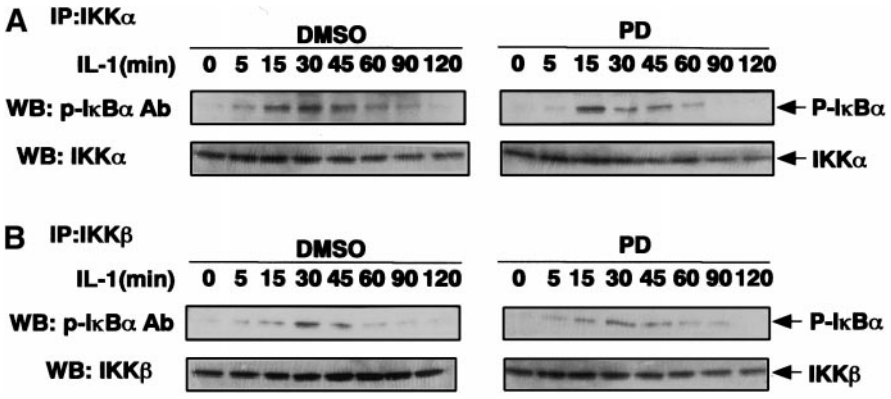

FIG. 4. PD98059 had no effect on IL-1-induced IKK activation. T98G cells were incubated with 10 μM PD98059 or DMSO for 30 min prior to stimulation with IL-1α for indicated times and IKKα (A) or IKKβ (B) was immunoprecipitated from cell extracts using specific Ab against IKKα or IKKβ. Relative activity of IKKα or IKKβ in the immunoprecipitates was measured by *in vitro* kinase assay using with GST-IκBα (1–180). IκBα phosphorylation at Ser-32 was determined by Western blot analysis using anti-phospho IκBα (Ser-32) Ab. Immunoprecipitates were also immunoblotted with anti-IKKα or IKKβ Ab.

DISCUSSION

We reported previously that inhibition of p38 MAP kinase did not reduce IL-1-induced NF-κB activation significantly, while it played a more important role in AP-1 activation (13). Here, we explored the role of MEK-ERK pathway in IL-1 signaling, using a specific MEK inhibitor, PD98059. This compound has been shown to block activation of the both MEK1 and MEK2 isoforms as a noncompetitive inhibitor, but has no effect on p38 MAP kinase or c-Jun NH2-terminal kinase (JNK) (18). Our initial finding was that PD98059 augmented IL-1-stimulated NF-κB activation which was rather an unexpected finding. Furthermore, ectopic expression of v-Raf effectively prevented IL-1-induced NF-κB activation. This may explain that MEK-ERK pathway has some negative role on NF-κB activation in IL-1 signaling. It should be noted that the augmentation was specific to the NF-κB activation, since the AP-1 activation was suppressed. We were interested in the target of PD98059 in the IL-1 signaling. Therefore, we constructed TRAF6-overexpressed cells and found that PD98059 acted at the downstream of TRAF6. In addition, the effect of PD98059 on the DNA binding activity was studied by the EMSA using a NF-κB consensus sequence. Inhibition of the MEK-ERK pathway by PD98059 had no discernible effect on the translocation and binding of NF-κB after IL-1 stimulation. However, we found that PD98059 sustained IL-1-induced NF-κB DNA binding activity at least until 2 h. This sustained DNA binding activity by PD98059 led to enhanced NF-κB-dependent gene expression, i.e., A20 and I κBα mRNA expression.

Activation of NF-κB appears to require the phosphorylation and degradation of IκB protein, which allow rapid translocation of NF-κB from the cytoplasm to the nucleus. Surprisingly, we observed that PD98059 augmented IκBα degradation without apparent activation of IKKs, which is regarded to be required for the

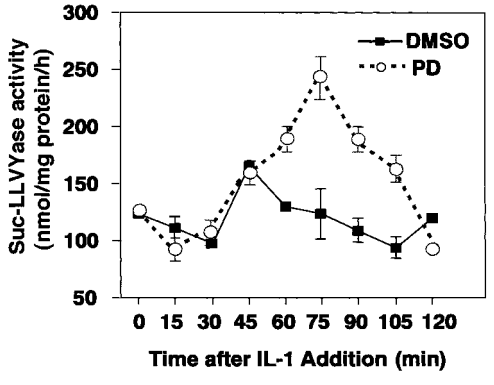

FIG. 5. PD98059 enhanced IL-1-induced proteasome activity. T98G cells were incubated with 10 μM PD98059 or DMSO for 30 min prior to stimulation with IL-1α for indicated times and washed twice with ice-cold PBS. Cytosolic fraction was prepared as described under Materials and Methods. The Suc-LLVYase activity hydrolyzing Suc-LLVY-4-methyl-coumaryl-7-amide was determined and expressed as nmol/mg protein/h.

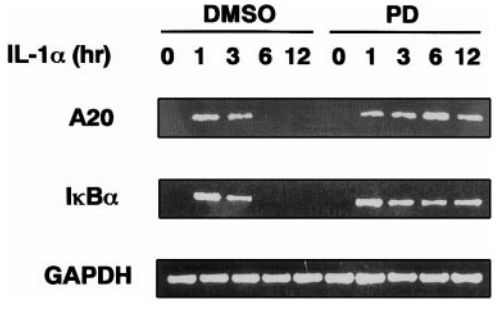

FIG. 6. PD98059 prolonged NF-κB-dependent gene expression. T98G cells were incubated with 10 μM PD98059 or DMSO for 30 min prior to stimulation with IL-1α for indicated times. Total cellular RNA was prepared and analyzed by RT-PCR.

IκB phosphorylation. As for the mechanism leading to IκB degradation, it is recognized that the phosphorylated IκBα is a target for ubiquitination at Lys-21 and Lys-22, which in turn, is processed for the degradation by 26 S proteasomes (22–26). Why PD98059 increases IκBα degradation without IκBα phosphorylation should be clarified. To answer this question, we determined Suc-LLVY cleaving activity in the cytosolic fraction. Since Suc-LLVY is a substrate for chymotrypsin (which is very low in glial cells), calpain, and proteasomes, the activity in the cytosolic fraction may be derived from these proteins. Uehara *et al.* demonstrated that there was no enhancement of calpain activity during IL-1 treatment (15). Moreover, a calpain inhibitor, E-64-d did not block IL-1-stimulated IκB degradation in T98G cells (data not shown), suggesting that the transient increase in Suc-LLVY cleaving activity is mostly due to the proteasome activity. We demonstrated that the transient enhancement of Suc-LLVYase by PD98059 has a time course similar to that of the translocation of NF-κB. Taken together, it may be that the enhanced proteasome activity contributes to the transient NF-κB activation. Li and Karin (31) have reported that UV-induced IκBα degradation occurs through the ubiquitin-mediated proteasome pathway without any activation of IKKs and phosphorylation of IκBα at Ser-32 and Ser-36. We described here that PD98059 also augmented NF-κB activation through enhancement of proteasome activity without apparent increase of IKK activity. Thus, our data support the Li and Karin's observation that NF-κB activation actually occurs independent on the phosphorylation of IκBα at serines by IKKs. Our observation also indicates that intrinsic MEK-ERK pathway has a regulatory role on NF-κB activation, through inhibition of proteasome activity.

MAP kinase signaling cascade, with three distinct members of the protein kinase family, MAPK, MAPKK, and MAPKKK, is a signal transduction pathway well conserved in cells from yeasts to vertebrates. Previous study reported that MAP kinase pathway selectively masks the ability of dorsal, which is a *Drosophila* homologue of mammals NF-κB (32). Here we demonstrated that MAP kinase pathway plays the regulatory role in the NF-κB activation in mammalian as well as *Drosophila*. Although we did not define the direct interaction of MEK-ERK with proteasome pathway, further studies should be delineated to solve this problem.

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