

# p38 and Extracellular Signal-regulated Kinase Mitogen-activated Protein Kinase Pathways Are Required for Nuclear Factor- $\kappa$ B p65 Transactivation Mediated by Tumor Necrosis Factor\*

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Interleukin-6 (IL-6) is a pleiotropic cytokine, which is involved in inflammatory and immune responses, acute phase reactions, and hematopoiesis. In the mouse fibrosarcoma cell line L929, the nuclear factor (NF)- $\kappa$ B plays a crucial role in IL-6 gene expression mediated by tumor necrosis factor (TNF). The levels of the activated factor do not, however, correlate with the variations of IL-6 gene transcription; therefore, other factors and/or regulatory mechanisms presumably modulate the levels of IL-6 mRNA production. Upon analysis of various deletion and point-mutated variants of the human IL-6 gene promoter coupled to a reporter gene, we screened for possible cooperating transcription factors. Even the smallest deletion variant, containing almost exclusively a NF- $\kappa$ B-responsive sequence preceding the IL-6 minimal promoter, as well as a recombinant construction containing multiple  $\kappa$ B-motifs, could still be stimulated with TNF. We observed that the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 was able to repress TNF-stimulated expression of the IL-6 gene, as well as of a  $\kappa$ B-dependent reporter gene construct, without affecting the levels of NF- $\kappa$ B binding to DNA. Furthermore, we clearly show that, using a nuclear Gal4 “one-hybrid” system, the MAPK inhibitors SB203580 and PD0980589 have a direct repressive effect on the transactivation potential of the p65  $\kappa$ B subunit. Therefore, we conclude that, in addition to cytoplasmic activation and DNA binding of NF- $\kappa$ B, the p38 and extracellular signal-regulated kinase MAPK pathways act as necessary cooperative mechanisms to regulate TNF-induced IL-6 gene expression by modulating the transactivation machinery.

Interleukin (IL)<sup>1</sup> 6 contributes to a multitude of physiological and pathophysiological processes. Among its many functions,

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<sup>1</sup> The abbreviations used are: IL, interleukin; MAPK, mitogen-activated protein kinase; NF, nuclear factor; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; CBP, cAMP responsive element binding protein binding protein; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

IL-6 plays an active role in immunological responses, bone metabolism, reproduction, inflammation, neoplasia, and aging. The cellular and molecular biology of IL-6 has been explored by a variety of approaches (1, 2). The regulation of expression of the IL-6 gene is adapted to the key function of this cytokine, namely a systemic alarm signal that recruits diverse host defense mechanisms that serve to limit tissue injury. Inflammation-associated cytokines such as tumor necrosis factor (TNF), IL-1, and platelet-derived growth factor, bacterial products such as endotoxin, and acute viral infections, all enhance IL-6 gene expression. The characterization of the IL-6 promoter revealed a complex control region that can be triggered by multiple activation pathways (3, 4).

In the case of TNF (and of IL-1), the main transcriptional activator for IL-6 gene induction is the nuclear factor (NF)- $\kappa$ B (5–7), which is typically a dimer between p50 and the transactivating subunit p65 (RelA). In unstimulated cells, NF- $\kappa$ B resides in the cytoplasm. Here, the DNA-binding dimer is bound to the inhibitory molecule I $\kappa$ B, from which it is released upon cell stimulation. NF- $\kappa$ B then migrates into the nucleus, where it effects expression of its numerous target genes (8).

We have shown previously that in the mouse fibrosarcoma cell line L929sA, the quantities of activated NF- $\kappa$ B do not correlate with the variations of IL-6 mRNA levels in the cell. We therefore concluded that other cooperative factors or regulatory mechanisms are necessary for modulating the levels of NF- $\kappa$ B-dependent IL-6 mRNA production (9).

Studies over the last few years have shown that different mitogen-activated kinase (MAPK) cascade pathways contribute to the transmission of extracellular signals that can finally result in direct or indirect phosphorylation of various transcription factors and alterations in gene expression (10, 11). More particularly, we reported that abrogation of the p38 MAPK pathway represses TNF-mediated IL-6 gene expression, but not NF- $\kappa$ B activation and DNA binding (12).

In the present article, we report on the essential role of NF- $\kappa$ B to trigger IL-6 gene activation in response to TNF in L929sA cells. Furthermore, we show that, apart from TNF-induced cytoplasmic NF- $\kappa$ B activation and nuclear DNA binding, the TNF-activated p38 and ERK MAPK pathways contribute to transcriptional activation by modulating the transactivation capacity of the NF- $\kappa$ B p65 subunit.

## MATERIALS AND METHODS

*Cell Culture, Cytokines, and Inhibitors*—Murine fibrosarcoma L929sA cells (13) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. 24 h before induction, cells were seeded in multiwell dishes such that they were subconfluent at the time of the experiment.

Secreted IL-6 levels were assayed on the basis of the proliferative response of 7TD1 cells (14). Recombinant murine TNF, produced in *Escherichia coli* and purified to at least 99% homogeneity in our laboratory, had a specific activity of  $1.95 \times 10^8$  IU/mg of protein, as determined in a standardized TNF cytotoxicity assay on 164 WEHI cl 13 cells, and contained <24 EU of endotoxin/mg of protein. Reference TNF (code 88/532) was obtained from the National Institute for Biological Standards and Control (Potters Bar, United Kingdom).

The pyridinyl imidazole SB203580, a specific inhibitor of the p38 MAPK cascade, was a gift from SmithKline Beecham (15); a 20 mM stock solution was prepared in dimethyl sulfoxide. The inhibitor PD098059, which specifically blocks the ERK pathway, was purchased from Calbiochem-Novabiochem International (San Diego, CA; Ref. 16); a 25 mM stock solution was also prepared in dimethyl sulfoxide. Control experiments showed that the final quantities of organic solvent used did not interfere with any of the assays.

**Plasmids and Oligonucleotides**—The 1168-base pair human IL-6 promoter was isolated from pGEM1gHIL61 (17) and inserted as a *Bam*HI-*Xho*I fragment into an intermediate vector. A series of 5'-terminal deletion variants was generated by subcloning the respective fragments with the indicated enzymes (Fig. 1) and was transferred into the multicloning site of pGL3 basic (Promega Biotec, Madison, WI) in front of the luciferase cDNA, giving rise to the plasmids p1168hu.IL6P-luc+, p234hu.IL6P-luc+, p110hu.IL6P-luc+ and p50hu.IL6P-luc+. The recombinant plasmids p(IL6 $\kappa$ B)<sub>3</sub>50hu.IL6P-luc+ and p(GAL4)<sub>2</sub>-50hu.IL6P-luc+ have been described previously (18). The vector pPGK $\beta$ GeobpA, constitutively expressing a neomycin-resistant/ $\beta$ -galactosidase fusion protein under control of the pPGK promoter from the mouse housekeeping enzyme 3-phosphoglycerate kinase, was a kind gift of Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA) (19). The expression plasmids pGal4, pGal4-p65<sup>1-551</sup>, pGal4-p65<sup>1-285</sup>, pGal4-p65<sup>286-551</sup>, pGal4-p65<sup>521-551</sup>, pGal4-p65<sup>286-521</sup>, pGal4-p65<sup>286-551</sup> $\Delta$ 443-476, pGal4-p65<sup>286-521</sup> $\Delta$ 443-476, and pGal4-VP16, expressing the DNA-binding domain of the yeast Gal4 protein either alone, or fused to various parts of the  $\kappa$ B p65 subunit or the transactivation domain of the viral protein VP16, were described previously (20, 21).

**Site-directed Mutagenesis**—Site-directed mutagenesis of the IL-6 promoter was carried out by the gapped heteroduplex method (22), using a transformer site-directed mutagenesis kit (CLONTECH). The following mutator oligonucleotides, each containing a specific restriction site, were used (altered nucleotides are underlined): 5'-AP1, 5'-ATGCCAAAGTGTCTGCAGCAGCACTAATAAAGAA-3'; CRE, 5'-GCGATGCTAAAGGGATCCACATTGCCAAT-3'; NFIL6, 5'-AAAGGACGT-CACAGATATCAATCTTAATAAG-3'; ETS, 5'-CAATCTTAATAAGTC-GACCAATCAGCCCCA-3'; GATA, 5'-CTCCAACAAAGATTCTAGAA-ATGTGG-3'; NF- $\kappa$ B, 5'-CAAATGTGAGATCTTCCCATGAGTCTC-3'; 3'-AP1, 5'-GGGATTTCCCAAGAATCTCAATATTAG-3'.

Mutant clones were screened for the presence of a newly created restriction site and confirmed by sequence analysis. All designed mutations have been described to abolish respective transcription factor binding (3) and were also tested for their biological effect on gene induction in corresponding, stably transfected clones.<sup>2</sup>

**Transfection Procedure**—Stable transfection of L929sA cells was performed by the calcium phosphate precipitation procedure according to standard protocols (23, 24), using a 10-fold excess of the plasmid of interest over the selection plasmid pPGK $\beta$ GeobpA. Transfected cells were selected in 500  $\mu$ g/ml G418 for 2 weeks, after which the resistant cell clones were pooled for further experiments. In this way, the individual clonal variation in expression was averaged, thus providing a reliable response upon induction. The cotransfected plasmid pPGK $\beta$ GeobpA, conferring resistance to G418 and expressing constitutive  $\beta$ -galactosidase enzymatic activity, was further used as an internal control for calculating the protein concentration.

For transient transfections, approximately  $10^5$  exponentially growing L929sA cells were transfected with 1.6  $\mu$ g of the reporter gene plasmid of interest, using the diethylaminoethyl-dextran procedure, essentially as described (25). After 4 h, the transfection mixture was replaced with a 10% dimethyl sulfoxide/Hank's balanced salt solution for 2 min, after which the solution was immediately diluted with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Finally, fresh medium containing 10% serum was put on the cells for another 65 h until experiments were carried out. All transient transfections were performed at least in triplicate.

**Reporter Gene Analysis**—Luciferase assays were carried out accord-

ing to the instructions of the manufacturer (Promega Biotec). Light emission was measured in a luminescence microplate counter (Top-Count; Packard Instrument Co., Meriden, CT). Luciferase assay reagent comprised 270  $\mu$ M coenzyme A and 470  $\mu$ M luciferin (both from Sigma) plus 530  $\mu$ M adenosine triphosphate (Boehringer Mannheim, Mannheim, Germany) in a reaction buffer containing 10 mM Tricine, 0.54 mM (MgCO<sub>3</sub>)<sub>4</sub>Mg(OH)<sub>2</sub>, 1.34 mM MgSO<sub>4</sub>, 0.05 mM EDTA, and 16.7 mM dithiothreitol (all from Sigma). Luciferase activity, expressed in arbitrary light units, was corrected for the protein concentration in the sample, as indicated, by normalization to the co-expressed  $\beta$ -galactosidase levels or by Bradford's protein determination (26).  $\beta$ -galactosidase protein levels were quantified with a chemiluminescent reporter assay Galacto-Light kit (TROPIX, Bedford, MA).

## RESULTS

**TNF-mediated IL-6 Gene Induction Is Exclusively Dependent on Activated NF- $\kappa$ B**—Several hIL-6 promoter deletion variants and point mutants were coupled to the luciferase reporter gene (Fig. 1) and stably transfected into the mouse fibrosarcoma cell line L929sA.

The corresponding cell pools were induced with TNF, and the lysates were assayed for reporter gene expression. A gradually decreasing promoter activity was observed upon truncation of the IL-6 promoter from the 5' end, corresponding to a sequential removal of various enhancer elements in the promoter sequence (Fig. 2A). On the other hand, the relative inducibility by TNF remained comparable for the various deletion variants, which contained the  $\kappa$ B motif (Fig. 2B). By contrast, deletion of the NF- $\kappa$ B sequence environment, as in the reporter construct p50hu.IL-6P-luc+, led to a complete loss of inducibility by TNF. These results clearly indicate an essential role of NF- $\kappa$ B for the gene response to TNF in L929sA cells.

A similar conclusion can be drawn from the results obtained with the different point-mutated hIL-6 promoter variants (Fig. 2C). Mutation of the potential binding sites of a variety of transcription factors did not significantly affect the inducibility by TNF. Only the specific NF- $\kappa$ B mutation could no longer be stimulated by TNF. Proof for the essential role of NF- $\kappa$ B was obtained by inserting multiple NF- $\kappa$ B-binding sites in front of the non-responsive construct p50hu.IL-6P-luc+ (Fig. 2B). This insertion conferred again TNF inducibility (Fig. 2B). Taken together, these data point to a key function of the  $\kappa$ B-element for IL-6 gene activation in response to TNF and do not show the primary need for cooperation by other DNA-binding factors.

**Involvement of p38 MAPK Pathway in the Transcriptional Activity of p65**—We have shown previously that inhibition of the p38 MAPK pathway abrogates TNF-mediated IL-6 gene expression without affecting the levels of TNF-induced NF- $\kappa$ B release and DNA binding (12). The involvement of this pathway in NF- $\kappa$ B-mediated transactivation has now been further studied using the Gal4 "one-hybrid" technique in eukaryotic cells, as explained below in more detail. This system has the advantage that the Gal4-transactivator fusion proteins are exclusively nuclear and are regulated independently of I $\kappa$ B (20, 27, 28).

As transient transfection experiments with vectors expressing the  $\kappa$ B subunit p50 or p65, showed activation of p(IL6 $\kappa$ B)<sub>3</sub>50hu.IL-6P-luc+ only with  $\kappa$ B p65 (data not shown), we have continued our further studies with the analysis of the  $\kappa$ B p65 transactivation mechanism. This fits with the fact that, in contrast to p50, the p65 subunit has two strong transcriptional activating domains (20). The plasmids pGal4, pGal4-p65<sup>1-551</sup>, and pGal4-VP16 were transfected into L929sA cells and stable pools were expanded. These were assayed by transient transfection with a reporter gene under control of two Gal4-binding sites (p(GAL4)<sub>2</sub>50hu.IL-6P-luc+). The transactivation potential of the chimeric proteins, driving the promoter of p(GAL4)<sub>2</sub>50hu.IL-6P-luc+, was determined by measuring the corresponding luciferase activity. The same inductions

<sup>2</sup> W. Vanden Berghe, S. Plaisance, E. Boone, K. De Bosscher, M. L. Schmitz, W. Fiers, and G. Haegeman, unpublished results.

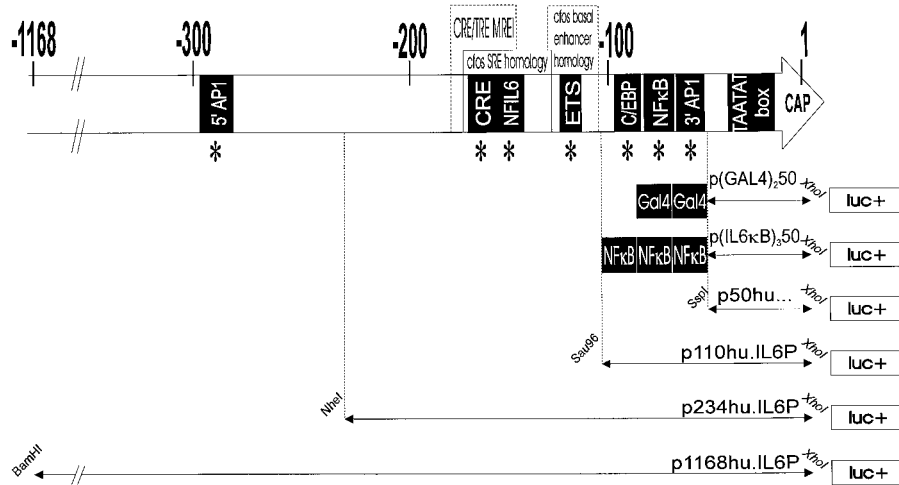


FIG. 1. Representation of the various IL-6 promoter-derived recombinant reporter gene constructions. The IL-6 promoter is shown on top, position 1 corresponding to the first nucleotide of the major RNA transcript (17, 47). Black boxes represent binding sites for the indicated transcription factors. Asterisk indicates that the particular transcription factor site has been mutated to yield the corresponding point-mutated version of the wild-type promoter reporter gene construction p1168hu.IL6P-luc+. The different 5'-terminal shortened promoter variants obtained by the restriction enzymes *Bam*HI, *Nhe*I, *Sau*96, and *Ssp*I, respectively, and on the 3' end *Xho*I, are presented as thin double-headed arrows; these promoter constructs were coupled to the luciferase gene as described under "Materials and Methods." The 50-base pair minimal promoter has been used as such (p50hu.IL6P-luc+) or preceded by three NF- $\kappa$ B sites (the indication IL6 $\kappa$ B refers to the NF- $\kappa$ B site as it occurs in the IL-6 promoter) or two Gal4-binding sites.

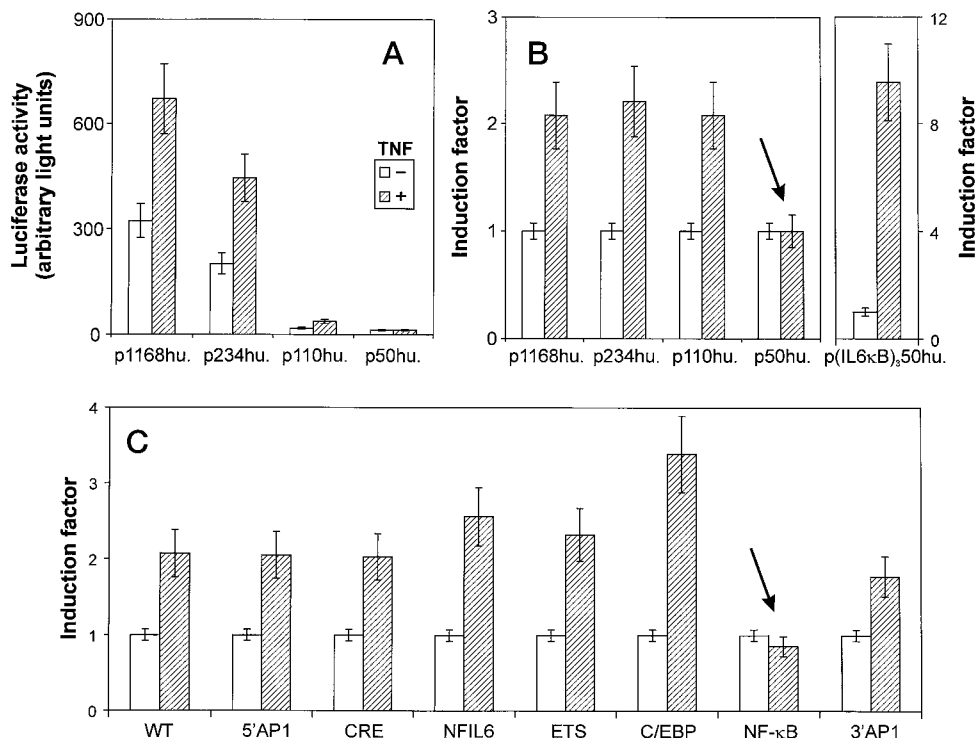
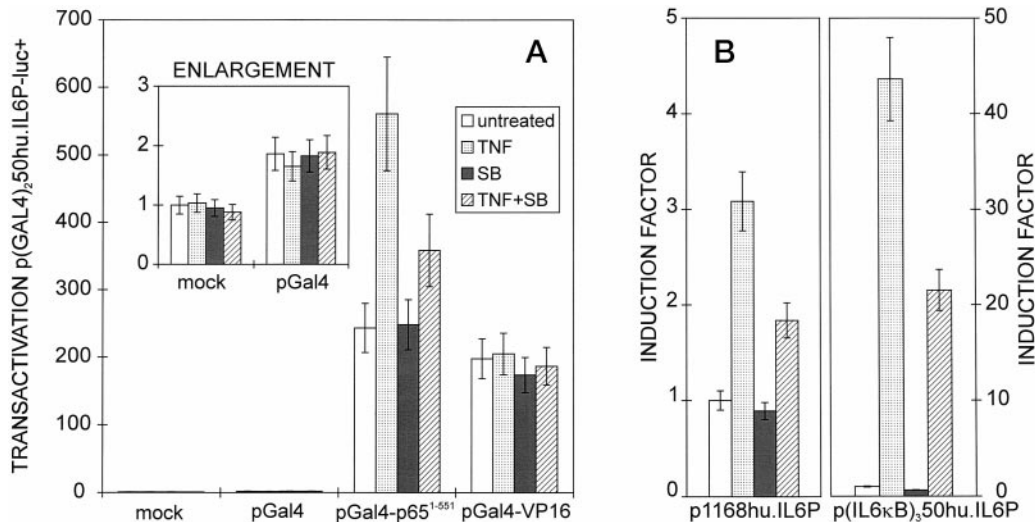


FIG. 2. TNF-mediated IL-6 gene induction is dependent on activated NF- $\kappa$ B. Stable cell pools of various deleted and point-mutated hIL-6 promoter/reporter gene constructions, were left untreated or induced with 2000 IU/ml TNF for 6 h, followed by assaying the lysates for luciferase activity. The different deletion variants tested (A and B) are described in Fig. 1. Point-mutated variants of p1168hu.IL-6P-luc+ tested (C) are indicated by their respective mutated transcription factor-binding site as described in Fig. 1. Luciferase activity is expressed in arbitrary units (A) or as a relative "induction factor," i.e. the ratio between the expression levels of the induced and the untreated state, taken to 1 (B and C). The arrows point to the absence of a TNF induction effect when NF- $\kappa$ B is absent or mutated.

were carried out on stable pools of p1168hu.IL-6P-luc+ and p(IL6 $\kappa$ B)<sub>3</sub>50hu.IL-6P-luc+, as a control experiment.

Fig. 3A displays the constitutive level of transactivation by the nuclear fusion proteins Gal4-p65 and Gal4-VP16, as compared with mock-transfected cells or pools expressing Gal4 alone. Interestingly, the basal and constitutive transactivation by Gal4-p65 could be further up-regulated (2–3 times) with TNF, and this enhanced activation could be down-modulated by the inhibitor SB203580. This finding suggests modulatory

effects on the transactivation potential of the  $\kappa$ B p65 subunit by the TNF-activated p38 MAPK pathway. Moreover, the specificity of the observed modulation was further ensured by the lack of a TNF effect on the constitutive activity of Gal4-VP16. When the activity of the IL-6 promoter (p1168hu.IL-6P-luc+) or a NF- $\kappa$ B-dependent reporter construct (p(IL6 $\kappa$ B)<sub>3</sub>50hu.IL-6P-luc+) was tested, we found a similar responsivity to TNF and SB203580, demonstrating that regulation of  $\kappa$ B-dependent gene activation runs in parallel with modulation of the trans-



**FIG. 3. Involvement of the p38 MAPK pathway in NF- $\kappa$ B p65 transactivation.** *A*, untransfected cells (mock) or stable pools expressing the nuclear proteins Gal4, Gal4-p65<sup>1-551</sup>, or Gal4-VP16 were transiently transfected with the reporter plasmid p(GAL4)<sub>3</sub>,50hu,IL6P-luc+. 65 h after transfection, cells were left untreated or induced with 2000 IU/ml TNF. Some cultures were treated with 10  $\mu$ M SB203580, starting at -2 h, as indicated. The transactivation potential of the different Gal4 fusion proteins was determined by measuring the luciferase activity and normalization to galactosidase levels expressed by the internal control plasmid. The results in *A* are expressed in arbitrary light units to allow comparison of the transactivation potential of the various Gal4 constructs. *B*, in parallel, similar inductions were carried out on stable pools of luciferase constructs, either controlled by the wild-type IL-6 promoter (p1168hu,IL6P-luc+) or the construct containing three NF- $\kappa$ B sites in front of the IL-6 minimal promoter (p(IL6 $\kappa$ B)<sub>3</sub>,50hu,IL6P-luc+). The experiment was performed in triplicate and has been repeated twice.

activation potential of the nuclear fusion protein Gal4-p65. In conclusion, our results show that in addition to cytoplasmic activation of NF- $\kappa$ B, TNF regulates the transcriptional activity of the nuclear NF- $\kappa$ B complex by a p38 MAPK-dependent phosphorylation-step, involving a protein of the transactivation complex.

*The p38 and ERK MAPK Pathways Cooperate in the p65 Transactivation Mechanism in Response to TNF*—In previous studies, the TNF-induced IL-6-mRNA and protein levels of the endogenous IL-6 gene could be nearly completely inhibited by SB203580 (12), presumably because of potentiation of transcriptional effects by post-transcriptional and translational regulatory events on AUUUA repeated motifs located in the 3'-untranslated region of IL-6 mRNA (29, 30). In contrast, at the transcription level, in absence of this regulatory motifs, using the luciferase reporter gene system, the activation of p65 by TNF could not be totally counteracted by SB203580, either on the NF- $\kappa$ B-dependent promoter constructs (p1168hu,IL6-Pluc+ and p(IL-6 $\kappa$ B)<sub>3</sub>,50hu,IL6-luc+; Fig. 3*B*) or on the nuclear fusion protein Gal4-p65<sup>1-551</sup> (Fig. 3*A*). Hence, phosphorylation-dependent mechanisms other than the p38 MAPK cascade activation may also contribute to the TNF-mediated activation of the promoter-transcription complex. In this respect, we and others have previously shown that the ERK pathway is also subject to activation by TNF (31, 32). Therefore, we also tested the effect of the inhibitor PD98059 on TNF-mediated gene activation. Indeed, PD98059 partially inhibited TNF-mediated gene activation both for a  $\kappa$ B-dependent promoter (Fig. 4*B*) as well as for the reporter gene driven by Gal4-p65<sup>1-551</sup> (Fig. 4*A*). Moreover, the inhibition by both compounds was additive and decreased TNF-induced activity to basal level without affecting the levels of the activated NF- $\kappa$ B complex or the expression levels of the nuclear protein Gal4-p65, respectively (data not shown). Again, these effects were highly specific for gene activation by NF- $\kappa$ B, in particular for the transactivator subunit p65, as they did not affect a different promoter reporter gene system (Fig. 4*C*). These data suggest that (at least) two different activated MAPK pathways cooperate to regulate  $\kappa$ B-dependent gene activation in response to TNF.

*Role of the Transactivation Domains of p65 in TNF-stimulated Transcriptional Activity*—It has been reported that the

p65 subunit contains at least two strong transactivation domains within its C terminus. The first domain, TA1, comprises the last 30 amino acids of p65, whereas TA2 comprises the adjacent 90 amino acids (20). To delineate the p65 domain, responsible for TNF-mediated transactivation, we tested various fusion proteins of Gal4 coupled to different parts of the p65 subunit (Fig. 5*B*). Fig. 5*A* shows that, upon TNF treatment, up-regulation of transactivation was observed with Gal4-p65<sup>1-551</sup>, as was already shown above, as well as with Gal4-p65<sup>286-551</sup> containing an intact C-terminal part of p65. By contrast, activation by TNF was absent with the variant Gal4-p65<sup>286-521</sup>, in which the TA1 domain is missing. This indicates the essential role of the TA1 domain for transactivation by p65 in response to TNF. However, transactivation could only be partially restored by fusing the TA1 domain to the Gal4 segment (Gal4-p65<sup>521-551</sup>), indicating that other protein domains might also be of importance in TNF-mediated signaling. As a matter of fact, the chimeric form p65<sup>286-551</sup> $\Delta$ 443-476, in which the TA1 domain is entirely present, but which lacks the TA2 domain, also showed a strongly reduced transactivation capacity, suggesting a cooperative role of the TA2 segment. Indeed, in the construction Gal4-p65<sup>286-521</sup> $\Delta$ 443-476, where both activation domains are removed, TNF-induced transactivation was totally abolished. Evidently, the same was true for Gal4-p65<sup>1-285</sup>, which lacks the entire C-terminal half of the p65 molecule. These results defined the two transactivation domains as the protein segments necessary for mediating the p65 transcriptional activation by TNF.

## DISCUSSION

In this paper, we have focused on the transcriptional activation by the factor NF- $\kappa$ B in response to TNF. By the use of various deleted and point-mutated versions of the IL-6 promoter, we have documented the key role of the  $\kappa$ B motif for induction by TNF. A number of previous reports have already described the necessity of cooperation and association of NF- $\kappa$ B with other DNA-bound transcription factors for optimal gene activation (33-36). The deletion analysis of the IL-6 promoter shows that such factors have indeed a co-activating and integrating function for full stimulation of the IL-6 promoter. How-

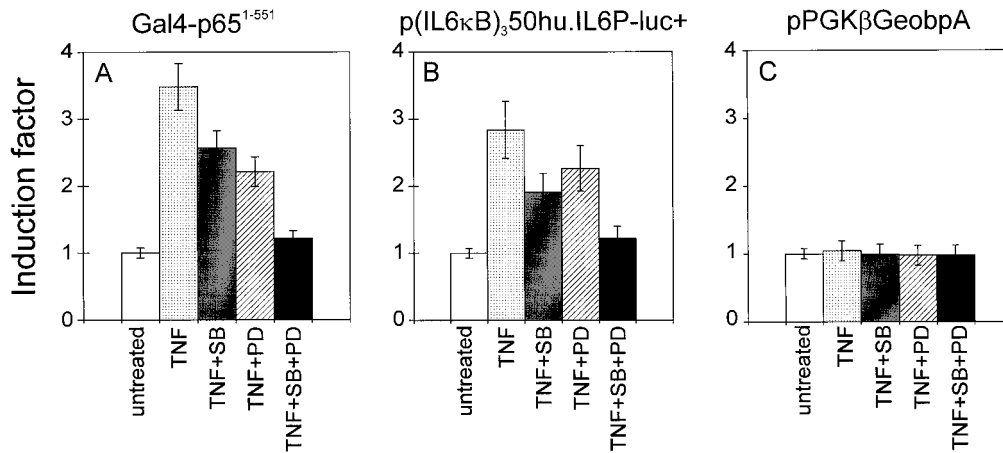


FIG. 4. Both the p38 and the ERK MAPK pathway cooperate in the NF- $\kappa$ B p65 transactivation potential in response to TNF. A stable pool expressing the nuclear fusion protein Gal4-p65<sup>1-551</sup> was transiently transfected with a Gal4-dependent reporter plasmid (p(GAL4)<sub>2</sub>50hu.IL-6P-luc+). 65 h after transfection, cells were either untreated, induced with 2000 IU/ml TNF for 6 h, or treated starting at -2 h, with 10  $\mu$ M SB203580, with 10  $\mu$ M PD098059, or with both compounds together. The transactivation potential of the Gal4-p65<sup>1-551</sup> fusion protein was determined by measuring the luciferase activity (A). In parallel, similar inductions were carried out on a stable pool of p(IL6 $\kappa$ B)<sub>3</sub>50hu.IL-6P-luc+ (B) or the control plasmid pPGK $\beta$ GeobpA (C). The experiment, carried out in triplicate, has been repeated at least four times.

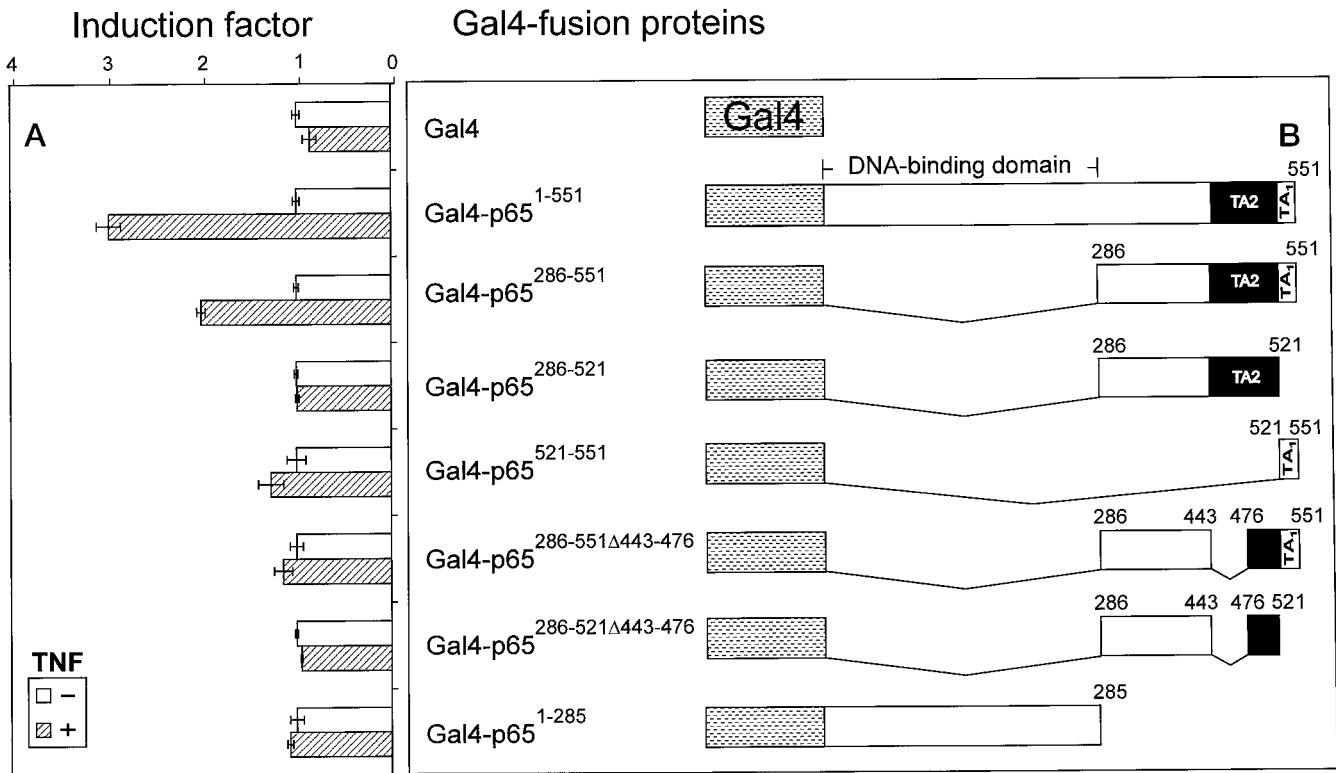


FIG. 5. Involvement of the transactivation domains of NF- $\kappa$ B p65 in TNF-stimulated gene activation. A, stable pools of the complete and of the various deleted versions of the fusion protein Gal4-p65 were transiently transfected with p(GAL4)<sub>2</sub>50hu.IL-6P-luc+. 65 h after transfection, cells were induced with 2000 IU/ml TNF for 6 h, followed by measuring the luciferase activities, expressed as a relative "induction factor," *i.e.* the ratio between the expression levels of the induced and the untreated state, taken to 1. Data shown are mean of an experiment carried out in quadruplicate and repeated twice. B, schematic overview of the various Gal4-p65 deletion constructs.

ever, using "loss-of-function" mutants of the IL-6 promoter, the crucial role of NF- $\kappa$ B is obvious, without the primary need for other associating DNA-bound factors. Using the "gain-of-function" approach by inserting multiple  $\kappa$ B sites in front of an unresponsive promoter, the TNF response could be restored.

Activation of NF- $\kappa$ B and its binding to DNA is, however, not sufficient for IL-6 gene activation by TNF; the requirement of additional activating mechanisms has already been described previously (4, 9). More particularly, recently, we have established the importance of the p38 MAPK pathway as a necessary mechanism for transcriptional activity of the IL-6 promoter

(12). Our present data with the nuclear fusion protein Gal4-p65 show that the basal constitutive transcriptional activity of NF- $\kappa$ B p65, but not that of another acidic transactivator like VP16, could be specifically enhanced by TNF, independently of effects involving the cytoplasmic activation of NF- $\kappa$ B. This increased transcriptional activity is the result of the activation of MAPK pathways by TNF. The p38 as well as the ERK MAPK pathway contribute to the specific up-regulation by TNF without affecting the basal TNF-independent activity. This suggests a signaling system of at least two steps from the TNF receptor to the gene, in which, first, NF- $\kappa$ B becomes activated

in the cytoplasm by a I $\kappa$ B $\alpha$ -specific kinase, as recently reported (37), and second, the "nuclear" transactivation potential of the DNA-bound complex is modulated by (an) additional phosphorylation event(s) via different TNF-activated MAPKs. Such a multilevel regulation allows fine tuning and/or gene specific modulation of the transcriptional activity.

Whether MAPKs act directly on the p65 transcription activation complex, or via intermediate kinases, remains an open question. Nevertheless, by *in vitro* kinase assays it was shown that neither I $\kappa$ B $\alpha$ , nor the NF- $\kappa$ B DNA-binding subunits p50, nor the transactivating C-terminal half of p65 became phosphorylated by p38. However, it is still conceivable that, *in vivo*, a kinase downstream in the p38 and ERK pathways phosphorylates NF- $\kappa$ B subunits (38).

Furthermore, whether MAPK-dependent phosphorylation effectively takes place on the p65 subunit itself is also not proven. Evidence for phosphorylation-dependent regulation of NF- $\kappa$ B has already been reported by Naumann and Scheidereit (39), who found increased binding of NF- $\kappa$ B upon phosphorylation of the p65 subunit. Recently, Zhong and co-workers (40) showed a strongly increased transcriptional activity after phosphorylation of p65 on a consensus cAMP-dependent protein kinase site, which is located in the p65 Rel homology domain. However, since this site is clearly different and distinct from the p65 transactivation domains TA1 and TA2, our data point to another phosphorylation system. Schmitz *et al.* (21) also observed increased transcriptional activity upon treatment of HeLa cells with phorbol ester and suggested a possible phosphorylation in the p65 TA2 domain by a protein kinase C-dependent mechanism. It cannot, however, be excluded that different signals and/or stimuli converge into the same activation region of the p65 subunit.

Recent data connect transcriptional activity of the  $\kappa$ B p65 subunit with the versatile coactivator/cointegrator proteins p300 and CBP (41, 42). Extensive protein-protein interactions have been mapped between the N- and C-terminal regions of CBP/p300, and the C terminus of p65, containing both transactivation domains. Interestingly, since our results with the Gal4-p65 fusion proteins demonstrate a crucial role of these domains of p65 for TNF inducibility, the possible phosphorylation status of these domains in p65-CBP interaction may be of particular interest. Furthermore, the coactivator proteins CBP/p300 are subject themselves to phosphorylation control and were described as a nuclear target for S6 kinase pp90<sup>rsk</sup> and for cyclin-dependent kinases (43). Other targets for MAPK phosphorylation are part of the RNA polymerase complex (28, 44, 45). Since RNA polymerase II is constitutively associated with CBP/p300, interaction of the coactivator with NF- $\kappa$ B in the IL-6 promoter complex may efficiently recruit the polymerase complex, to trigger subsequent IL-6 gene expression (46).

In summary, our data show that p38 and ERK MAPK signaling pathways constitute an additional level of gene regulation by the transcription factor NF- $\kappa$ B, more particularly of the p65 subunit, in response to TNF. Modulation of the p65 transactivation occurs in the nucleus and independent from I $\kappa$ B regulation, since it is faithfully reproduced with the Gal4 fusion proteins. However, whether *in vivo* the  $\kappa$ B p65 subunit itself is a direct or indirect substrate of TNF-activated p38 and/or ERK MAPK pathways, or else is part of an integrated transcriptionally active complex, which is subject to modulation by MAPK phosphorylation, needs further study.

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#### REFERENCES

- Hirano, T., and Kishimoto, T. (1992) *Res. Immunol.* **143**, 689–788
- Keller, E. T., Wanagat, J., and Ershler, W. B. (1996) *Frontiers Biosci.* **1**, d340–d357
- Dendorfer, U., Oettgen, P., and Libermann, T. A. (1994) *Mol. Cell. Biol.* **14**, 4443–4454
- Haegeman, G., and Fiers, W. (1995) in *Signalling Mechanisms: From Transcription Factors to Oxidative Stress* (Packer, L., and Wirtz, K., eds) pp. 375–382, Springer Verlag, Berlin
- Libermann, T. A., and Baltimore, D. (1990) *Mol. Cell. Biol.* **10**, 2327–2334
- Shimizu, H., Mitomo, K., Watanabe, T., Okamoto, S., and Yamamoto, K. (1990) *Mol. Cell. Biol.* **10**, 561–568
- Zhang, Y., Lin, J.-X., and Vilček, J. (1990) *Mol. Cell. Biol.* **10**, 3818–3823
- Baeuerle, P. A., and Henkel, T. (1994) *Annu. Rev. Immunol.* **12**, 141–179
- Patestos, N. P., Haegeman, G., Vandevoorde, V., and Fiers, W. (1993) *Biochimie* **75**, 1007–1018
- Treisman, R. (1996) *Curr. Opin. Cell Biol.* **8**, 205–215
- Cohen, P. (1997) *Trends Cell Biol.* **7**, 353–361
- Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J. C., Haegeman, G., Cohen, P., and Fiers, W. (1996) *EMBO J.* **15**, 1914–1923
- Vanhaesebroeck, B., Decoster, E., Van Ostade, X., Van Bladel, S., Lenaerts, A., Van Roy, F., and Fiers, W. (1992) *J. Immunol.* **148**, 2785–2794
- Landegren, U. (1984) *J. Immunol. Methods* **67**, 379–388
- Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) *FEBS Lett.* **364**, 229–233
- Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 27489–27494
- Haegeman, G., Content, J., Volckaert, G., Derynck, R., Tavernier, J., and Fiers, W. (1986) *Eur. J. Biochem.* **159**, 625–632
- Plaisance, S., Vanden Berghe, W., Boone, E., Fiers, W., and Haegeman, G. (1997) *Mol. Cell. Biol.* **17**, 3733–3743
- Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991) *Cell* **64**, 693–702
- Schmitz, M. L., and Baeuerle, P. A. (1991) *EMBO J.* **10**, 3805–3817
- Schmitz, M. L., dos Santos Silva, M. A., and Baeuerle, P. A. (1995) *J. Biol. Chem.* **270**, 15576–15584
- Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., and Fritz, H.-J. (1989) *Nucleic Acids Res.* **17**, 4441–4453
- Graham, F. L., and van der Eb, A. J. (1973) *Virology* **52**, 456–467
- Vanhoenacker, P., Fiers, W., and Haegeman, G. (1994) *Eur. Cytokine Netw.* **5**, 283–291
- Lopata, M. A., Cleveland, D. W., and Sollner-Webb, B. (1984) *Nucleic Acids Res.* **12**, 5707–5717
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Schmitz, M. L., Indorf, A., Limbourg, F. P., Städtler, H., Traencker, E. B.-M., and Baeuerle, P. A. (1996) *Mol. Cell. Biol.* **15**, 4052–4063
- Bellier, S., Dubois, M.-F., Nishida, E., Almouzni, G., and Bensaude, O. (1997) *Mol. Cell. Biol.* **17**, 1434–1440
- Kruys, V., and Huez, G. (1994) *Biochimie* **76**, 862–866
- Lee, J. C., and Young, P. R. (1996) *J. Leukocyte Biol.* **59**, 152–157
- Van Lint, J., Agostinis, P., Vandevoorde, V., Haegeman, G., Fiers, W., Merlevede, W., and Vandenheede, J. R. (1992) *J. Biol. Chem.* **267**, 25916–25921
- Saklatvala, J., Davis, W., and Guesdon, F. (1996) *Phil. Trans. R. Soc. Lond. Biol. Sci.* **351**, 151–157
- Kazubaska, W., Hooft van Huijsduijnen, R., Ghersa, P., DeRaemy-Schenk, A.-M., Chen, B. P. C., Hai, T., DeLamar, J. F., and Whelan, J. (1993) *Mol. Cell. Biol.* **13**, 7180–7190
- Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T., and Akira, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10193–10197
- Stein, B., Baldwin, A. S., Jr., Ballard, D. W., Greene, W. C., Angel, P., and Herrlich, P. (1993) *EMBO J.* **12**, 3879–3891
- Ray, A., Hannink, M., and Ray, B. K. (1995) *J. Biol. Chem.* **270**, 7365–7374
- Régnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (1997) *Cell* **90**, 373–383
- Wesselborg, S., Bauer, M. K. A., Vogt, M., Schmitz, M. L., and Schulze-Osthoff, K. (1997) *J. Biol. Chem.* **272**, 12422–12429
- Naumann, M., and Scheidereit, C. (1994) *EMBO J.* **13**, 4597–4607
- Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) *Cell* **89**, 413–424
- Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2927–2932
- Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) *Science* **275**, 523–527
- Shikama, N., Lyon, J., and La Thangue, N. B. (1997) *Trends Cell Biol.* **7**, 230–236
- Venetianer, A., Dubois, M. F., Nguyen, V. T., Bellier, S., Seo, S. J., and Bensaude, O. (1995) *Eur. J. Biochem.* **233**, 83–92
- Marshall, N. F., Peng, J., Xie, Z., and Price, D. H. (1996) *J. Biol. Chem.* **271**, 27176–27183
- Kee, B. L., Arias, J., and Montminy, M. R. (1996) *J. Biol. Chem.* **271**, 2373–2375
- Ray, A., LaForge, K. S., and Sehgal, P. B. (1990) *Mol. Cell. Biol.* **10**, 5736–5746