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Immunology

Phosphorylation at threonine-235 by a *ras*-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6

(C/EBP/gp130/cytokine/signal transduction)

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ABSTRACT **NF-IL6, a member of the basic leucine zipper (bZIP) family transcription factors, is involved in expression of inducible genes involved in immune and inflammatory responses. We observed that coexpression of oncogenic $p21^{ras}$ stimulated the transactivating activity of NF-IL6 and induced phosphorylation of Thr-235 located just N-terminal to the DNA binding domain of NF-IL6. Recently, mitogen-activated protein (MAP) kinases have been shown to be implicated in the cellular response to activated *ras*. Purified MAP kinases specifically phosphorylated Thr-235 of NF-IL6 *in vitro*. Mutation of Thr-235 abolished the *ras*-dependent activation of NF-IL6. From these results, we conclude that NF-IL6 is regulated through phosphorylation by MAP kinases in response to activated *ras*.**

NF-IL6 was initially identified as a nuclear factor binding to the interleukin 1 (IL-1) responsive element in the IL-6 gene (1). The cloned NF-IL6 exhibits a homology with CCAAT/enhancer binding protein (C/EBP), a member of the basic leucine zipper (bZIP) family of transcription factors (2). This protein has recently been reported by other groups under the names AGP/EBP, LAP, IL-6DBP, rNFIL-6, C/EBPβ, and CRP2 (3–8). NF-IL6 activates several acute-phase protein genes through the IL-6 responsive elements, implying that it is a nuclear target for IL-6 signal transduction (5, 9). NF-IL6 has also been shown to be responsible for regulation of the genes encoding albumin, c-fos, and several adipocyte-specific proteins. Furthermore, NF-IL6 has been implicated in activation of various genes involved in inflammatory and immune responses, including the IL-8, granulocyte/colony-stimulating factor, IL-1, and immunoglobulin genes (10). Thus, NF-IL6 has turned out to be a pleiotropic transactivator involved in signal transduction and cell differentiation.

The signal transduction pathway mediated by growth factors and cytokines is initiated by activation of tyrosine kinases. Several cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, and granulocyte–macrophage/colony-stimulating factor activate intracellular tyrosine kinases (11). Recently, the *ras*-encoded protein has been shown to act downstream of several tyrosine kinases (12–14). However, signaling events subsequent to *ras* are still poorly understood except that they involve the activation of several serine/threonine kinases. In the case of the IL-6 signaling process, IL-6-triggered association of IL-6 receptor and gp130 activates *ras* in addition to an as yet unidentified tyrosine kinase (14–17). Recently, gp130 has been shown to be a common signal transducer for several cytokines, including IL-6, oncostatin M, leukemia inhibitory factor, and ciliary neurotrophic factor, suggesting that NF-IL6 may be the target of a common signal transduction pathway shared by several cytokines (18–20).

As a first step toward understanding the gp130-mediated signaling pathway, we examined the effect of $p21^{ras}$ expression on NF-IL6-mediated transactivation and observed that coexpression of oncogenic $p21^{ras}$ stimulated the transcriptional activity of NF-IL6. Furthermore, we demonstrated that the ras-dependent activation of NF-IL6 was mediated through phosphorylation by mitogen-activated protein (MAP) kinases.

MATERIALS AND METHODS

Cells and Plasmids. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). P19 cells were grown in minimal essential medium (α modification) (GIBCO) supplemented with 10% FCS. An IL-6 promoter luciferase reporter plasmid and vectors expressing NF-IL6 and NF-IL6Δ(41–205) have been described (21). An oncogenic *ras* expression vector (pCDSRαras^{Val12}) was a gift from K. Kaibuchi (Kobe University). For construction of the site-directed mutants of NF-IL6, the coding region of NF-IL6 was cloned into an M13 vector, and a single-stranded M13 DNA was prepared for the mutagenesis reactions. Mutations were introduced by an oligonucleotide-directed *in vitro* mutagenesis system according to the manufacturer's instructions (Amersham).

Luciferase Assays. Cells were transfected with 5 μg of the reporter plasmid and 0.5 μg of CMV-NFIL6 expression vector, and/or 1 μg of the oncogenic *ras* expression vector by the calcium phosphate coprecipitation method. Cells were harvested 48 hr after transfection and measured for luciferase activity (21).

Assays for MAP Kinase Activity. MAP kinases were partially purified with a DEAE-cellulose (DE52) column and phenyl-Sepharose column as described (22). A kinase assay was performed in 18 mM Hepes, pH 7.5/50 μM ATP/10 mM $Mg(OAc)_2$/1 mg of myelin basic protein (MBP) per ml/10 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq)/5 μl of samples for 10 min at 30°C. Phosphorylated MBP was separated on a SDS/15–25% polyacrylamide gradient gel.

Cell Labeling and Immunoprecipitation. Cells were labeled with $^{32}P_i$ (3 mCi/ml) in phosphate-free DMEM for 5 hr. Prior to labeling, cells were serum starved in DMEM supplemented with 0.5% fetal bovine serum for 24 hr. Nuclei were isolated from labeled cells by hypotonic lysis and lysed in boiling hot SDS lysis buffer (50 mM Tris·HCl, pH 7.5/0.5% SDS/5 mM dithiothreitol) (23). The lysates were diluted with 4 vol of

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Abbreviations: IL, interleukin; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein.

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RIPA buffer lacking SDS (0.15 M NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate). NF-IL6 proteins were immunoprecipitated from nuclear lysates by using 2 μg of affinity-purified polyclonal antibody per ml and were fractionated on SDS/4–20% polyacrylamide gradient gels. The antibody used for immunoprecipitation was raised against the 17 amino acids (RRERNNIAVRKSRDKAK) in the DNA binding domain of NF-IL6 (21).

Peptide Mapping and Phosphoamino Acid Analyses. For peptide mapping, the immunoprecipitated NF-IL6 proteins were eluted from an SDS/polyacrylamide gel and digested in 50 mM NH_4HCO_3 containing 100 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin per ml. The phosphotryptic peptides were oxidized and analyzed in the first dimension by cellulose thin-layer electrophoresis at pH 1.9 and in the second dimension by ascending chromatography in 1-butanol/pyridine/acetic acid/distilled water (75:50:15:60). For phosphoamino acid analysis, the labeled peptide was hydrolyzed in 6 M HCl for 2 hr at 106°C and analyzed by cellulose thin-layer electrophoresis at pH 3.5.

***In Vitro* Phosphorylation Assays.** The phosphorylation reaction was performed in 50 mM Tris·HCl/20 mM $MgCl_2$/100 μM unlabeled ATP/10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol)/100 μM each synthetic peptide/2 ng of MAP kinases purified from *Xenopus* oocytes at 25°C for 30 min. The phosphorylated peptide was fractionated on an SDS/polyacrylamide gel.

Other Procedures. Synthetic peptides were prepared based on the primary sequence of NF-IL6 protein (amino acids 209–242; AYLGYQAVPSGSSGSLSTSSSSSPPGTPSPADAK). Peptides for the epidermal growth factor receptor (amino acids 661–681, KRELVEPLTPSGEAPNQALLR), MBP (amino acids 89–107, FKNIVTPRTPPPSQGKGRG), and Myc protein (amino acids 51–66, KKFELLPTPPLSPSRR) were gifts from R. J. Davis (University of Massachusetts Medical School). For mass spectrometry electrospray, ionization mass and tandem mass spectra of both phosphorylated and unphosphorylated peptides were recorded on the TSQ-700 instrument with the Finnigan-M electrospray ion. Collision-activated dissociation MS/MS analyses were performed on the Finnigan TSQ 70 with a 10-eV collision energy and 1.5×10^{-3} torr (1 torr = 133.3 Pa) of argon as the collision gas.

RESULTS

Oncogenic *ras* Augments the Transcriptional Activity of NF-IL6. Fibroblast NIH 3T3 and embryonic carcinoma P19 cells were cotransfected with an IL-6 promoter–luciferase gene reporter construct and with vectors expressing NF-IL6 and/or oncogenic p21^{ras} (Fig. 1). Expression of oncogenic p21^{ras} alone stimulated transcriptional activity of the reporter gene a few times above background, while expression of NF-IL6 alone resulted in a similar, somewhat smaller increase in reporter gene activity. However, simultaneous expression of both NF-IL6 and oncogenic p21^{ras} resulted in 10- to 20-fold stimulation of the reporter gene. This result shows that oncogenic p21^{ras} markedly enhances the transactivating function of NF-IL6.

Oncogenic *ras* Augments the Phosphorylation of NF-IL6 at Thr-235. We considered the possibility that oncogenic p21^{ras} might augment transactivation of NF-IL6 by stimulating its phosphorylation, as has been reported in the case of c-jun (24). NIH 3T3 cells were transfected with both NF-IL6 and oncogenic p21^{ras} expression vectors, and ^{32}P-labeled proteins were immunoprecipitated with anti-NF-IL6 antibody. These proteins were subsequently analyzed by two-dimensional tryptic peptide mapping. In this case, we used NF-IL6Δ(41–205), a deletion mutant of NF-IL6 instead of wild-type NF-IL6, in order to separate it from endogenous NF-IL6 on

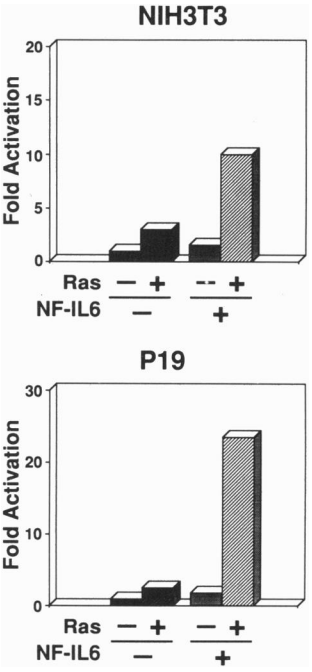


FIG. 1. Stimulation of NF-IL6 transcriptional activity by cotransfected oncogenic p21^{ras}. NIH 3T3 and P19 cells were transfected with the human IL-6 promoter–luciferase reporter gene and CMV-NFIL6 in the presence or absence of oncogenic p21^{ras} expression vector by the calcium phosphate procedure. Transcriptional activity was determined by assaying the luciferase activity of cellular extracts prepared 48 hr after transfection. One unit of relative activity represents the luciferase activity obtained after transfection of the reporter gene. This experiment was repeated three times and similar results were obtained.

SDS/polyacrylamide gels. In parallel with this experiment, we examined the phosphorylation sites of NF-IL6 in Hep3B cells. By transient expression of a series of site-directed mutants of NF-IL6 and subsequent phosphopeptide map-

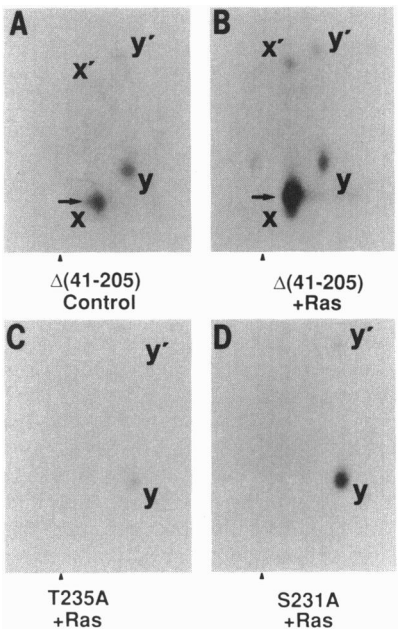


FIG. 2. Effect of oncogenic p21^{ras} on NF-IL6 phosphorylation. NIH 3T3 cells were transfected with CMV-NFIL6Δ(41–205) or site-directed mutant derivatives in the presence of *raf* mutant EHneo301(Control) or oncogenic p21^{ras} expression vector. Two-dimensional phosphopeptide mapping was performed.

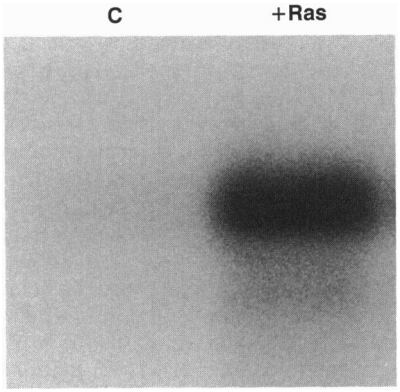
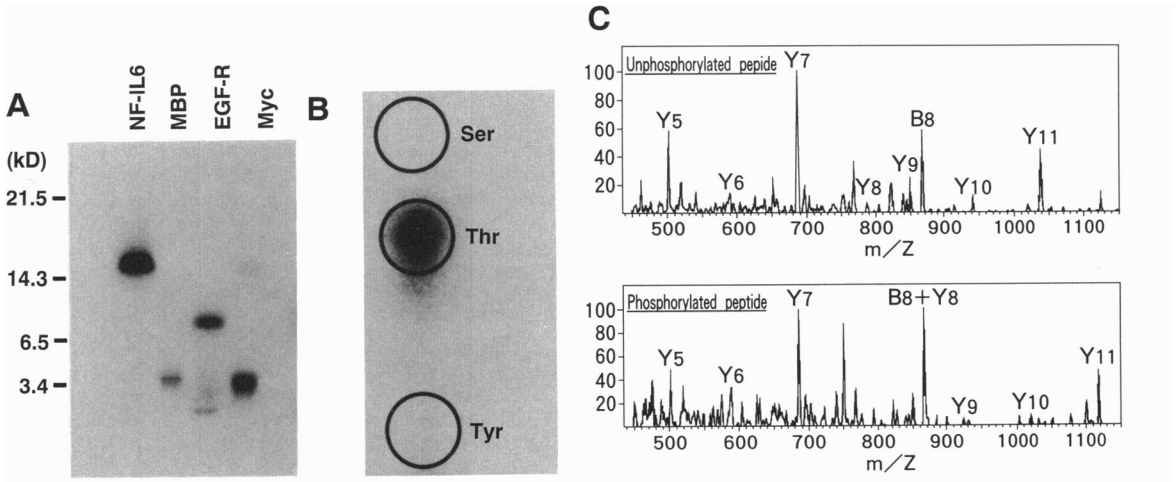


FIG. 3. Activation of MAP kinases by transfection of oncogenic p21^{ras} into NIH 3T3 cells. MAP kinases were partially purified from the extracts of NIH 3T3 cells transiently transfected with *raf* mutant EHneo301(C) or oncogenic p21^{ras} expression vector (+Ras), and an *in vitro* kinase assay was performed with bovine MBP. Phosphorylated MBP was analyzed on an SDS/polyacrylamide gel.

ping, we have identified three phosphorylated residues in NF-IL6, all of which are present in the NF-IL6Δ(41–205) mutant: Ser-231 and Thr-235, both located within the serine-rich domain (SRD) adjoining bZIP; and Ser-325, located within the leucine zipper (data not shown). As shown in Fig. 2 *A* and *B*, oncogenic p21^{ras} expression dramatically augmented the phosphorylation of peptide x. Peptides x and y reside within a single tryptic peptide containing the SRD and correspond to a doubly phosphorylated form (Ser-231 + Thr-235) and a monophosphorylated form (Ser-231), respectively, of this tryptic peptide [peptides x′ and y′ are derived from x and y and may be derived by the alkylation of peptides during sample handling, as has been observed in the case of c-jun (24)]. To determine whether peptide x is indeed a target for phosphorylation, we generated derivatives (S231A and T235A) of NF-IL6Δ(41–205) by *in vitro* mutagenesis in which Ala was substituted at Ser-231 or Thr-235 and repeated the phosphopeptide mapping analysis. Each substitution resulted in the disappearance of peptide x (Fig. 2 *C* and *D*). In the case of S231A, hyperphosphorylation of peptide y, the monophosphorylated form, was observed, which was a result of *ras*-induced phosphorylation of Thr-235 (data not shown).

***In Vitro* Phosphorylation of NF-IL6 by MAP Kinases.** Recently, it has been demonstrated that MAP kinases are involved in the cellular response to many growth factors and to activated *ras* (25–27). Indeed, the partially purified MAP kinase preparation from the NIH 3T3 cells transfected with the oncogenic *ras* expression vector, phosphorylated MBP, demonstrating that transient transfection of oncogenic p21^{ras} induced activation of MAP kinases in NIH 3T3 cells (Fig. 3). A consensus sequence for MAP kinase substrates has been determined to be -Pro-Xaa-Ser/Thr-Pro (28). Closer inspec-



D

Calculated mass of Y fragment

	residue		U	P
34	Lys	Y1	147.1	147.1*
33	Ala	Y2	218.1*	218.1*
32	Asp	Y3	333.2*	333.2*
31	Ala	Y4	404.2*	404.2*
30	Pro	Y5	501.3*	501.3*
29	Ser	Y6	588.3*	588.3*
28	Pro	Y7	685.4*	685.4*
★ 27	**Thr**	**Y8**	**786.4***	**866.4***
26	Gly	Y9	843.4*	923.4*
25	Pro	Y10	940.5*	1020.5*
24	Pro	Y11	1037.5*	1117.5*
23	Ser	Y12	1124.6*	1204.6*
22	Ser	Y13	1211.6	1291.6
21	Ser	Y14	1298.6*	1378.6*
20	Ser	Y15	1385.7*	1465.7*
19	Ser	Y16	1472.7*	1552.7
⋮	⋮		⋮	⋮
2	Tyr		3127.5	3207.5
1	Ala		3198.5	3278.5

* detected signal on CAD spectrum

FIG. 4. Phosphorylation of synthetic peptides by purified MAP kinases. (*A*) *In vitro* phosphorylation by MAP kinases. The phosphorylation of synthetic peptides based on the primary sequence of the NF-IL6 (amino acids 209–242), the epidermal growth factor receptor (EGF-R) (amino acids 661–681), MBP (amino acids 89–107), and Myc protein (amino acids 51–66) was performed with purified MAP kinases. (*B*) Phosphoamino acid analysis. The labeled peptide was eluted from the polyacrylamide gel, hydrolyzed, and analyzed by cellulose thin-layer electrophoresis at pH 3.5. (*C* and *D*) Electrospray collision-activated dissociation (CAD) mass spectrum. In the CAD spectrum of peptide $(M + 2H)^{2+}$ ion recorded under the low energy collision conditions used in quadrupole instruments, two types of fragment ions are observed: sequential N-terminal type B and C-terminal type Y fragments. The spectra revealed the common signals Y5–Y7 in both peptides. In the phosphorylated peptide spectrum, the atomic mass unit values of Y8–Y12 displayed 80.0 higher than those from the unphosphorylated peptide. All of the detected B-type fragments were observed at the same positions in both spectra. In addition, in the first step electrospray ionization mass spectrum, observed molecular mass in deconvoluted spectrum of the phosphorylated peptide was 3279 Da, which is 80 Da higher than the unphosphorylated peptide (data not shown), suggesting that the peptide is monophosphorylated. All these facts suggest that residue 27 of threonine is phosphorylated.

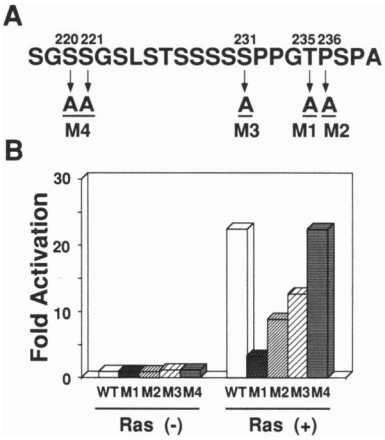


FIG. 5. Effect of site-directed mutations on *ras*-dependent NF-IL6 transcriptional activity. CMV-NFIL6 [wild type (WT)] and its site-directed mutants (M1–4 in *A*) were cotransfected into P19 cells with the reporter luciferase gene in the presence or absence of oncogenic $p21^{ras}$. One unit of relative activity represents the luciferase activity obtained after transfection of the reporter gene. This experiment was repeated three times and similar results were obtained.

tion of the primary sequence of NF-IL6 revealed the presence of this consensus sequence in the region immediately surrounding Thr-235 (SSPPGTPSP).

We examined *in vitro* whether NF-IL6 is an efficient substrate for MAP kinases by using a synthetic peptide corresponding to the predicted tryptic peptide containing Ser-231 and Thr-235. We observed that this peptide was phosphorylated *in vitro* by purified MAP kinases and was in fact a better substrate than several other synthetic peptides derived from known substrates (29) (Fig. 4*A*). A combination of phosphoamino acid analysis and mass spectrometry of the phosphopeptide showed that phosphorylation of the synthetic peptide occurred on Thr-235 (Fig. 4 *B–D*).

Effect of Site-Directed Mutations of NF-IL6 on *ras*-Dependent Transcriptional Activity. We next investigated whether mutation of the phosphorylation sites affected the transcriptional activity of NF-IL6. Several site-directed mutant derivatives of NF-IL6 were cotransfected into P19 cells (which have undetectable levels of endogenous NF-IL6) together with the oncogenic $p21^{ras}$ expression vector. Substitution of Ala for Thr-235 resulted in the loss of *ras*-dependent activation of NF-IL6 (Fig. 5). A similar substitution at Pro-236, which is important for substrate recognition by MAP kinases, or substitution at Ser-231 also impaired *ras*-dependent activation. These results demonstrated that phosphorylation of Thr-235 by MAP kinases is essential for *ras*-dependent activation of NF-IL6. Although Ser-231 is constitutively phosphorylated irrespective of cotransfection by oncogenic $p21^{ras}$ and the kinase that phosphorylates Ser-231 remains unknown, our results suggest that phosphorylation of this site is nevertheless necessary for *ras*-dependent activation of NF-IL6 to occur.

DISCUSSION

Our present studies provide evidence that MAP kinases are directly responsible for the phosphorylation and activation of NF-IL6, a transcription factor involved in cytokine signal transduction. MAP kinases, also known as the extracellular signal-regulated kinases, are a family of serine/threonine kinases that are activated very rapidly in response to many extracellular stimuli, including insulin, nerve growth factor, epidermal growth factor, platelet-derived growth factor, activators of protein kinase C, and antigen stimulation of T cells (25–27). MAP kinases are shown to be activated in mouse hepatocytes by IL-6 stimulation (unpublished data). The activation of MAP kinases by these diverse stimuli implies its importance in signal transduction, but c-myc and c-jun are at present the only transcription factors reported to be substrates for MAP kinases. The phosphorylation site of c-myc protein is located in the N-terminal region, which is important for the transcriptional activation of c-myc (28, 30). In the case of c-jun, oncogenic $p21^{ras}$ has been shown to cause the phosphorylation of its N-terminal activation domain via activation of MAP kinases (31), although data contrary to this have also been presented, showing that MAP kinases do not phosphorylate the c-jun activation domain *in vitro* (32, 33).

Recently, Wegner *et al.* (34) have demonstrated that C/EBPβ, the mouse homolog of human NF-IL6, is phosphorylated within the leucine zipper in response to increased intracellular calcium concentrations via the activation of a calcium/calmodulin-dependent kinase. Thus, NF-IL6 harbors two independent functional domains, one that is phosphorylated by a *ras*-dependent MAP kinase cascade and another that is activated by Ca^{2+}-mediated signals. Signals mediated via gp130 exert diverse effects depending on cell type, including cell growth stimulation or inhibition, acute-phase protein synthesis in hepatocytes, immunoglobulin synthesis in B cells, and neural differentiation of PC12 cells. We imagine that these diverse effects may be mediated by differential phosphorylation of three amino acids (Ser-231, Thr-235, and the Ca^{2+}-stimulated Ser-325) in NF-IL6. Further studies on NF-IL6 phosphorylation should reveal more about the mechanisms by which cytokines, including IL-6, exert their pleiotropic biological effects.

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