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Nerve Growth Factor Induces *zif268* Gene Expression *via* MAPK-Dependent and -Independent Pathways in PC12D Cells[1]

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In this study we examined the contribution of MAPK1 and 2 [also known as extracellular signal-regulated kinases (ERK)-1 and 2] to the induction of *zif268* mRNA in PC12D cells by using two methods to block the activation of these kinases. In one set of experiments, we inhibited the activation of MAPK by pretreating cells with PD098059, a specific inhibitor of MEK (MAPKK), the immediate upstream activator of MAPK. In the second set of experiments, we blocked the activation of MAPK by overexpressing N17Ras, a dominant-negative form of Ha-Ras. These two approaches yielded similar results and showed that inhibition of MAPK blocks less than half of the induction of *zif268* mRNA by NGF. Much of the residual induction of *zif268* mRNA is blocked by low concentrations of wortmannin, an inhibitor of phosphatidylinositol (PI) 3-kinase. Since PI 3-kinase was previously shown to function upstream in epidermal growth factor (EGF)-mediated activation of c-Jun N-terminal kinase (JNK), and JNK is known to phosphorylate and activate transcription factors that regulate the expression of *zif268*, we investigated the role of JNK in the induction of *zif268* mRNA by NGF. Stimulation of PC12D cells with NGF weakly activates JNK, but this activation is enhanced rather than inhibited by pretreatment with wortmannin, suggesting that JNK does not function downstream of PI 3-kinase in the induction of *zif268* mRNA. A role for JNK in the induction of the *zif268* gene is indicated, however, by the fact that cotransfection of expression vectors encoding JIP-1 or the JNK binding domain of JIP-1, which act as dominant-negative inhibitors of JNK, partially blocks the NGF-mediated induction of a luciferase reporter gene linked to the *zif268* promoter. Together, these results suggest that MAPK, PI-3 kinase and JNK each play a role in the induction of *zif268* gene expression by NGF in PC12D cells.

Key words: JNK, MAPK, NGF, wortmannin, *zif268*.

Zif268 (also designated as *Egr-1, NGFI-A, Krox-24*, and *TIS 8*) is an immediate early gene that encodes a transcription factor containing three zinc fingers in its DNA binding domain (for a review see Ref. *1*). *Zif268* mRNA has been shown to be rapidly induced in a large number of cell lines and tissues by growth factors (*2-6*), cytokines (*7*), neurotransmitters (*8-11*), phorbol ester (*11*), increases in intracellular calcium (*11, 12*), and agents that induce cellular stress (*13*). Although the genes regulated by Zif268 under physiologic conditions remain unknown, it has been proposed that the Zif268 transcription factor functions as a "third messenger" in genetic programs that change the long-term properties of cells in which it is expressed (*14, 15*). Of particular interest is the observation that induction of *zif268* mRNA in hippocampal neurons is highly correlated with the induction of long-term potentiation (LTP) of synaptic transmission (*15-18*). Gene expression has been proposed to play a role in maintaining LTP for extended periods of time, *i.e.*, for longer than several hours (*19-23*). Recent experiments using monkeys have also shown that expression of *zif268* protein in the anterior temporal lobe of the brain is highly correlated with visual stimulus-stimulus associative learning (*24*). Since *zif268* gene expression is induced by diverse stimuli, it is possible that the role that it plays in a given cell depends upon the stimulus. For example, different stimuli may induce distinct sets of transcription factor genes, which act in concert to induce specific responses in the cell. Insights into this process may be gained by determining the intracellular signaling path-

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Abbreviations: CRE, cAMP-response element; CREB, cAMP-response element binding protein; DMSO, dimethylsulfoxide; JNK, c-Jun N-terminal kinase; LY294002, (2-[4-morpholinyl]-8-phenyl-1[4*H*]-benzopyran-4-one); MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MLK, mixed lineage kinase; NGF, nerve growth factor; PD098059, 2-(2′-amino-3′-methoxyphenyl)oxanaphthalen-4-one; RSK, ribosomal S6 kinase; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor.

ways used by different stimuli to induce gene expression.

We (*11*) and others (*2, 6, 25*) previously showed that *zif268* mRNA is rapidly and transiently induced by nerve growth factor (NGF) in PC12D cells (*26*), a rapidly differentiating subline of the rat pheochromocytoma-derived cell line PC12 (*27*). Extensive studies on another immediate-early gene, *c-fos*, have shown that the rapid induction of its mRNA by NGF is mediated primarily *via* activation of the MAPK cascade, *i.e.*, Ras-Raf-MEK-MAPK (reviewed in Ref. *28*). Activated MAPK enters the nucleus and phosphorylates ternary complex factors (TCF), including elk-1, Sap-1a, and Sap-2/Erp/Net (*29*), which stimulate transcription of the *c-fos* gene expression by forming a ternary complex with serum response factor (SRF) bound to a single serum response element (SRE) in the *c-fos* promoter (*29-34*). Although the pathway for the induction of *zif268* mRNA by NGF has not yet been defined, is thought that the MAP kinase cascade plays a major role, since the *zif268* promoter is known to contain six SREs (*35-37*).

Although the above reasoning makes a plausible case for the MAPK cascade playing a central role in the linking of NGF receptors to the activation of *zif268* gene expression, other pathways for this activation are also possible. For example, stimulation of NGF receptors in PC12 cells has been shown to activate a variety of kinases including Akt/PKB (*38*), p70S6K (*39*), PLC-gamma (*40*), PKC (*41-43*), PI 3-kinase (*44-46*), and JNK (*47, 48*). Among these, JNK was previously proposed to function in the induction of *zif268* mRNA by agents that induce cellular stress, including UV radiation, arsenite, anisomycin, and osmotic shock (*13*).

In this study, we investigated the role of MAPK in the induction of *zif268* mRNA by NGF in PC12D cells. Two methods were used to block the activation of MAPK: (i) pretreatment of the cells with PD098059, a specific inhibitor of MEK (*49, 50*), and (ii) overexpression of N17Ras, a dominant-negative form of Ha-Ras (*51*) previously shown to block the activation of MAPK in PC12 cells (*52, 53*). In both cases, complete or nearly complete inhibition of MAPK was found to result in only partial inhibition of *zif268* mRNA induction. Additional experiments revealed possible roles for both PI 3-kinase and JNK in the induction of *zif268* mRNA. Together these results show that NGF induces *zif268* mRNA *via* both MAPK-dependent and -independent pathways in PC12D cells.

MATERIALS AND METHODS

Materials—Initial experiments were carried out with PD098059 generously provided by Alan Satiel (Park-Davis). In subsequent investigations we used PD098059 purchased from Calbiochem. NGF and wortmannin were obtained from Wako Chemical Industries. Dexamethasone was from Nacalai Tesque. LY294002, acetyl-CoA and *S*-acetyl coenzyme A synthetase were from Sigma. γ-[^{32}P]-ATP and α-[^{32}P]dCTP were obtained from Amersham and ^{3}H-NaAcetate was from NEN. Anti-MAPK1 and -2 antibodies were obtained from Santa Cruz Biotechnology. Restriction enzymes and other reagents for modification of DNA were obtained from Toyobo, Takara Shuzo, and New England Biolabs. Murine *zif268* cDNA (ATCC #63027), murine *c-fos* genomic DNA (ATCC #41041), and expression vector pBLCAT2 were obtained from the American Tissue Culture Collection. Human cyclophilin cDNA was a gift from Toshio Watanabe (Tohoku University), and pEF-BOS (*54*) was a gift from Shigeki Nagata (Osaka Bioscience Institute). A plasmid encoding dominant-negative Ras (N17Ras) under the control of a dexamethasone-inducible promoter [pMMTVrasH-Asn17 (*51*)] was a gift from Dr. G. Cooper (Harvard University). Plasmids encoding full-length JIP-1 [pCMV5-Flag-JIP-1 (*55*)] or the c-Jun kinase binding domain of JIP-1 [pCDNA3-JBD-JIP-1 (*55*)] were gifts from Dr. Roger Davis (University Massachusetts Medical Center).

Cell Culture and Selection of Ras Dominant-Negative Expressing Cell Lines—PC12D cells (*26*) were a gift from Mamoru Sano (Institute for Developmental Research, Aichi Prefectural Colony, Japan), and were cultured as previously described (*11*). Non-differentiated PC12D cells were used in all of the experiments. Drugs were added directly to the culture medium and were present until the time at which the cells were harvested. The corresponding vehicle (water, DMSO, or ethanol) was added to control cells. Stable cell lines that express N17Ras under the control of the dexamethasone-inducible MMTV LTR promoter were obtained by transfecting PC12D cells with the pMMTVras H-Asn17 plasmid using LipofectAMINE™ (Life Technologies/Gibco-BRL) followed by selection for neo^{R} colonies in DMEM containing 400 μg/ml G418 (selection was initiated 5 days after transfection). After two weeks of selection, G418-resistant colonies were isolated and screened for the ability of dexamethasone pretreatment to block the activation of MAPK by NGF. Cell lines that showed complete or nearly complete inhibition of NGF-mediated MAPK activation (cell lines 37 and 55) were chosen for further study. Ras dominant-negative cell lines were maintained in DMEM containing 100 μg/ml G418. Overexpression of dominant-negative N17Ras in these cells was induced by treating the cells with 0.5 μM dexamethasone for 19 h.

RNA Purification and Northern Blot Analysis—RNA was isolated from PC12D cells and Northern analysis was carried out as previously described (*11*). The RNA in each sample was quantified by optical spectroscopy and the integrity of the RNA was assessed by examining the ethidium bromide stained RNA in the formaldehyde-containing gel used for Northern blot analysis. Unless noted otherwise, 10 μg total cellular RNA was analyzed in each lane. After transfer to Pall Biodyne type B membranes (0.45 μm pore size), hybridization was carried out simultaneously using DNA probes prepared from *zif268*, *c-fos* (coding regions), and cyclophilin DNA fragments isolated from agarose gels and labeled using a Pharmacia Oligolabelling kit and α-[^{32}P]dCTP. The intensities of the bands on Northern blots were quantified using a Fuji Bioimaging analyzer BAS2000.

MAPK Assay—MAPK immunoprecipitation assays were carried out essentially as described by Cook and McCormick (*56*). PC12D cells grown to 80-90% confluency in 3.5 cm uncoated plastic culture dishes were exposed to various agents for 10 min, and then lysed by the addition of 200 μl lysis buffer [20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 20 μM leupeptin, and 10 μg/ml aprotinin]. After removing cellular debris by centrifugation, 0.1 μg anti-ERK1 and 0.1

μg anti-ERK2 antibodies (Santa Cruz Biotechnology) were added to the supernatant fractions, followed by incubation for 1 h at 4°C with rotation to allow mixing. Protein-A agarose (10 μl resin suspension; Santa Cruz Biotechnology) was then added to each sample and the incubation continued with rotation at 4°C for an additional hour. The resin in each sample was collected by centrifugation (2,500 rpm), and washed twice with 200 μl lysis buffer and once with 200 μl (2×) reaction buffer. [2×reaction buffer: 25 mM MOPS (pH 7.2), 25 mM sodium β-glycerophosphate, 15 mM $MgCl_2$, 1 mM EGTA, 0.1 mM NaF, 4 mM DTT, and 1 mM Na_3VO_4.] Twenty-two microliter reaction mixture containing 25 μM ATP, 1 μCi [γ-^{32}P]ATP, and 15 μM myelin basic protein in 1×reaction buffer was added to the resin and the mixture incubated at 30°C for 30 min. The reaction mixtures were spotted onto Whatman phosphocellulose filters and the filters were washed 6 times (5 min/wash) in 1% phosphoric acid.

Electrophoretic mobility shift-up MAPK assays were carried out as described in Ref. *57*. Briefly, cells were grown to 80–90% confluency in 3.5 cm diameter uncoated plastic dishes and stimulated with NGF or water for the indicated times. After rapidly removing the culture medium, the cells were lysed in 200 μl 1×SDS-PAGE sample buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromphenol blue). The lysates were then transferred to 1.5 ml Eppendorf tubes and boiled for 5 min. The cell lysates (10 μl/lane) were then resolved by SDS-PAGE [5% polyacrylamide stacking gel containing 125 mM Tris-Cl, pH 6.8, 0.1% SDS; 10% polyacrylamide resolving gel (prepared from 29.5% acrylamide +0.5% bis-acrylamide stock solution) containing 375 mM Tris-Cl, pH 8.8, 0.1% SDS; running buffer containing 25 mM Tris, 250 mM glycine, 0.1% SDS]. Electrophoresis was carried out at 15 mA per gel until the tracking dye entered the resolving gel, after which the current was increased to 30 mA per gel. Proteins were then electrophoretically blotted (0.5 A, 45 min) onto polyvinylidene difluoride (PVDF) membranes (Immobilon™ transfer membrane, Millipore) using a Nihon Eido Western blotting apparatus (20×20 cm) in buffer containing 100 mM Tris, 192 mM glycine, 10% methanol, and 0.02% SDS. Following transfer, the membranes were blocked by incubation in phosphate-buffered saline (PBS) containing 0.5% skim milk and 1% Tween-20 for 60 min at room temperature. The membranes were then exposed to 0.02 μg/ml (anti-MAPK) or 0.1 μg/ml (anti-Raf) antibodies in the same buffer for 2 h at room temperature, washed 3-times with buffer, and incubated in buffer containing anti-rabbit IgG antibodies cross-linked with horseradish peroxidase (Seikagaku Kogyo, Cat No. 286765; 2,000-fold final dilution) for 1 h at room temperature. After washing 3 times with buffer, immune complexes were visualized by staining the membranes with a freshly prepared solution containing 0.25 mg/ml 3,3′-diaminobenzidine, 50 mM Tris-Cl, pH 7.5, 0.01% H_2O_2, and 0.04% $NiCl_2$.

JNK Assays—JNK assays were performed using a SAPK/JNK assay kit (New England BioLabs). Briefly, PC12D cells grown to 80–90% confluency in 3.5 cm dishes were stimulated with 20 ng/ml NGF for 15 min and then lysed by the addition of 200 μl lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM PMSF). After brief centrifugation to remove cellular debris, 1 μg c-Jun fusion protein cross-linked beads were added to the supernatant fractions, which were then incubated with rotation overnight at 4°C. After brief centrifugation (2,500 rpm, 30 s), the beads were washed 2 times with 200 μl lysis buffer and 2-times with 200 μl 1×kinase buffer [25 mM Tris-Cl (pH 7.5), 5 mM β-glycero-phosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$], and then were resuspended in 20 μl 1×kinase buffer supplemented with 0.1 mM ATP and incubated at 30°C for 30 min. The kinase reaction was terminated by adding 5 μl 5×SDS sample buffer and boiling for 5 min. The samples were resolved by SDS-PAGE (10% gel) and then transferred to PVDF membranes (Immobilon™ transfer membrane, Millipore) by electrophoretic transfer. Phosphorylated c-jun was detected using phospho-c-Jun specific antibodies and the immune complexes visualized by enhanced chemiluminescense (ECL kit, Amersham).

Plasmid Construction—The expression vector 3R110, containing a firefly luciferase reporter gene linked to the rat *zif268* promoter, was constructed as follows: synthetic oligonucleotide primers were used to PCR amplify the *zif268* promoter region (from −525 bp to +110 bp) containing 6 SRE sites and 2 CRE sites [the forward primer contained a synthetic *Nhe*I site followed by nucleic acid residues 10–26 of the rat *zif268* promoter (Ref. *14*; GenBank assession #J04154): 5′-[GCGCTAGC]CTCAGC-TCTACGCGCCT-3′; the backward primer contained a synthetic *Bgl*II site followed by nucleic acid residues 634–648 (*14*) of the rat *zif268* gene: 5′-[CGAGATCT]GGTGG-ACGCAGGGCT-3′]. The PCR product was digested with *Nhe*I and *Bgl*II, and then cloned between the *Nhe*I and *Bgl*II sites of pGL3. Plasmid 4×SRE containing the distal 4 SRE sites of the *zif268* promoter was constructed by digesting 3R110 with *Apa*I, which cuts at a site immediately upstream from the TATA box, and *Sma*I, which cuts at a site between the distal and proximal SRE sites. The digested plasmid was blunt-ended and then self-ligated to produce the 4×SRE plasmid. plasmid +Sma, containing the proximal SRE sites and two flanking CRE-like sites was constructed by digesting 3R110 with *Sac*I, which cuts upstream of the *zif268* promoter within the multi-cloning site of pGL3, and with *Sma*I. The digested plasmid was blunt-ended and then self-ligated to obtain the +Sma plasmid. An expression vector containing the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the human elongation factor 1α promoter was constructed as follows: pBLCAT2 was digested with *Bam*HI and *Bgl*II, blunt-ended, and then self-ligated. Digestion of the resulting plasmid with *Sal*I and *Sma*I yielded a 1.5 kb fragment containing the CAT gene and a polyadenylation signal derived from SV40. This fragment was cloned between the *Sal*I and *Sma*I sites of pBluscript SK(+). The fragment was then re-isolated by digestion with *Xba*I, and cloned in the *Xba*I site of pEF-BOS (*54*). The resulting vector, pEF-CAT, was used as an internal control in transfection experiments.

Transfection of PC12D Cells and Assaying of Reporter Genes—Transfections were performed using Lipofectamine™ reagent (Life Technologies) essentially as recommended by the manufacturer. Cells were seeded in 6 cm plastic dishes at a density of 4×10^6 cells/dish and then

cultured for 1 day prior to transfection. 0.92 μg pEF-CAT DNA, 2.3 μg *zif268* promoter/luciferase expression vector DNA, 2.3 μg pCDNA3, and pCMV5-Flag-JIP-1 or pCDNA3-JBD-JIP-1 DNA, 13.8 μl Lipofectamine™ reagent were added to each dish of cells, followed by incubation for 4 h, after which medium containing twice the normal concentration of serum was added. Following incubation overnight, the cells in each 6 cm dish were resuspended and distributed into 12×1.1 cm wells. The next day, the medium was replaced with normal DMEM and the cells were cultured for one more day. Drugs were added directly to the culture medium and the cells were harvested after 4 h. Luciferase expression was carried out using a Promega Luciferase or Packard Lucite Assay System, and luciferase activities were quantified using a Packard Tri-Carb liquid scintillation counter or a Packard Top count microplat scintillation counter as described in the manuals supplied by Promega and Packard, respectively. Background luciferase expression was determined using cells transfected with pGL2, which lacks a promoter for luciferase gene expression. Transfection efficiency was determined by cotransfection with pEF-CAT. Chloramphenicol acetyltransferase (CAT) activities were measured as described by Nordeen *et al.* (*58*), and the values were used to calculate the normalized luciferase activity for each sample.

RESULTS

MAPK Activation Precedes zif268 mRNA Induction Following Stimulation of PC12D Cells with NGF—To determine whether or not MAPK participates in the induction of *zif268* gene expression by NGF, we first compared the time course of MAPK activation with that of *zif268* mRNA induction in PC12D cells. Following the addition of NGF to the growth medium, *zif268* mRNA was rapidly and transiently induced, reached maximal levels at about 45 min, and returned to the baseline within three to 4 h (Fig. 1, A and B). Western blot analysis showed that stimulation with NGF induced the rapid appearance of more slowly migrating forms of MAPK1 (Fig. 1C). These "shifted-up" forms of MAPK were previously shown to correspond to the

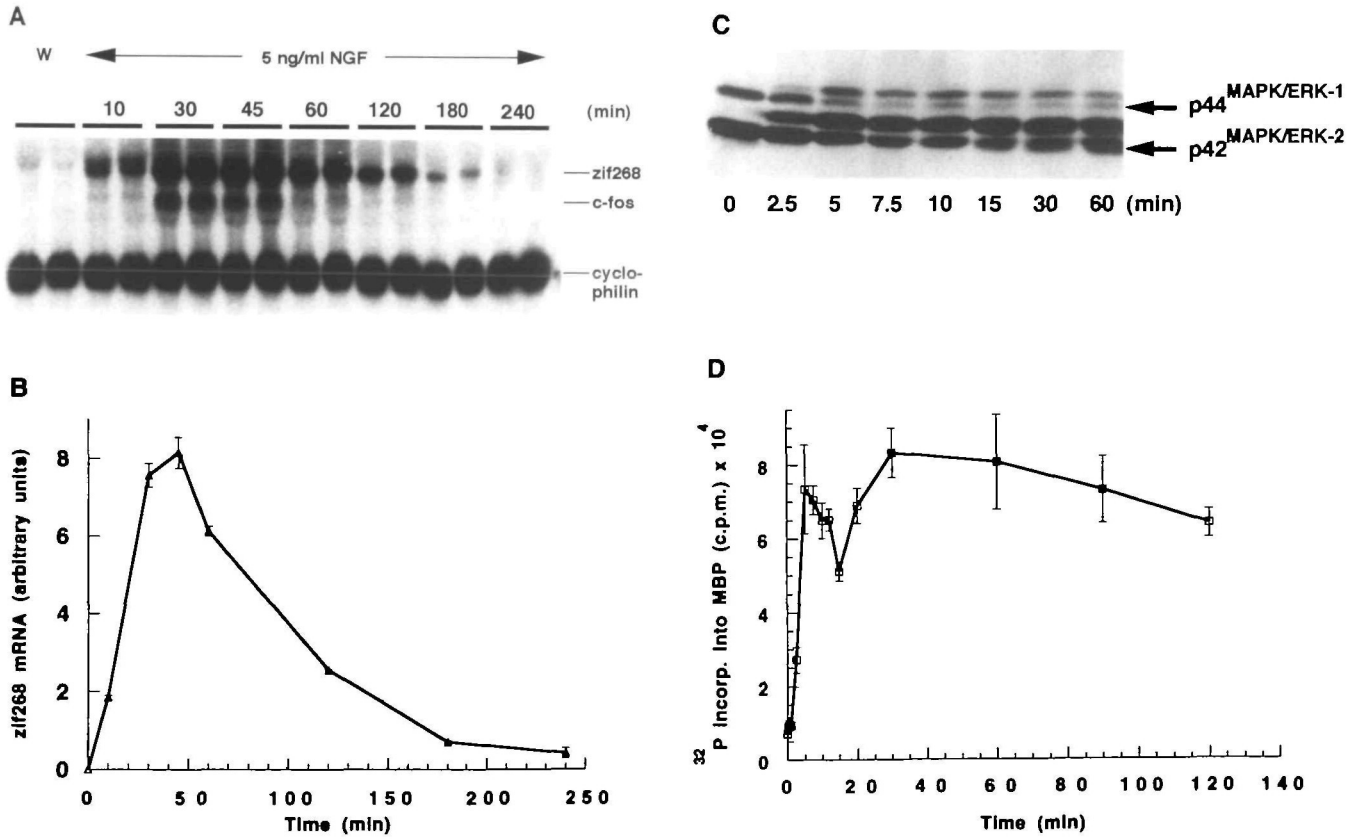


Fig. 1. **NGF stimulates the rapid induction of *zif268* mRNA and the activation of MAPK.** (A, B) Northern blot analysis showing the time courses of *zif268* and *c-fos* mRNA induction following exposure to NGF. PC12D cells were stimulated with 5 ng/ml NGF or water (W) for the indicated times and then total RNA was harvested. (A) Autoradiogram of Northern blot prepared as described under "MATERIALS AND METHODS" using 10 μg total RNA per lane, and hybridized with ^{32}P-labeled *zif268*, *c-fos*, and cyclophilin DNA probes. (B) *Zif268* mRNA (normalized with respect to the level of cyclophilin mRNA at each time point) was quantified using a BAS2000 Imaging analyzer. (C, D) MAPK assays. (C) PC12D cells were exposed to 5 ng/ml NGF for the indicated times, after which cell extracts were prepared and Western blot analysis was performed using antibodies that recognize ERK1 (p44$^{MAPK/ERK-1}$) and ERK2 (p42$^{MAPK/ERK-2}$) as described under "MATERIALS AND METHODS." (D) PC12D cells were exposed to 5 ng/ml NGF for the indicated times after which cell extracts were prepared and immunoprecipitated with anti-ERK1 and anti-ERK2 antibodies. *In vitro* phosphorylation of the MAPK substrate, myelin basic protein (MBP), in the presence of γ-[^{32}P]ATP was carried out as described under "MATERIALS AND METHODS." Each point represents the average of two independent measurements of the incorporation of ^{32}P into myelin basic protein. These data are representative of experiments performed 2 times.

phosphorylated, activated forms of these kinases (*57*). A rapid increase in MAPK activity following exposure to NGF was also demonstrated in quantitative immuno-precipitation assays (Fig. 1D). These assays revealed two distinct phases of activation by NGF: initial activation that peaked at about 5 min, and then sustained activation that peaked at about 30 min and remained high for several hours. The kinetics of MAPK activation by NGF revealed by both the shift-up and immunoprecipitation assays show that activation is sufficiently rapid for MAPK to function in the signaling cascade that induces *zif268* mRNA.

Activation of MAPK and Induction of zif268 mRNA Occur with Similar Concentrations of NGF—The dose-dependencies for activation of MAPK and induction of *zif268* mRNA by NGF are shown in Fig. 2. The close correlation between these inductions is also consistent with MAPK playing a role in the induction of *zif268* mRNA induction by NGF.

The MEK Inhibitor PD098059 Nearly Completely Inhibits MAPK Activation by NGF but Only Partially Blocks the Induction of zif268 mRNA—To determine if MAPK is required for *zif268* gene expression by NGF, we blocked its activation by pretreating the cells with PD098059, a specific inhibitor of MEK, the immediate upstream activator of MAPK (*49, 50*). As shown in Fig. 3A, PD098059 dose-dependently blocked NGF-mediated activation of MAPK. By contrast, *zif268* mRNA induction by NGF was only partially blocked even when cells were pretreated with high concentrations of PD098059 (Fig. 3B). Quantitative analysis of Northern blot data revealed that *zif268* mRNA induction is inhibited by only 30 to 40% of the maximal response when cells are pretreated with 200 μM PD-098059, a concentration at which MAPK activation by NGF is completely abolished (Fig. 3C and data not shown). PD098059 pretreatment by itself does not stimulate *zif268* gene expression (data not shown). These results suggest that the induction of *zif268* mRNA takes place *via* both MAPK-dependent and -independent pathways in PC12D cells.

The PI 3-Kinase Inhibitor Wortmannin Partially Blocks MAPK-Independent zif268 mRNA Induction Stimulated by NGF—To determine if other known kinases function in the MAPK-independent pathway, we examined the effects of several protein kinase inhibitors on the NGF-mediated induction of *zif268* mRNA in PC12D cells pretreated with PD098059. These experiments showed that much of the residual *zif268* mRNA induction could be blocked by low concentrations of wortmannin, an inhibitor of PI 3-kinase (*59-61*). The dose-dependent inhibition of *zif268* mRNA induction by wortmannin is shown in Fig. 4A. Inhibition of

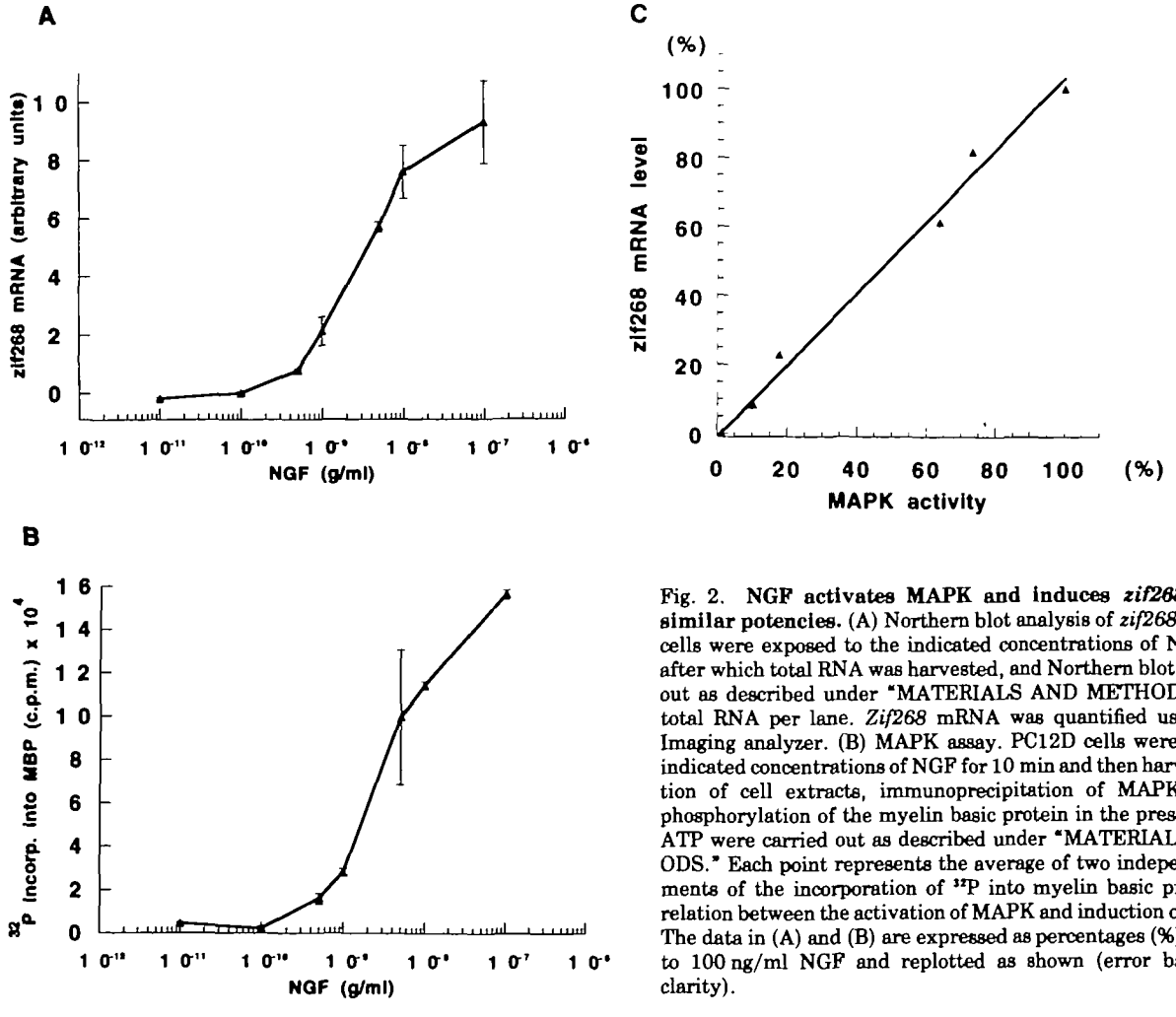


Fig. 2. **NGF activates MAPK and induces *zif268* mRNA with similar potencies.** (A) Northern blot analysis of *zif268* mRNA. PC12D cells were exposed to the indicated concentrations of NGF for 45 min, after which total RNA was harvested, and Northern blot analysis carried out as described under "MATERIALS AND METHODS" using 10 μg total RNA per lane. *Zif268* mRNA was quantified using a BAS2000 Imaging analyzer. (B) MAPK assay. PC12D cells were exposed to the indicated concentrations of NGF for 10 min and then harvested. Preparation of cell extracts, immunoprecipitation of MAPK, and *in vitro* phosphorylation of the myelin basic protein in the presence of γ-[^{32}P]-ATP were carried out as described under "MATERIALS AND METHODS." Each point represents the average of two independent measurements of the incorporation of ^{32}P into myelin basic protein. (C) Correlation between the activation of MAPK and induction of *zif268* mRNA. The data in (A) and (B) are expressed as percentages (%) of the response to 100 ng/ml NGF and replotted as shown (error bars omitted for clarity).

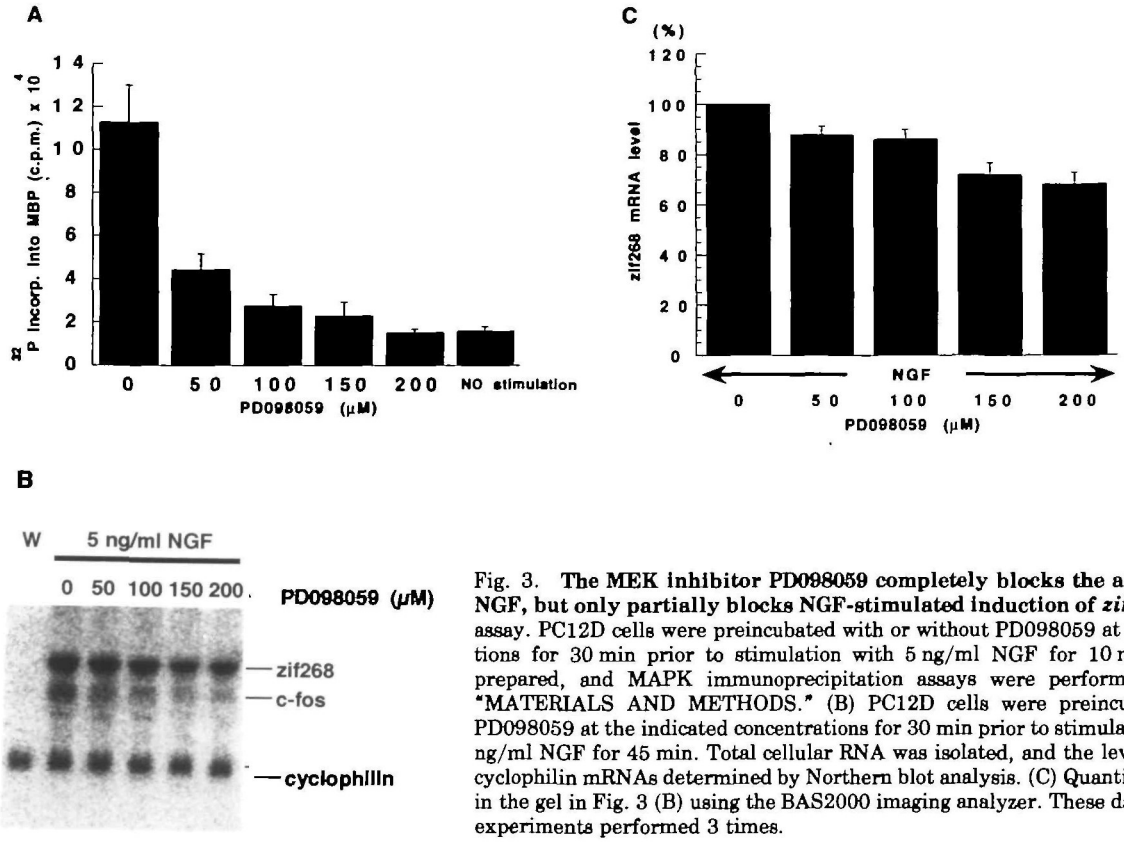
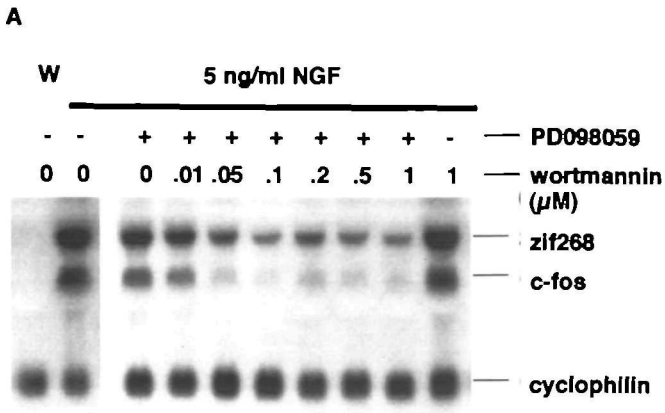


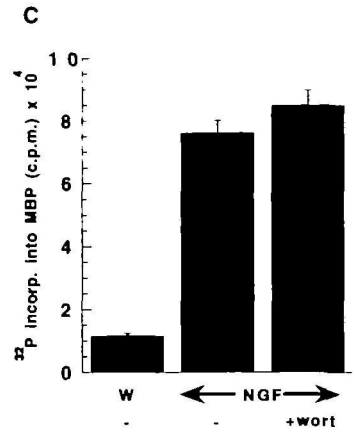
Fig. 3. **The MEK inhibitor PD098059 completely blocks the activation of MAPK by NGF, but only partially blocks NGF-stimulated induction of *zif268* mRNA.** (A) MAPK assay. PC12D cells were preincubated with or without PD098059 at the indicated concentrations for 30 min prior to stimulation with 5 ng/ml NGF for 10 min. Cell extracts were prepared, and MAPK immunoprecipitation assays were performed as described under "MATERIALS AND METHODS." (B) PC12D cells were preincubated with or without PD098059 at the indicated concentrations for 30 min prior to stimulation with water (W) or 5 ng/ml NGF for 45 min. Total cellular RNA was isolated, and the levels of *zif268*, *c-fos*, and cyclophilin mRNAs determined by Northern blot analysis. (C) Quantification of *zif268* mRNA in the gel in Fig. 3 (B) using the BAS2000 imaging analyzer. These data are representative of experiments performed 3 times.

zif268 mRNA induction was maximal when cells were pretreated with 100 nM wortmannin, a concentration sufficient to completely inhibit PI-3 kinase in PC12 cells (*62*). Induction of *zif268* mRNA was also blocked, but less potently, in a dose-dependent manner by the PI-3 kinase inhibitor LY294002 (*63*) in PC12D cells pretreated with PD098059 (Kumahara and Saffen, unpublished observation). These results suggest that PI 3-kinase may function in the MAPK-independent pathway for *zif268* gene induction by NGF. As shown in Fig. 4B, wortmannin pretreatment by itself did not induce *zif268* mRNA and also had no effect on the induction of *zif268* mRNA by NGF in the absence of PD098059. Wortmannin also had no effect on the activation of MAPK activity by NGF in the absence of PD098059 (Fig. 4 C). The fact that wortmannin fails to block NGF-stimulated *zif268* mRNA induction suggests that the wortmannin-sensitive pathway contributes little to the induction of *zif268* by NGF when MAP kinase is also stimulated.

Expression of Dominant-Negative Ras in PC12D Cells Completely Blocks MAPK Activation but Only Partially Blocks zif268 mRNA Induction—It is now well established that activation of the MAPK cascade by NGF in PC12 cells is mediated by Ras (*28*). We therefore examined if overexpression of a dominant-negative form of Ha-Ras, N17Ras, blocks *zif268* mRNA induction by NGF. For this purpose, we constructed two independent sublines of PC12D that express N17Ras under the control of the dexamethasone-inducible promoter in the mouse mammary tumor virus (MMTV) long terminal repeat (LTR). As shown in Fig. 5A, activation of MAPK by NGF in these cells is completely blocked by pretreatment with dexamethasone. As shown in Fig. 5B, however, *zif268* gene expression by NGF is only partially blocked by dexamethasone pretreatment of these cells. This is consistent with the experimental results described above, *i.e.* pretreatment with the MEK inhibitor PD098059 completely blocked the activation of MAPK by NGF, but only partially blocked the induction of *zif268*. Also consistent with the previous results, wortmannin blocked a significant portion of the dexamethasone-resistant *zif268* mRNA induction in cell lines 37 and 55 (Fig. 5B).

It was recently reported that overexpression of dominant-negative Ras blocks the initial phase of activation of MAPK by NGF in PC12 cells, but fails to block the sustained phase of activation (*64*). Rather, the sustained phase of MAPK activation was said to be maintained through a Ras-independent pathway involving the low molecular weight GTP-binding protein Rap1 and B-Raf, another upstream activator of MEK (*64*). Because delayed dexamethasone-resistant activation of MAPK by NGF would affect the interpretation of the experimental results described above, we examined the effect of dexamethasone induction of N17Ras on the time course of activation of MAPK. As shown in Fig. 5C, there was only slight activation of MAPK at late time points in cells expressing N17Ras. This level of late MAPK activation by NGF is less than that reported for PC12 cells expressing dominant-negative N17Ras (*64*), and may reflect differences in the strains of cells. The slight activation observed in cell line 37 cells is not expected to be sufficient to account for the observed *zif268* mRNA induction, although it may contrib-





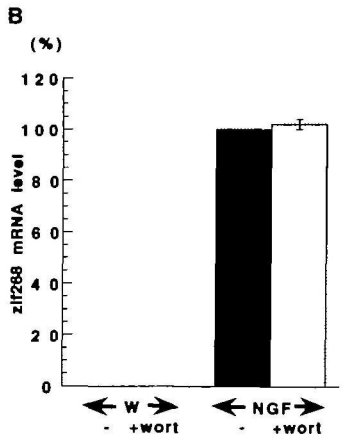


Fig. 4. **The PD098059-resistant component of NGF-induced *zif268* mRNA is partially blocked by pretreatment with the PI 3-kinase inhibitor wortmannin.** (A) Northern blot of RNA isolated from PC12D cells pretreated with DMSO (−) or 200 μM PD098059 (+) and wortmannin at the indicated concentrations for 30 min prior to stimulation with water (W) or 5 ng/ml NGF for 45 min. Total cellular RNA was isolated, and levels of *zif268*, *c-fos*, and cyclophilin mRNAs were determined by Northern blot analysis as described under "MATERIALS AND METHODS." (B) Northern blot analysis. Cells were pretreated with DMSO (−) or 1 μM wortmannin (+wort) for 30 min prior to stimulation with water (W) or 5 ng/ml NGF for 45 min ($n=2$). (C) MAP kinase assays. Cells were pretreated with DMSO (−) or 200 nM wortmannin (+wort) for 30 min prior to stimulation with water (W) or 5 ng/ml NGF for 10 min. MAPK immunoprecipitation assays were performed as described under "MATERIALS AND METHODS."

ute to the dexamethasone- and wortmannin-resistant component of induced *zif268* mRNA.

JNK Does Not Function Downstream of PI3-Kinase in the Induction of zif268 mRNA—JNK has been shown to function downstream of PI 3-kinase following activation of EGF receptors in NIH3T3 cells (*65*), following aggregation of FcepsilonRI on mast cells (*66*), and following intracellular injection of the activated form of PI 3-kinase (*67*). JNK is also a potential activator of *zif268* gene expression since it is known to activate *c-fos* gene expression by phosphorylating the TCF's elk-1 (*68, 69*) and Sap-1a (*69, 70*). We therefore examined whether or not JNK is activated following exposure to NGF and whether or not this activation could be blocked by wortmannin. The results presented in Fig. 6 show that JNK is weakly activated by NGF, and that this activation is not blocked by pretreatment with wortmannin. Wortmannin, in fact, weakly activates JNK, so that combined exposure to wortmannin and NGF enhances JNK activation. Similar results were obtained with PC12D cells pretreated with PD098059 (data not shown). These results strongly imply that JNK does not function downstream of PI 3-kinase in the induction of *zif268* mRNA in PC12D cells.

JNK Plays a Role in the Induction of zif268 mRNA by NGF—Although the results presented above indicate that JNK does not play a role downstream from PI 3-kinase in the induction of *zif268*, they do not rule out a role for JNK in some other NGF-activated pathway. As mentioned above, JNK has been shown to be involved in the activation of *zif268* by agents that induce cellular stress (*13*). Because there are no known specific chemical inhibitors for JNK, we examined the effects of proteins that act as dominant-negative inhibitors of JNK on *zif268* promoter activity in expression plasmids containing luciferase reporter genes. The dominant-negative proteins used were full-length JIP-1 (*55*), a structural protein thought to function as a scaffold for the (MLK)-(MKK)-JNK cascade (*71*), and a truncated form of JIP-1 comprising only the JNK binding domain (JBD) (*55*). Over-expression of JIP-1 or JBD-JIP-1 was previously shown to inhibit the activation of JNK-mediated gene expression, possibly by preventing the translocation of JNK into the nucleus (*55, 71*). The structures of three expression vectors containing various segments of the rat *zif268* promoter linked to the bacterial lucifierase reporter gene are depicted in Fig. 7A. The plasmid 3R110 contains approximately 635 bp of the *zif268* promoter, including the TATA box, and distal and proximal clusters of SREs. The plasmids 4×SRE and +sma contain the distal and proximal SREs, respectively. Two CRE-like sequences that flank the proximal SRE's are included in plasmids 3R110 and +sma. The results shown in Fig. 7, B and C, show that cotransfection of plasmids encoding JIP-1 or JBD-JIP-1 partially inhibits the activity of the *zif268* promoter in the 3R110 and 4×SRE plasmids. By contrast, overexpression of JIP-1 had no effect on the activity of the *zif268* promoter fragment containing the proximal SREs and flanking CRE-like sites. These data show that JNK does play a role in the induction of *zif268* mRNA by NGF and

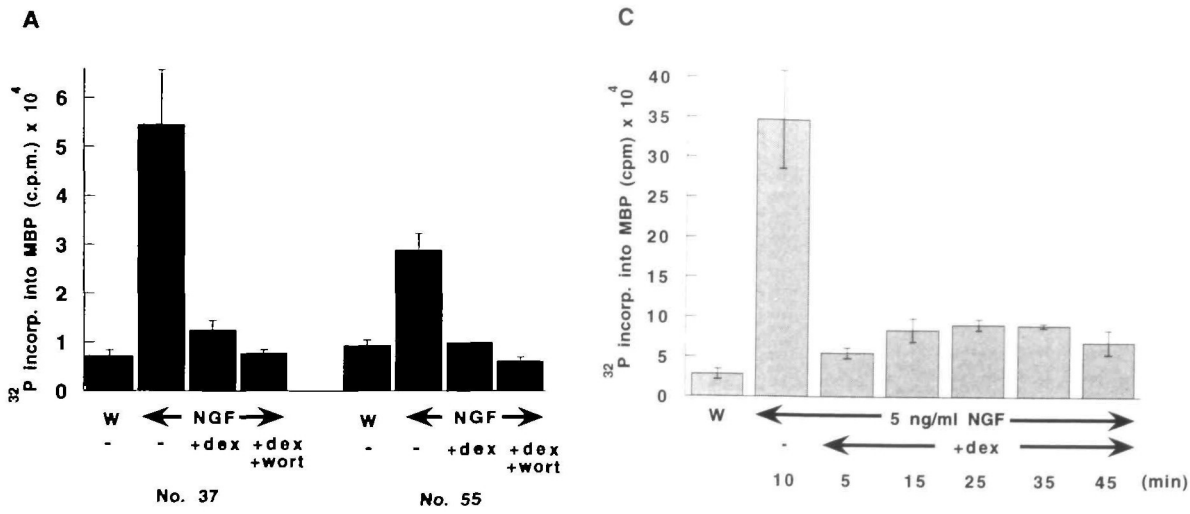


Fig. 5. **Overexpression of dominant-negative Ras nearly completely blocks MAPK activation by NGF but only partially blocks the induction of *zif268* mRNA; the remaining induction is partially blocked by wortmannin.** (A) MAPK assays. PC12D-derived cell lines 37 and 55, which stably express dexamethasone-inducible dominant-negative N17Ras, were pretreated with 0.5 μM dexamethazone (+dex) for 19 h and then with DMSO (−) or 200 nM wortmannin (+wort) for 30 min prior to stimulation with water (W) or 5 ng/ml NGF for 10 min. The MAPK immunoprecipitation assays were performed as described under "MATERIALS AND METHODS." (B) Northern blot analysis of *zif268* induction by NGF in cell lines 37 and 55. Cells were treated as described in (A) and then stimulated with water (W) or 5 ng/ml NGF for 45 min. Total cellular RNA was isolated, and Northern blot analysis of *zif268*, *c-fos*, cyclophilin mRNAs was performed as described under "MATERIALS AND METHODS." (C) Time course of MAPK activation by NGF in cell line 37 pretreated with dexamethasone to induce dominant negative N17Ras. Cells were pretreated with 0.5 μM dexamethasone (+dex) for 19 h prior to stimulation with water (W) or 5 ng/ml NGF for the indicated times. MAPK immunoprecipitation assays were performed as described under "MATERIALS AND METHODS."

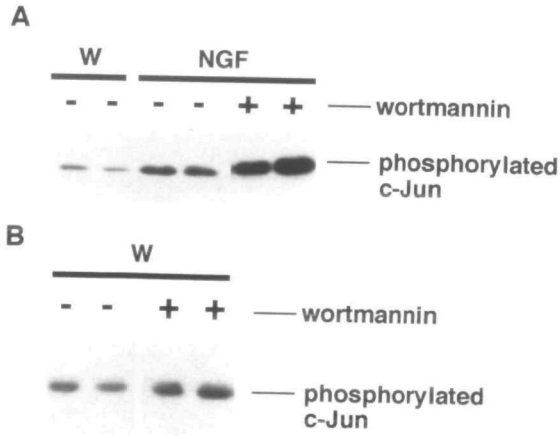


Fig. 6. **Wortmannin augments rather than blocks the activation of JNK by NGF.** JNK assays: Western blot analysis of phospho-c-Jun phosphorylated by JNK isolated from cell extracts was performed as described under "MATERIALS AND METHODS." (A) Cell line 37 was pretreated with DMSO (−) or 200 nM wortmannin (+) for 30 min prior to stimulation with water (W) or 5 ng/ml NGF for 15 min. (B) PC12D cells were pretreated with DMSO (−) or 200 nM wortmannin (+) for 30 min prior to stimulation with water (W).

suggest that it is very important for transcription driven by the distal SREs.

DISCUSSION

In this study, we showed that the induction of *zif268* mRNA by NGF is mediated not only *via* a MAPK-dependent pathway, but also *via* a wortmannin-sensitive pathway that is independent of, and possibly inhibited by, MAPK. The fact that wortmannin was able to block the induction of *zif268* mRNA at low concentrations suggests that PI 3-kinase may play a role in the MAPK-independent pathway. We also showed that although JNK does not function in the wortmannin-sensitive pathway, it is required for full activation of the *zif268* promoter by NGF. This study is the first to directly assess the importance of the MAPK cascade in the induction of *zif268* mRNA by NGF, and the first to suggest a possible role for PI 3-kinase in this induction.

Evidence that the MAPK cascade plays a role in the induction of *zif268* and *c-fos* mRNAs by NGF was previously obtained using PC12 cells expressing dominant-negative Ras. Expression of high levels of dominant-negative Ha Ras ASN17 (N17Ras) results in significant, but partial inhibition of the induction of *zif268* and *c-fos* mRNAs by NGF (*51, 72*). Although the results of these experiments are consistent with a role for MAPK in the induction of *zif268*

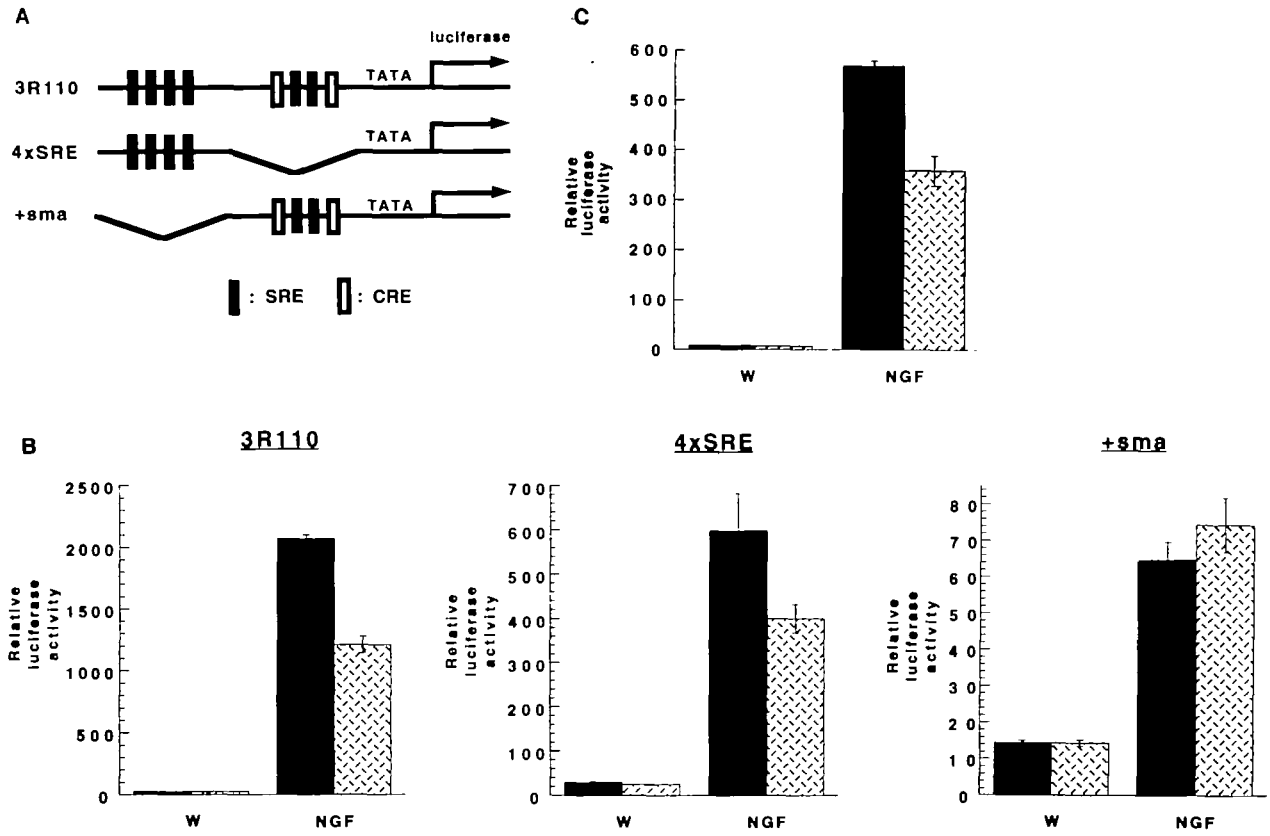


Fig. 7. **JNK contributes to the activation of the *zif268* promoter by NGF.** (A) Structures of expression plasmids 3R110, 4× SRE and +sma containing the indicated segments of the *zif268* promoter linked to the luciferase reporter gene. Filled-in vertical boxes represent SRE elements and open vertical boxes represent CRE-like elements. The extents of deletion of the *zif268* promoter in the 4×SRE and +sma vectors are indicated by diagonal lines. (B) Expression vectors (3R110, 4×SRE, and +sma) were transfected into PC12D cells in combination with pCDNA3 (solid bars) or pJBD (hatched bars), which expresses the JNK interacting protein-1 (JIP-1) binding domain (JBD). After two days, cells were stimulated with water (W) or 5 ng/ml NGF, and luciferase assays were performed as described under "MATERIALS AND METHODS." (C) PC12D cells were transfected with the 4×SRE vector in combination with pCDNA3 (solid bars) or a plasmid expressing the JNK interacting protein, JIP-1 (hatched bars). Two days later, the cells were stimulated with water (W) or 5 ng/ml NGF, and luciferase assays performed. The heights of the bars represent the averages of two independent measurements of luciferase activity.

and *c-fos* mRNAs, the interpretation of the results is complicated by the fact that (i) there are downstream Ras effectors that are unrelated to the MAPK cascade (*72, 73*), and (ii) NGF-mediated activation of MAPK can take place *via* activation of B-Raf in a manner independent of Ras (*64*). Because of the existence of branching and alternative pathways upstream of MAPK, there has been a need to directly assess the contribution of ERK-1 and 2 to the induction of *zif268* and *c-fos* mRNAs. This study is the first to attempt to directly investigate the role of MAPK (ERK1/2) in the induction of *zif268* and *c-fos* mRNAs by NGF using the MEK inhibitor PD089059. It is also the first study to directly compare the effects of expression of dominant negative Ras on MAPK activation and induction of *zif268* mRNA.

The results presented in Fig. 1 confirm and extend the results of previous studies showing that NGF induces *zif268* (*2, 6, 11, 25*) mRNA and activates MAPK (ERK-1 and -2) (*28, 47, 52, 53*) in PC12 cells. As previously described (*2, 6, 11, 25*), the induction of *zif268* is rapid and transient, with levels peaking within 30 to 45 min (Fig. 1, A and B). MAPK (ERK-1 and -2) is also rapidly activated after the addition of NGF to the culture medium, with kinetics sufficiently rapid to allow it to function in the induction of *zif268* and *c-fos* mRNAs (Fig. 1 C). Quantification of MAPK activation by NGF revealed two phases of activation: an initial peak of activity at about 5 min and a second one at about 30 min, that decreases very slowly over several hours. The reasons for the two phases of activation are not understood, but they could be related to a recently described pathway for NGF-mediated MAPK activation that is Ras-independent but dependent upon B-Raf and rap-1 (*64*). It should be noted that in the continued presence of NGF, high levels of MAPK activity are maintained even after the induced levels of *zif268* and *c-fos* mRNA have significantly decreased.

The data in Fig. 2 show a close correlation between the extent of activation of MAPK and the induction of *zif268* mRNA. Blocking the activation of MAPK with PD098059, however, shows that *zif268* mRNA induction is only partially inhibited under conditions where activation of MAPK is totally blocked (Fig. 3, B and C). This implies that *zif268* mRNA can be induced by NGF *via* pathways that do not involve MAPK.

Once we had established that blocking of the MAPK cascade blocked less than half of the *zif268* mRNA induc-

tion, we decided to test various protein kinase inhibitors for the ability to block the residual induction. Among the inhibitors examined, the fungal metabolite wortmannin (*59-62*) showed the most consistent inhibition and was effective at concentrations at which it is known to inhibit PI 3-kinase (Figs. 4A and 5, A and B). Partial inhibition of residual *zif268* mRNA induction was also obtained by pretreating cells with LY294002 (*63*), a PI 3-kinase inhibitor that is structurally unrelated to wortmannin (Kumahara and Saffen, data not shown). Together these observations suggest that PI-3 kinase may play a role in the MAPK-independent induction of *zif268* mRNA by NGF. Previous studies have established that PI 3-kinase is activated following the stimulation of PC12 cells by NGF (*44-46*). Two pathways of activation are possible for NGF: (i) activation mediated *via* indirect coupling of the high affinity NGF receptor (p140trk) to the SH2 domain of the PI 3-kinase p85 regulatory subunit and subsequent recruitment of the PI 3-kinase catalytic subunit (*45, 46*), and (ii) Ras (GTP)-mediated activation of PI 3-kinase (*74, 75*). Our observation that residual NGF-stimulated *zif268* mRNA induction is sensitive to wortmannin in cells overexpressing dominant-negative N17Ras (Fig. 5, A and B) suggests that a Ras-independent pathway for PI-3 activation may also be functioning in PC12D cells.

The data presented so far are consistent with PI 3-kinase playing a role in the MAPK-independent induction of *zif268* mRNA by NGF. There is, however, one aspect of this data that is rather puzzling. That is, although wortmannin is effective in blocking residual *zif268* mRNA induction in PC12D cells in which the activation of MAPK is blocked by pretreatment with PD098059 or expression of N17Ras, wortmannin has no measurable effect on the induction of *zif268* mRNA by NGF in the absence of blocking MAPK activation (Fig. 4B). These results suggest that induction of *zif268* mRNA by NGF in PC12D cells does not occur through the simple summation of MAPK-dependent and PI 3-kinase-dependent pathways. Rather, the PI 3-kinase pathway seems to make little or no contribution to *zif268* mRNA induction under conditions where MAPK can be activated by NGF. This could be explained if the MAPK-dependent pathway induces *zif268* mRNA more efficiently than the PI 3-kinase pathway, so that the contribution of the latter is masked. Another possibility is that activation of MAPK somehow inhibits the PI 3-kinase-dependent pathway for *zif268* mRNA induction. As yet, we have obtained no evidence that allows us to decide between these possible mechanisms.

We next attempted to determine the downstream target of PI 3-kinase that plays a role in the induction of *zif268* mRNA. Several kinases have been proposed to function downstream of PI-3 kinase including the serine/threonine kinases, Akt/PKB (*76, 77*), p70S6K (*78*), the atypical PKC isoforms, PKC-zeta (*79*) and PKC-lambda (*80*), and JNK (*65, 81, 82*). [See Ref. *83* for additional references.] Among these kinases, JNK seemed to be the most likely candidate to transmit the signal from PI 3-kinase to the *zif268* promoter. EGF stimulation of JNK in HeLa cells has been shown to be inhibited by wortmannin (*65*), and expression of a constituently active form of PI 3-kinase activated JNK in PC12 cells (*67*). Furthermore, there is now considerable evidence that activated JNK can phosphorylate and activate ternary complex factors, and thereby activate promoters containing SREs (*68-70*). Based upon these considerations, we decided to determine whether or not JNK is activated by NGF in PC12D cells and whether or not this activation is blocked by wortmannin. The data presented in Fig. 6 show that JNK is weakly activated by NGF in PC12D cells, confirming previous reports of the activation of JNK by NGF in PC12 cells (*47,*

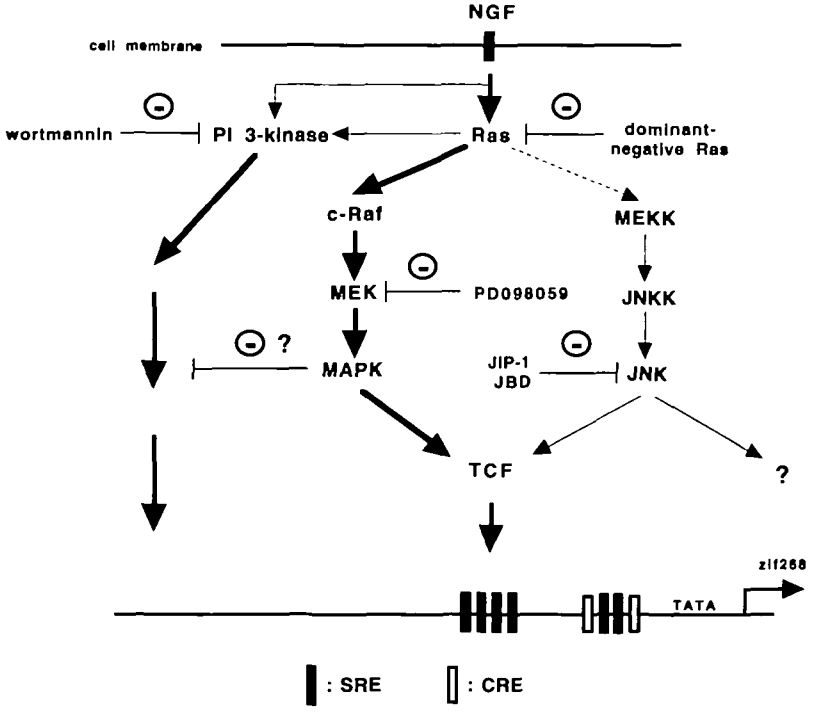


Fig. 8. **Model for induction of *zif268* mRNA by NGF.**

48). This activation, however, is not blocked by wortmannin, but rather enhanced, probably due to weak stimulation of JNK by wortmannin itself. These data argue against JNK playing a role in the wortmannin-sensitive pathway for *zif268* mRNA induction.

Because JNK is weakly activated by NGF in PC12D cells we wondered whether or not this activation contributes to the induction of *zif268* mRNA. The observations that wortmannin weakly activates JNK (Fig. 6), but does not by itself induce *zif268* mRNA (Fig. 4B), imply that this level of JNK activation is not sufficient for *zif268* mRNA induction. Still, we considered that it is possible that a certain level of JNK activation may be necessary, if not sufficient, for induction of *zif268* mRNA by NGF. Although there are currently no known chemical inhibitors that are specific for JNK, overexpression of the JNK-binding protein JIP-1 or its JNK binding domain (JBD-JIP-1) has been shown to block gene expression mediated by JNK (*47*). To determine if JNK plays a role in the induction of *zif268* mRNA in PC12D cells, we co-transfected PC12D cells with expression vectors encoding JIP-1 or JBD-JIP-1 with reporter plasmids containing segments of the *zif268* promoter linked to luciferase reporter genes, and examined the ability of NGF to induce luciferase. The data presented in Fig. 7 show that overexpression of JNK binding proteins partially blocked the induction of luciferase activity for two of the three expression vectors examined. The two expression vectors sensitive to expression of the JNK binding protein both contain the distal SREs of the *zif268* promoter, suggesting that JNK may function in *zif268* gene expression mediated by these elements. Examination of the DNA sequences of the distal and proximal SREs showed the presence of potential binding sites for TCF (*i.e.*, ets-like binding sites) upstream from the first and second SREs in the distal cluster, and inverted ets-like binding sites located upstream from the two proximal SREs (unpublished observations). Thus, differences in the sequences of individual SRE elements in the *zif268* promoter suggest that TCF may be particularly important for gene induction mediated by the distal SRE cluster.

The experiments described in this paper show that NGF induces *zif268* mRNA *via* MAPK-dependent and -independent pathways in PC12D cells, the latter being possibly mediated by PI 3-kinase. Our study also provides evidence for a role for JNK in the induction of *zif268* mRNA by NGF. A working model for *zif268* mRNA induction by NGF in PC12D cells, based upon these observations, is depicted in Fig. 8. This is the first report suggesting that PI 3-kinase may play a role in the induction of *zif268* gene expression by NGF. A role for PI-3 kinase in the induction of *c-fos* has previously been reported, but these studies suggested that PI 3-kinase functions upstream of Ras and the MAPK cascade (*84, 85*), or demonstrated *c-fos* expression following over-expression of the catalytic subunit of PI 3-kinase, without specifying the pathway of induction (*82*). By contrast, our data indicate that the wortmannin-sensitive pathway functions independently of Ras and MAPK. The observations that wortmannin and LY294002 partially block the residual *zif268* mRNA induction by NGF when activation of MAPK is inhibited are consistent with a role for PI 3-kinase, but the recent report (*86*) that wortmannin inhibits other kinases, including PLA_2, PLC, and PLD, at the concentrations used in this study shows that conclusive evidence for or against a role of PI 3-kinase must await studies using dominant-negative or activatable forms of PI-3 kinase.

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