Collaboration of JNKs and ERKs in Nerve Growth Factor Regulation of the Neurofilament Light Chain Promoter in PC12 Cells*

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Nerve growth factor (NGF) induces transcription-dependent neural differentiation of PC12 cells, and the ERK family of MAPKs has been implicated as the dominant signal pathway that mediates this response. We employed a neurofilament light chain (NFLC) promoter-luciferase (NFLC-Luc) reporter to define the role of the ERKs as well as additional MAPK pathways in NGF induction of this neural specific gene. Constitutive active forms of c-Raf-1, MEKK1 and MKK6, proximal regulators of the ERKs, JNKs, and p38 MAPKs, respectively, all stimulated NFLC-Luc activity. NFLC-Luc activity stimulated by NGF, however, was partially (~50%) inhibited by the MEK inhibitor, PD98059, or by co-transfection of kinase-inactive MEK1 but not by the p38 MAPK inhibitor, SB203580, indicating a role for the ERKs, but not the p38 MAPKs, in NGF regulation of the NFLC promoter. Importantly, a gain-of-function MKK7-JNK3 fusion protein stimulated NFLC-Luc and synergized with gain-of-function c-Raf-1 to activate the NFLC promoter. In addition, transfection of kinase-inactive forms of MEK1 and MKK7 produced an additive inhibition of NGF-stimulated NFLC-Luc relative to either inhibitor alone. These findings indicate that the ERK and JNK pathways collaborate downstream of the NGF receptor for regulation of the NFLC promoter. Truncation analysis and electromobility shift assays established the requirement for a cAMP-response element/activating transcription factor-like site in the NFLC promoter that minimally interacts with constitutively expressed cAMP-response element-binding protein and JunD as well as c-Jun which is induced by NGF in an ERK-dependent manner. Cumulatively, these findings indicate that the ERK pathway requires collaboration with the JNK pathway for maximal activation of the NFLC gene in PC12 cells through the integrated control of c-Jun function.

Members of the mitogen-activated protein (MAP)1 kinase family including the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAP kinases mediate cell growth, adaptation, and differentiation, in part, by phosphorylating and activating transcription factors that regulate genes required for execution of these programs (1). The PC12 pheochromocytoma cell line (2) provides a useful cell culture system in which to explore the mechanism by which MAP kinases control cell differentiation. Incubation of PC12 cells with nerve growth factor (NGF) leads to growth arrest and cellular hypertrophy as well as the acquisition of a neural morphology typified by extension of neurites. A molecular correlate of the morphologic differentiation of PC12 cells is induction of neurofilaments (3) that are composed of heavy, medium, and light polypeptide chains (4). Increased expression and phosphorylation (3, 5) of neurofilament proteins is thought to contribute to the extension and stabilization of neuronal processes. Moreover, the rat NFLC promoter has been characterized and shown to be induced by NGF in PC12 cells (6), indicating that transcriptional induction contributes to the increased content of neurofilament proteins.

Mutational analysis of the NGF receptor has defined the requirement for collaborative signaling through phospholipase Cγ (PLCγ) and the monomeric G protein, Ras, for morphologic differentiation of PC12 cells (7, 8). Similar mutational studies with ectopically expressed βPDGF receptors demonstrated the requirement for Ras signaling in combination with signaling through the Src family tyrosine kinases or regulation of PLCγ (9, 10). A clearly defined action of Ras within cells is the stimulation of MAP kinase cascades including the ERK pathway (11). The ability of constitutive active forms of proximal regulators of the ERK pathway to induce morphological differentiation suggests that the ERK pathway is necessary and perhaps sufficient for morphologic differentiation of PC12 cells (12). However, mutant βPDGF receptors that can activate the ERK pathway, but not Src or PLCγ, failed to induce morphologic PC12 cell differentiation (10), indicating that the ERK pathway is not sufficient for growth factor-induced PC12 cell differentiation and requires collaboration with other signals. Expression of GTPase-deficient, constitutive active forms of Gaαi proteins or incubation with bone morphogenetic protein-2 induced PC12 cell differentiation accompanied by persistent activation of the JNK family of MAP kinases (13, 14). Significantly, the ERKs were not activated in these settings, indicating that PC12 cell differentiation can sometimes occur independently of the ERK pathway and that the JNK pathway may...
contribute as well to transcriptional induction of PC12 cell differentiation.

In this study, we have employed a luciferase reporter construct controlled by the neurofilament light chain (NFLC) promoter to define MAP kinase pathways required for regulation of differentiation. We found that the NFLC promoter is activated upon NGF withdrawal, NGF, EGF, and IGF-1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The neurofilament light chain antibody was purchased from Sigma. Antibodies to CREB and phospho-CREB (Glu-Gly)5 linker between MKK7 and JNK1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Expression Vectors and Reporters
NFLC-Luciferase Reporters—Based on the reported sequence of the rat NFLC promoter (6), the forward primer 5′-GTACCGTTACCCTGCTCCTATGTGATAGAGGAATTCATTATCAGTG-3′ and reverse primer 5′-CTTCAAGCCTGGGGGCGGGAGATAGGGGACCT-3′ were synthesized and used to amplify by PCR the NFLC promoter sequences (~655 to ~82 bp) from rat PC12 cell genomic DNA. The PCR product was cloned into the pBluescript II Blue (Novagen) vector and submitted to DNA dideoxy sequencing, revealing a sequence identical to that deposited under the GenBank accession number X53981 and previously reported by Reuben et al. (6). The NFLC promoter was excised from pBluescript II Blue with KpnI (cuts at ~646 bp of the promoter) and HindIII (cuts in the polylinker of pBluescript II Blue) and ligated into pA3Luc, a promoterless luciferase vector, that had been double-digested with KpnI and HindIII, thereby generating NFLC-Luc. The CRE/ATF site at −46 to −36 bp (6) was converted to CTGGCTCAG to CCAACACAGAG to CAAATCAGAG (CRE-Mut) within the −650 to −82 NFLC promoter by PCR-based mutagenesis.

To generate progressively 5′-truncated NFLC promoter fragments, the pA3Luc construct was employed as a template for PCR using forward primers that annealed at −401 (5′-GGTACCGGAAAGGGGAGCGCCGGGG-3′), −201 (5′-GGTACCGGAAAGGGGAGCGCCGGGGGCC-3′), −128 (5′-GGTACCTTCTTCTGTGCCGGGATC-3′), −98 (5′-GGTACCGCTGAGGAGCCAGCTGC-3′), −88 (5′-GGTACCGCTGAGGAGCCAGCTGC-3′), and −19 bp (5′-GGTACCGGAAAGGGGAGCGCCGGGGGCC-3′) of the NFLC promoter and a reverse primer that annealed within the luciferase coding sequences of pA3Luc (5′-GTCGAGGAG-TATATCAGTG-3′). The amplified products were sequenced and ligated into pA3Luc that had been double-digested with KpnI and EcoRI.

MKK7-JNK3 Fusion Construct—Zheng et al. (15) have reported that a fusion protein consisting of MKK7 and JNK1 exhibited a gain-of-function phenotype. We created a cDNA encoding a similar polypeptide by fusing the murine MKK7b1 cDNA (16) to the rat p46 JNK3 cDNA (17), creating a MKK7b1-JNK3 fusion protein. To introduce an in-frame (Glu-Gly) linker between MKK7b1 and p46 JNK3 proteins, the MKK7b1 cDNA was submitted to PCR with a forward primer, 5′-GATACTCGAGTGGATCCGTC-3′, which contains the single MKK7b1 XhoI site (underlined), and the reverse primer 5′-CCACCGGAGAAGAGAGAGAT-3′, which encodes the (Glu-Gly) linker, as a replacement for the MKK7b1 stop codon and an AgeI site (underlined) to facilitate ligation to an N-terminal JNK3 PCR product. A PCR product containing an AgeI site and the sequences encoding the N-terminal portion of p46 JNK3 (up to the BsrXI site) was generated using the forward primer, 5′-CCACCGGAGACAGAGAGAGAT-3′ and the reverse primer 5′-GATCCAGCAGGAGGTTTGTTGC-3′. The MKK7b1 (Glu-Gly), and the p46JNK3 fragments were cloned into the pGEMT Easy vector (Promega) for DNA sequencing. The N-terminal fragment of p46JNK3 was excised from the pGEMT vector with AgeI and BsrXI and ligated into pGEMT-MKK7b1 (Glu-Gly), previously digested with AgeI and BsrXI. The resulting MKK7b1 (Glu-Gly)-N-terminal JNK3 fragment was excised with XhoI-BsrXI and ligated along with the BsrXI-ClaI fragment of p46JNK3 (JNK3 C terminus) from LNCX-PA46JNK3 (18) into XhoI-ClaI-digested LNCX-FLAG-MKK7b1, thereby generating FLAG-MKK7b1 (Glu-Gly)-p46JNK3 in the pLNCX retroviral expression vector (19). The XhoI, AgeI, and BsrXI ligation junctions were confirmed by DNA dideoxynucleotide sequencing.

Expression Vectors for c-Jun and c-Jun-DBD—An HindIII-Nor fragment of human c-JUN cDNA was excised from pRSV-c-Jun and ligated into pLNCX digested with HindIII and HpaI. Sequences encoding the DNA-binding domain of c-Jun (residues 224–331) were amplified from the human c-JUN cDNA and epitope-tagged (hemagglutinin) by PCR, sequenced, and ligated into the HindIII and ClaI sites of pLNCX.

Transient Transfections
Aliquots of PC12 cells (2 million cells in 100 μl) were electroporated (220 V and 250 microfarads with a GeneZapper (IBI, New Haven, CT)) in Gene Pulser 0.4-cm electrode gap cuvettes (Bio-Rad). PC12 cells were transfected with 2 μg of NFLC-Luc, 1 μg of pCMV-βGal for determination of transfection efficiency, 10 μg of salmon sperm DNA as a carrier, and other expression plasmids as indicated in the figure legends. Following electroporation, the PC12 cells were plated on collagen-coated dishes in full media for 24 h at 37 °C. The growth medium was subsequently replaced with DMEM containing 1% horse serum and antibiotics with growth factors or protein kinase inhibitors as indicated in the figure legends, and the transfected cells were returned to the incubator for 3 days. The transfected PC12 cells were then collected, washed once with ice-cold phosphate-buffered saline, and resuspended in 250 μl of Luciferase Reporter Lysis Buffer (Promega). The cell lysates were centrifuged in a microcentrifuge, and aliquots (80 μl) of the extracts were assayed for luciferase activity with a Monolith 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) and luciferase assay substrate from Promega. Aliquots (15 μl) of the extracts were also assayed for β-galactosidase (β-Gal) to correct for transfection efficiency. The data are presented as relative light units (RLU) per milliunit of β-galactosidase (RLU/milliunit β-Gal).

Electromobility Shift Assays
For preparation of nuclear extracts, PC12 cells were cultured for 4 days in DMEM containing 1% horse serum with or without 100 ng/ml NGF. The cells were then harvested and nuclear extracts prepared as follows. Cell pellets were resuspended in 5 volumes of 10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 0.2 mM dithiothreitol (DTT), 0.2 mM PMSF at 4 °C for 10 min, homogenized in a Dounce homogenizer, and centrifuged at 25,000 × g for 20 min. The pellets containing the nuclei were resuspended in 20 μM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF, homogenized with a Dounce homogenizer, and incubated at 4 °C for 30 min. Samples were centrifuged for 20 min at 25,000 × g and the supernatants recovered. Extracts were dialyzed against 20 mM HEPES-KOH (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 20% glycerol and stored at −70 °C.

Overlapping sense and antisense oligonucleotides encompassing the CRE/ATF site located at −56 to −28 within the NFLC promoter sequence (6) (5′-CGATCACAGTTCGCGCAGAGT-3′ and 5′-CGCCGGGACCTTCGACAGCAGAGT-3′) or the CRE-Mut (5′-CGATCACAGTTCACAGATTAG-3′ and 5′-CGCCGGGACCTTCGACAGCAGAGT-3′) sequences were annealed, the 5′-extensions filled with DNA polymerase (Klenow fragment) and deoxynucleotide triphosphates containing [α-32P]dCTP, and purified by gel filtration. Aliquots (80,000 cpm) of the 32P-labeled oligonucleotides were incubated (30 min, 4 °C) in a total volume of 20 μl with 5 μg of nuclear extracts, 0.125 μg of poly(dI-dC) (20 μl of HEPES-KOH (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, and 20% glycerol. For competition studies, the indicated concentrations of unlabeled double-stranded oligonucleotides were added to the reaction prior to addition of the radiolabeled probe. In the supershift experiments, antibodies were preincubated at 4 °C for 30 min prior to the addition of 32P-labeled DNA. Protein-DNA complexes were electrophoresed on 5% acrylamide gels at 25 mA/gel for 2 h in 5 mM Tris, 1.0 mM EDTA, 190 mM glycine. The gels were dried and exposed to x-ray film for 12–24 h.

RT-PCR Analyses
RNA was isolated with TRIzol reagent (Invitrogen) from PC12 cells that had been cultured for 1–4 days in DMEM containing 1% horse serum with or without 100 ng/ml NGF. Reverse transcription of total RNA (5 μg) was performed in a volume of 20 μl using random hexamers and murine leukemia virus-reverse transcriptase according to the man-

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Materials
PD098059 and SB203580 were purchased from Biomol (Plymouth Meeting, PA). NGF, EGF, and IGF-1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The neurofilament light chain antibody was purchased from Sigma. Antibodies to CREB and phospho-JNK1 c-Jun and JunD was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

"Experimental Procedures"
RESULTS

Induction of NFLC mRNA and Protein by NGF in PC12 Cells—The NFLC protein is a defined marker of neural cells that is involved in neurite outgrowth and function (4). In this study, we explored whether the NFLC gene is a target of NGF-stimulated MAP kinase pathways in PC12 cells. As determined by RT-PCR, the NFLC mRNA was induced in a delayed fashion with a maximal observed increase of ~10-fold after ~3–4 days of NGF treatment (Fig. 1, A and B). This finding is consistent with a previous report (21) of increased NFLC mRNA and protein accumulation in NGF-treated PC12 cells. The content of the GAPDH mRNA did not change over the same time course (Fig. 1C). Similar to the delayed induction of NFLC mRNA, the NFLC protein as detected by immunoblot

manufacturer’s protocol (PerkinElmer Life Sciences). Aliquots (10 \( \mu l \)) of the reverse transcription reactions were then submitted to PCR with oligonucleotides specific for the rat NFLC mRNA (forward primer, 5'-TCAGCAGGTTGGTAGCATA-3'; reverse primer, 5'-TGGGCTCAA-TTTGCTTCTGAG-3') yielding a 436-bp product or specific for rat GAPDH mRNA (forward primer, 5'-ATGACATCCTTCCAGGAGCAG-3'; reverse primer, 5'-CACGGATACATTGGGTTAGG-3') yielding a 508-bp product. The PCR conditions for amplification of NFLC were 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, and the GAPDH PCR conditions were 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. Aliquots (20 \( \mu l \)) of the PCRs were electrophoresed on 15%–20% agarose gels, stained with ethidium bromide, and photographed.

To confirm that the NFLC PCR amplified authentic NFLC cDNA, a Southern blot was performed. The agarose gel containing the NFLC PCR products was denatured for 30 min in 0.2 \( M \) NaOH, 0.5 \( M \) NaCl and neutralized by two washes in 50 \( mM \) Tris acetate (pH 7.8), 2.5 \( mM \) EDTA followed by one wash in 10 \( mM \) Tris acetate (pH 7.8), 0.5 \( mM \) EDTA. The gel was electrophoretically transferred to a Zeta-Probe (Bio-Rad) membrane, UV cross-linked, and pre-hybridized at 42 °C in 10 ml of Rapid-hob buffer (Amersham Biosciences). A single-strand oligonucleotide probe (5'-TCAGCAGGTTGGTAGCATA-3') corresponding to NFLC base pairs 1428–1448 was labeled with T4 polynucleotide kinase and \( \gamma \)-\( ^{32}P \)-ATP and incubated with the membrane at a concentration of 1 \( \times 10^6 \) cpm/ml for 1 h at 42 °C. The membrane was subsequently washed at room temperature in 5X SSC, 0.1% SDS and twice in 1X SSC, 0.1% SDS at 42 °C and exposed to x-ray film for 2 h.

Immunoblot Analysis

Sample of cells extracts prepared in MAP kinase lysis buffer (0.5% Triton X-100, 50 \( mM \) \( \beta \)-glycerophosphate (pH 7.2), 0.1 \( mM \) sodium vanadate, 2 \( mM \) MgCl\(_2\), 1 \( mM \) EGTA, 1 \( mM \) DTT, 2 \( \mu g/ml \) leupeptin, and 4 \( \mu g/ml \) aprotinin) supplemented with 300 \( mM \) NaCl were resolved by 10%–30% PAGE and transferred to nitrocellulose. The filters were blocked in Tris-buffered saline (10 \( mM \) Tris-Cl (pH 7.4), 140 \( mM \) NaCl, 5 \( mM \) KCl, 1 \( mM \) EDTA, 0.1% NaN$_3$) and incubated with the membrane at a concentration of 1 \( \times 10^4 \) cpm/ml for 1 h at 4 °C. The membrane was subsequently washed at room temperature in 5X SSC, 0.1% SDS and twice in 1X SSC, 0.1% SDS at 42 °C and exposed to x-ray film for 2 h.

Analysis of JNK and ERK Activity

PC12 cells were lysed in MAP kinase lysis buffer and clarified by a 5-min microcentrifugation (10,000 \( \times g \)). For assay of JNK activity, aliquots of the extracts containing 200 \( \mu g \) of protein in a volume of 0.5 ml were incubated for 2 h at 4 °C with 1 \( \mu g \) of anti-JNK1 (JNK1 (C-17) from Santa Cruz Biotechnology, Santa Cruz, CA) and 10 \( \mu l \) (packed bead volume) of protein A-Sepharose. The complexes were washed three times by repetitive centrifugation in lysis buffer and then incubated for 5 min with 100 \( \mu l \) of incubation buffer (20 \( mM \) Tris-HCl (pH 7.4), 150 \( mM \) NaCl, 0.1% Tween 20, 10 \( mM \) Na$_2$HPO$_4$, 5 \( mM \) EDTA, 1 \( mM \) EGTA, 1 \( mM \) DTT, 5 \( \mu g/ml \) aprotinin) supplemented with 300 \( mM \) NaCl were resolved by 10%–30% PAGE and transferred to nitrocellulose. The filters were blocked in Tris-buffered saline (10 \( mM \) Tris-Cl (pH 7.4), 140 \( mM \) NaCl, 5 \( mM \) KCl, 1 \( mM \) EDTA, 0.1% NaN$_3$) and incubated with the membrane at a concentration of 1 \( \times 10^4 \) cpm/ml for 1 h at 4 °C. The membrane was subsequently washed at room temperature in 5X SSC, 0.1% SDS and twice in 1X SSC, 0.1% SDS at 42 °C and exposed to x-ray film for 2 h.

Fig. 1. Kinetics of induction of neurofilament light chain mRNA and protein by NGF in PC12 cells. PC12 cells were treated for 0–4 days with NGF, and total RNA was prepared as described under “Experimental Procedures.” A, aliquots (5 \( \mu g \)) of total RNA were submitted to RT-PCR analysis using primers specific for NFLC. Portions of the PCRs were resolved by agarose gel electrophoresis and stained with ethidium bromide. B, the agarose gel shown in A was Southern-blotted with a \( ^{32}P \)-labeled oligonucleotide specific for NFLC. The washed filter was exposed to x-ray film for 2 h. C, aliquots of total RNA were submitted to reverse transcriptase-PCR analysis using primers specific for GAPDH, and the products were resolved by agarose gel electrophoresis. D, PC12 cells were incubated with NGF for the indicated number of days and dissolved in MAPK lysis buffer to generate a whole-cell extract (see under “Experimental Procedures”). Aliquots containing 100 \( \mu g \) of total cellular protein were resolved by SDS-PAGE and immunoblotted with anNFLC antibody.

The analysis was increased by 2 days and maximally increased after 3–4 days of NGF treatment. This time course of NFLC mRNA and protein accumulation coincides with the onset of neurite outgrowth in NGF-stimulated PC12 cells.

Stimulation of the NFLC Promoter by Growth Factors Is Correlated with Their Activity as Differentiation Factors and Dependent on Ras—We constructed a luciferase reporter containing NFLC promoter sequences (655 to +82) (see “Experimental Procedures”) to identify signal pathways that mediate NGF-stimulated NFLC induction as a marker for PC12 cell differentiation. Transient transfection of the NFLC-Luc plasmid into PC12 cells revealed a pronounced ~8-fold induction of luciferase activity (Fig. 2) following 3 days of incubation with NGF, similar to the degree of NFLC mRNA induction observed in Fig. 1. A weaker ~3.5-fold induction of NFLC-luciferase activity was measured in response to EGF, consistent with the relative ability of these two growth factors to induce morphologic differentiation in our PC12 cell cultures. IGF-1, a potent trophic factor but inactive differentiative agent for PC12 cells (22), failed to stimulate NFLC-luciferase over the basal activity and also failed to increase NFLC protein content (Fig. 2, inset).

Morphologic differentiation of PC12 cells by NGF requires signal transduction through Ras, and expression of constitutive active Ras elicits many of the morphologic and molecular actions of NGF in PC12 cells. Expression of constitutive active Ha-Ras (RasG12V) stimulated the NFLC promoter activity 4.5-fold from 144 ± 30 to 654 ± 64 RLU/milliunit \( \beta \)-Gal, similar to the 6-fold NGF response (850 ± 157 RLU/milliunit \( \beta \)-Gal) in these experiments. By contrast, transfection of the dominant-negative Ras molecule, RasN17, yielded basal and NGF-stimulated luciferase activities of 252 ± 81 and 422 ± 118 RLU/milliunit \( \beta \)-Gal, respectively, a significantly reduced 1.7-fold stimulation. Thus, the growth factor selectivity (NGF > EGF > IGF-1) and Ras requirement of NGF-stimulated NFLC-luciferase activity are consistent with the previously established growth factor sensitivities and Ras requirement of PC12 cell differentiation.
Evidence for Collaboration of the JNK and ERK MAP Kinases in NGF-stimulated NFLC-Luciferase Induction in PC12 Cells—Ras is a proximal regulator of multiple signal pathways including the ERK and JNK MAPK pathways (11). Transfection of constitutive active c-Raf (BXB-Raf), an upstream activator of the ERK pathway, significantly increased NFLC-Luc activity in the absence of NGF (Fig. 3, A and B). The BXB-Raf-stimulated NFLC-Luc activity was completely inhibited by the MEK inhibitor, PD098059 (Fig. 3A), or co-transfected kinase-inactive MEK1 (MEK1-K97M) (Fig. 3B). However, a concentration of PD098059 or MEK1-K97M sufficient to inhibit completely the BXB-Raf response only partially (~40–50%) inhibited NGF-induced NFLC-Luc activity (Fig. 3, A and B). This is consistent with the reduction, but not elimination, of the NGF-stimulated neurite outgrowth response in our PC12 cell cultures by PD098059 (not shown). The partial reduction of NFLC-Luc induction by PD098059 was not due to a partial MEK inhibition in these experiments because the concentration of drug employed completely inhibited persistently increased ERK activation measured after 24 h of NGF treatment from 3.6-fold in control cells to 0.8-fold in cells treated with PD098059. Thus, two different ERK pathway inhibitors at levels sufficient to inhibit the Raf-1 response yielded only a partial inhibition of the NGF stimulation, indicating that additional signal pathways collaborate with the ERK pathway for full regulation of the NFLC promoter by NGF.

The p38 MAPK pathway has been reported to be regulated by NGF in PC12 cells and also proposed as a signal pathway that mediates some of the actions of NGF in PC12 cells (23). Expression of a constitutive active MKK6 cDNA in which the activation-associated phosphorylation sites are mutated to glutamic acid (MKK6-EE) significantly increased NFLC-Luc activity from 125 ± 26 to 671 ± 109 RLU/million unit β-Gal (p < 0.05), indicating that the p38 MAPK pathway can activate this NGF-responsive promoter. However, incubation of PC12 cells with the 5 μM SB203580, a p38 MAPK inhibitor, significantly (p < 0.05) reduced the MKK6-EE-stimulated luciferase activity (206 ± 34 RLU/million unit β-Gal) but had no significant effect on the induction of the promoter by NGF (1887 ± 497 and 1787 ± 607 RLU/million unit β-Gal for control and SB203580-treated, respectively). Thus, the p38 MAPK pathway does not significantly contribute to regulation of the NFLC promoter by NGF.

Transfection of an activated form of MEKK1 also significantly stimulated NFLC-Luc activity but was only modestly reduced by PD098059 (Fig. 3A), indicating that MEKK1 activated the NFLC promoter largely through MEK/ERK-independent pathways. MEKK1 is a known upstream activator of the JNK pathway (24), suggesting that MKK4 or MKK7, proximal activators of the JNK pathway, may mediate the increased NFLC-Luc activity. Although gain-of-function forms of MKK4 or MKK7 analogous to constitutive active MEK1 and MKK6 do not exist, a fusion protein of MKK7 and JNK1 expressed in vivo yielded a gain-of-function phenotype when transfected into cells and activates neither ERKs nor p38 MAPKs (15). We generated a similar construct with an N-terminal MKK7 cDNA fused in frame with a C-terminal JNK3 cDNA and observed constitutive activity as assessed in vivo with c-Jun-GAL4 transcriptional activation assays and in vitro with c-Jun protein kinase activity (data not shown). As shown in Fig. 4A, transfection of the MKK7-JNK3 construct alone activated the NFLC-Luc reporter ~3-fold. Moreover, co-transfection of MKK7-JNK3 and BXB-Raf yielded a more than additive response compared with ei-
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The NFLC promoter contains multiple predicted enhancer sites with unknown requirements for NGF regulation (6). As shown in Fig. 5, analysis of the NFLC promoter sequence with the MacVector (Oxford Molecular Group) transcription factor enhancer site data base identified an AP1-TRE site, several predicted Sp1 sites, a CCAAT/enhancer-binding protein (C/EBP) site, an NFκB site, an EGR-1 site, and a CRE/activating transcription factor (ATF)-like site. A recent study has highlighted C/EBPβ as a neuronal transcriptional regulator that is activated by NGF signaling (27). Ziff and co-workers (28) have identified the Sp1 transcription factor-binding sites in the cyclin D and p21WAF promoters as putative NGF-responsive regulatory elements. Finally, the AP1 and CRE/ATF-binding sites in the NFLC promoter are elements known to integrate MAPK signaling pathways (1). To begin to identify the sequences within the NFLC promoter that are involved in mediating induction by NGF, we generated NFLC promoter-luciferase constructs progressively truncated at the 5′ end (−401, −201, −128, −98, −38, and −19 bp) and containing sequences up to position +82 base pairs. As shown in Fig. 5, deletion of sequences up to −128, which removes the predicted Sp1 sites, did not significantly influence the induction of the promoter by NGF. Further truncation to −98 bp markedly reduced the induction of luciferase activity by NGF and truncation to −38 bp, which deletes the CRE/ATF site, completely abolished NGF induction of the NFLC promoter, and yielded a basal activity similar to that observed with the TATA-less −19 bp promoter. Thus, NFLC promoter sequences between −128 and −38 are necessary for NGF-dependent regulation of the NFLC promoter in PC12 cells, and the CRE/ATF site at −45 to −39 bp is highlighted as one putative enhancer site that may integrate the response.

EMSA were performed with an EMSA oligonucleotide that flanks NFLC promoter sequences from −60 to −28 as well a mutant oligonucleotide in which a 4-base pair change was introduced into the CRE/ATF site (in bold), see Fig. 6. The wild-type oligonucleotide probe was bound by proteins present in nuclear extracts prepared from control and NGF-stimulated PC12 cells (Fig. 6A). The binding was specific as defined by complete competition of the binding by inclusion of unlabeled (cold) oligonucleotides. A greater amount of binding in extracts

other MKK7-JNK3 or BXB-Raf alone. Thus, a JNK signal alone can weakly activate the NFLC promoter but can collaborate with the Raf/MEK/ERK pathway to yield additive or greater responses.

To test directly the role of the JNK pathway in regulation of the NFLC promoter by NGF, an expression vector encoding a kinase-inactive form of MKK7 (MKK7-KM) was co-transfected with NFLC-Luc. MKK7-KM inhibited NGF-stimulated NFLC-luciferase activity −30% (Fig. 4B), similar to the reduction achieved by MEK1-KM (Fig. 3B). Expression of both MKK7-KM and MEK1-KM produced an additive inhibition of NFLC-luciferase (Fig. 4B), indicating a collaboration of the ERK and JNK pathways in the induction of the NFLC promoter by NGF. A similar additive inhibition was observed with kinase-inactive MKK4 and MEK1-KM (data not shown). Note that the maximal inhibition of NGF-induced NFLC promoter induction by kinase-inactive MEK1 and MKK7 was −60%. It is possible that MKK7-KM is unable to inhibit completely signaling through the JNK pathway. However, simultaneous transfection of kinase-inactive forms of MKK7 and MKK4 did not yield a greater degree of inhibition than either alone (data not shown), and MEK1-KM is able to inhibit completely the induction of NFLC-Luc by BXB-Raf (Fig. 3B). Thus, an alternative explanation is that signal pathways in addition to the JNKs and ERKs that are presently undefined also integrate at the NFLC promoter for full regulation by NGF.

The findings in Figs. 1–4 confirm the NFLC promoter responsiveness to NGF previously shown (6) and indicate that the JNKs as well as the ERKs integrate to mediate the stimulatory action of NGF. Based on these findings, persistent activation of the JNK pathway as well as the ERK pathway by NGF in PC12 cells is predicted. We measured JNK activity in PC12 cells treated for 10 min or 3 days with NGF using a specific JNK-IP assay (see “Experimental Procedures”). The results revealed an −2- and 8-fold increase in JNK activity following a 10-min or 3-day NGF treatment, respectively. In the same extracts, ERK activity was increased 27- and 6-fold, respectively. The modest increase in JNK activity after brief exposures to NGF and the more robust increases in JNK activity observed after several days of NGF treatment are consistent with findings in our previous publications (13, 25) as well as those of Malek et al. (26). Thus, the requirement for the ERK and JNK pathways in NFLC regulation by NGF is consistent with their persistent activation by this neurotrophin.

Identification of Promoter Sequences and Cellular Transcription Factors Involved in NGF Regulation of the NFLC Promoter—The NFLC promoter contains multiple predicted enhancer sites with unknown requirements for NGF regulation (6). As shown in Fig. 5, analysis of the NFLC promoter sequence with the MacVector (Oxford Molecular Group) transcription factor enhancer site data base identified an AP1-TRE site, several predicted Sp1 sites, a CCAAT/enhancer-binding protein (C/EBP) site, an NFκB site, an EGR-1 site, and a CRE/activating transcription factor (ATF)-like site. A recent study has highlighted C/EBPβ as a neuronal transcriptional regulator that is activated by NGF signaling (27). Ziff and co-workers (28) have identified the Sp1 transcription factor-binding sites in the cyclin D and p21WAF promoters as putative NGF-responsive regulatory elements. Finally, the AP1 and CRE/ATF-binding sites in the NFLC promoter are elements known to integrate MAPK signaling pathways (1). To begin to identify the sequences within the NFLC promoter that are involved in mediating induction by NGF, we generated NFLC promoter-luciferase constructs progressively truncated at the 5′ end (−401, −201, −128, −98, −38, and −19 bp) and containing sequences up to position +82 base pairs. As shown in Fig. 5, deletion of sequences up to −128, which removes the predicted Sp1 sites, did not significantly influence the induction of the promoter by NGF. Further truncation to −98 bp markedly reduced the induction of luciferase activity by NGF and truncation to −38 bp, which deletes the CRE/ATF site, completely abolished NGF induction of the NFLC promoter, and yielded a basal activity similar to that observed with the TATA-less −19 bp promoter. Thus, NFLC promoter sequences between −128 and −38 are necessary for NGF-dependent regulation of the NFLC promoter in PC12 cells, and the CRE/ATF site at −45 to −39 bp is highlighted as one putative enhancer site that may integrate the response.

EMSA were performed with an EMSA oligonucleotide that flanks NFLC promoter sequences from −60 to −28 as well as a mutant oligonucleotide in which a 4-base pair change was introduced into the CRE/ATF site (in bold), see Fig. 6. The wild-type oligonucleotide probe was bound by proteins present in nuclear extracts prepared from control and NGF-stimulated PC12 cells (Fig. 6A). The binding was specific as defined by complete competition of the binding by inclusion of unlabeled (cold) oligonucleotides. A greater amount of binding in extracts...
from NGF-stimulated cells was consistently observed relative to extracts prepared from control cells. Analysis of nuclear proteins that bound to the mutant probe, which is predicted to abolish CREB binding, failed to exhibit specific binding of nuclear proteins in either extract. To determine whether the CRE/ATF site is important for NGF-dependent regulation of the NFLC promoter in PC12 cells, the mutant CRE/ATF sequence was introduced into the NFLC-Luc construct, and the ability of NGF to induce luciferase activity following transfection of PC12 cells was assessed. As shown in Fig. 6B, introduction of the mutant CRE/ATF sequence into the NFLC promoter reduced NGF stimulation of the reporter by 50-80%. This result supports the findings in Fig. 5, which indicate that the CRE/ATF sequence within the NFLC promoter is a necessary enhancer element involved in NFLC regulation by NGF in PC12 cells. However, the CRE/ATF site is probably not sufficient for NGF induction of the NFLC promoter since the NFLC promoter truncated to -98 bp exhibits markedly reduced induction by NGF.

We used EMSA coupled with antibody supershift analysis to identify transcription factors in PC12 cell nuclear extracts that bind the CRE/ATF sequence at -45 to -39 bp of the NFLC promoter. As shown in Fig. 7, inclusion of a CREB antibody in the assay resulted in the supershift of the majority of the complex detected with the CRE/ATF probe in extracts from control and NGF-treated PC12 cells. However, a residual amount of complex reproducibly remained un-shifted with the CREB antibody in extracts from NGF-stimulated cells, indicating that a factor distinct from CREB contributes to the binding of EMSA probe in extracts from NGF-treated cells. The c-Jun antibody shifted a minor amount of the complex formed with extracts from control cells and a significantly greater amount of complex in extracts from NGF-treated cells. Likewise, a JunD antibody induced a diffuse supershift of the complex detected in extracts from NGF-treated cells but not control cells. By contrast, antibodies to ATF2 and JunB failed to induce a supershift of the complexes detected with EMSA probes (data not shown). Combined, the data in Figs. 6 and 7 indicate that CREB, c-Jun, and JunD are transcription factors that interact with the CRE/ATF site in the NFLC promoter and that c-Jun and JunD are recruited into this complex following stimulation with NGF.

To determine whether the appearance of JunD in the EMSA
A 32P-labeled oligonucleotide probe corresponding to the promoter.

protein complex that binds to the CRE/ATF-site in the NFLC arrowheads terisks bodies specific for different transcription factors as indicated. The extracts from control or NGF-treated PC12 cells with or without anti-wild-type sequence shown in Fig. 6 was submitted to EMSA with transcription factor by NGF, extracts were prepared from PC12 complex from NGF-treated cells was due to induction of this pho-Ser-73-specific antibody, was stably increased (Fig. 8 ent with previous reports (29, 30). Moreover, phosphorylation of PC12 cells (see above and Refs. 13, 25, 26). Finally, incubation of PC12 cells with PD098059 completely inhibited the NGF-stimulated JNK activation in NGF-treated PC12 cells (31, 32). Compared with basal and NGF-stimulated NFLC promoter activities of 160 and 2069 RLU/milliunit -Gal, respectively. Thus, the data in Table I indicate that c-Jun is limiting for the induction of the NFLC promoter in PC12 cells, whereas CREB and JunD overexpression can significantly increase the basal promoter activity but not the NGF-stimulated activity. To test further the role of c-Jun in regulation of the NFLC promoter in PC12 cells, an expression vector encoding an inhibitory form of c-Jun (c-Jun-DBD) was employed. This construct is predicted to behave in a competitive inhibitory fashion toward endogenous c-Jun (31, 32). Compared with basal and NGF-stimulated NFLC promoter activities of 160 ± 28 and 2069 ± 331 RLU/milliunit β-Gal, respectively, in control PC12 cells, transfection of c-Jun-DBD yielded basal and NGF-stimulated promoter activities of 105 ± 26 and 1136 ± 176 RLU/milliunit β-Gal, respectively. Thus, the data in Table I coupled with the ~50% decrease in NGF-stimulated NFLC-Luc activity by c-Jun-DBD support the involvement of c-Jun in the regulation of the NFLC promoter by NGF in PC12 cells.

The effect of overexpression of c-Jun, JunD, and CREB and different combinations of these transcription factors was tested on the NFLC-luciferase reporter in PC12 cells (Table I). Transfection of c-Jun, JunD, and CREB, but not ATF2, resulted in significant increases in the basal luciferase activity, with c-Jun and CREB stimulating the greatest increases. Moreover, the combination of c-Jun and CREB or JunD and CREB resulted in nearly additive increases in basal promoter activity (Table I). Co-transfection of c-Jun and JunD or c-Jun, JunD, and CREB did not result in further increases in basal activity relative to c-Jun alone or c-Jun and CREB, respectively. Analysis of the action of transfected c-Jun, JunD, CREB, and ATF2 on NGF-stimulated luciferase activity revealed that c-Jun, but not JunD, CREB, or ATF2, enhanced NGF-stimulated activity. Moreover, none of the combinations were significantly different than c-Jun alone. The findings in Table I indicate that c-Jun is limiting for the induction of the NFLC promoter in PC12 cells, whereas CREB and JunD overexpression can significantly increase the basal promoter activity but not the NGF-stimulated activity. To test further the role of c-Jun in regulation of the NFLC promoter in PC12 cells, an expression vector encoding an inhibitory form of c-Jun (c-Jun-DBD) was employed. This construct is predicted to behave in a competitive inhibitory fashion toward endogenous c-Jun (31, 32). Compared with basal and NGF-stimulated NFLC promoter activities of 160 ± 28 and 2069 ± 331 RLU/milliunit β-Gal, respectively, in control PC12 cells, transfection of c-Jun-DBD yielded basal and NGF-stimulated promoter activities of 105 ± 26 and 1136 ± 176 RLU/milliunit β-Gal, respectively. Thus, the data in Table I coupled with the ~50% decrease in NGF-stimulated NFLC-Luc activity by c-Jun-DBD support the involvement of c-Jun in the regulation of the NFLC promoter by NGF in PC12 cells.

**DISCUSSION**

The ability of constitutive active forms of c-Raf-1 and MEK-1 (12, 33, 34) to induce morphologic differentiation of PC12 cells has led to the suggestion that the ERK pathway is a dominant, perhaps even sufficient, pathway mediating NGF-stimulated...
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differentiation of PC12 cells (12). However, our findings in this study provide novel evidence for the required collaboration in PC12 cells of the JNK MAP kinase pathway with the ERKs in the regulation of the NFLC gene by NGF. The required integration of Ras and either PLCγ or c-Src for NGF-stimulated morphological differentiation of PC12 cells has been proposed previously based on findings with mutant Trk and βPDGF receptors (7–10). These results are in keeping with our previously published βPDGF receptor transfection studies showing a requirement for signaling pathways in addition to the Raf/MEK/ERK pathway for neurite outgrowth in PC12 cells (9, 10).

Thus, in contrast to gain-of-function oncogenic forms of c-Raf and MEK1, our findings withNFLC promoter regulation by NGF are similar to those with the egr-1, cyclooxygenase-2, tumor necrosis factor α, and atrial natriuretic factor promoters that exhibit a required collaboration of multiple signal pathways for regulation by growth factors and physiologic stimuli (35–38).

We have observed previously that gene transfer of constitutive active forms of Gαq induced differentiation of PC12 cells as assessed by neurite extension and Na+ channel induction that was correlated with JNK activation (13); ERK activity was not increased in this setting. Likewise, bone morphogenetic protein-2 stimulated morphologic differentiation of PC12 cells, increased NFLC protein content, and was associated with JNK activation but not ERK activation (14). Also, studies with transfected adrenergic receptor sub-types indicate that only the α3-adrenergic receptor, which couples to both ERK and JNK MAPKs, is competent to signal PC12 cells differentiation (39). By using conditionally immortalized central nervous system hippocampal neural precursor cells, Rosner and co-workers (40) have established the requirement of Src, Ras, and c-Raf but not ERKs in fibroblast growth factor-induced neural differentiation. Activation of the JNK pathway by fibroblast growth factor was not measured in these studies, but the literature provides ample evidence that both Ras and Src can engage the JNK pathway in different cells (11, 41, 42). Importantly, the results of our present study move beyond a correlation of JNK activation with PC12 cell differentiation and provide direct evidence for the requirement of both ERK and JNK pathways in the induction of a marker of neural differentiation in PC12 cells.

Our results demonstrate that the NGF-responsive region of theNFLC promoter resides between −128 and −38 bp, a region encompassing a CRE/ATF site at −45 to −39 bp. Mutation of the CRE/ATF site strongly reduced induction of theNFLC promoter by NGF, indicating that it is necessary for growth factor action (Fig. 6). However, the CRE/ATF site does not appear to be sufficient for full activation since truncation of theNFLC promoter to −98 bp leaves the CRE/ATF sequence intact but markedly blunts NGF responsiveness of the promoter (Fig. 5). Although data base searches have failed to identify any known consensus enhancer sites betweenNFLC promoter sequences −128 and −98 bp, it is likely that an additional enhancer site or sites resides in this promoter region. The sequence CTGCCGTAG, which includes and flanks the CRE/ATF site in the ratNFLC promoter, is absolutely conserved in sequence and relative positioning to the TATA box in the human and mouseNFLC promoters (43, 44), suggesting that this response element functions in an evolutionarily conserved fashion inNFLC gene regulation in vivo. In this regard, several groups have shown that approximately −300 bp of the 5′-flanking sequence of theNFLC gene are sufficient to direct neural specific expression in transgenic mice (44–46). However, a humanNFLC promoter truncated to −55 bp did not drive neural expression (46), consistent with our finding that sequences proximal to the CRE/ATF site are also required for NGF responsiveness in PC12 cells.

CRE/ATF sites have been invoked as putative regulatory enhancer sites in promoters derived from a number of NGF-responsive genes including nur77, vgf, c-fos, and egr-1 (47–51). The NGF-responsive nur77 promoter contains two cis-acting elements with a core sequence TGGCGTCA identical to the CRE/ATF sequence in theNFLC promoter, where both are required for induction of the nur77 promoter by NGF (48). In addition, the vgf and c-fos promoters contain the sequence GAGGTCA, and the egr-1 promoter contains the sequence, TACGTCA, both of which are highly similar to theNFLC CRE/ATF site. With the exception of the nur77 promoter, where the CRE/ATF sites are sufficient to confer NGF responsiveness to a minimal thymidine kinase promoter (48), the CRE/ATF sites in the vgf, c-fos, and egr-1 promoters are not sufficient to confer NGF inducibility on their own and apparently function in a cooperative manner with other enhancer sites in these promoters. Whereas a CRE/ATF enhancer site may serve an important role in the NGF-dependent regulation of the aforementioned promoters, Sp1 elements appear to serve dominant roles in the control of the p21WAF1 and cyclin D1 promoters by NGF (28, 52). Sp1 sites were not required for NGF-dependent regulation of theNFLC promoter (Fig. 5). Thus, NGF-stimulated transcriptional regulation of the many genes induced during differentiation of PC12 cells likely involves multiple signaling pathways integrating at distinct categories of transcription factor complexes where CRE/ATF sites acting in concert with other cis-acting elements may control a subset of NGF-regulated genes.

Electromobility shift assays identify CREB, JunD, and c-Jun as components of a complex that assembles on theNFLC CRE/ATF site. CREB is a major factor that binds to the EMSA probe, regardless of whether the nuclear extracts were prepared from control or NGF-stimulated PC12 cells. By contrast, we observed NGF-stimulated recruitment of JunD into the putative regulatory complex that assembles on theCRE/ATF site (Fig. 7B) despite a high constitutive expression of JunD detected by immunoblot analysis (Fig. 8A). JunD has been identified previously as a key factor involved in the regulation of the nur77 gene by NGF in PC12 cells (48). Although the mechanism for JunD recruitment to theCRE/ATF site is not defined in our study, one possibility is heterodimerization with an inducibly expressed member of the bZIP family of transcription factors which is then targeted to this particular enhancer sequence.

Transcriptional activation of the c-jun gene in PC12 cells by NGF occurs in a protein synthesis-independent manner (30) and thus represents an immediate-early response. Evidence also indicates that c-Jun represents a key transcription factor involved in the neural differentiation response elicited by NGF in PC12 cells. We and others (13, 29) have reported previously that overexpression of c-Jun in PC12 cells results in morphological differentiation of PC12 cells independent of other upstream signals. Moreover, in the present study, transfection of c-Jun markedly stimulated both the basal and NGF-stimulated activity of theNFLC promoter (Table I), and the c-Jun–DBD construct reduced induction of theNFLC promoter by NGF. Based on the ERK-dependent induction of c-Jun protein by NGF in PC12 cells (Fig. 8) and the persistent phosphorylation of c-Jun on a JNK-specific site (serine 73) in NGF-treated cells (Fig. 8), we hypothesize that c-Jun serves as a point where the ERK and JNK pathways integrate to regulate theNFLC promoter. This model is consistent with our experiments demonstrating the collaboration of the ERK and JNK pathway at theNFLC promoter shown in Fig. 4, although additional studies will be required to define the precise mechanism by which
c-Jun regulates the NFLC promoter. In conclusion, this study suggests that control of gene expression involved in cellular differentiation requires integration of multiple signal pathways and not the action of a single dominant pathway.

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Collaboration of JNKs and ERKs in Nerve Growth Factor Regulation of the Neurofilament Light Chain Promoter in PC12 Cells
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