Nerve Growth Factor Uses Ras/ERK and Phosphatidylinositol 3-Kinase Cascades to Up-regulate the N-Methyl-d-aspartate Receptor 1 Promoter*

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We reported previously that nerve growth factor (NGF) up-regulates activity of the N-methyl-d-aspartate receptor 1 (NR1) promoter. We have explored the pathways and nuclear targets of NGF signaling in regulating the NR1 promoter. PD98059 and wortmannin, but not rapamycin, significantly attenuated NGF-induced transcriptional activity from an NR1 promoter-luciferase construct. Coexpressing constitutively active forms of Ras, Raf, or MAPK/ERK kinase 1 (MEK1) increased promoter activity dramatically. The MEK1-induced increase was largely prevented by mutations of the tandem GC boxes in the promoter. Promoter activity was also increased significantly by coexpressed GC box-binding proteins (Sp1, 3, or 4) in nonstimulated PC12 cells. Either an extracellular signal-regulated kinase-1 (ERK1)- or Sp1-specific antibody coprecipitated Sp1 with ERKs, and the coprecipitation was enhanced significantly by NGF treatment of PC12 cells. ERK2 also incorporated radioactivity of [γ32P]ATP into recombinant Sp1. However, ERK2-treated Sp1 and PC12 nuclear extracts or nuclear extracts from NGF-treated cells exhibited reduced binding to the promoter or a consensus GC box. Our results suggest that NGF utilizes both the Ras/ERK and phosphatidylinositol 3-kinase pathways to up-regulate NR1 promoter activity and that Sp1 is a novel substrate of NGF-activated ERKs. NGF-increased NR1 promoter activity may involve a complicated mechanism of Sp1 phosphorylation and possible transcription factor exchange.

The neurotrophin family of neurotrophic factors plays an important role in growth, survival, differentiation, and plasticity of cells in the nervous system. The prototype of this family is nerve growth factor (NGF), which interacts with a specific tyrosine kinase receptor, TrkA, on the cell surface, triggers multiple intracellular phosphorylation cascades, and alters cellular functions of the nervous system (1–3). One mechanism underlying NGF effects is an alteration in gene expression at the transcription level. A well defined Ras/extracellular signal-regulated kinase (ERK) cascade plays a major role in transducing NGF signals from the membrane to nuclear targets (4). Terminal effectors of this cascade, activated ERK1 and ERK2, are members of the mitogen-activated protein kinase (MAPK) family. They translocate to the nucleus (4, 5), or activate the pp90 ribosomal S6 kinase that then translocates to the nucleus (6), to modify proteins involved in gene transcription. Transcription factors identified as substrates of MAPK include TCF/Elk-1, ATF/CREB, C/EBPβ, c-Jun, c-Myb, c-Myc, and MEF2A (3, 7–12). Another important pathway of NGF signaling is the phosphatidylinositol 3-kinase (PI3K) cascade. Akt/protein kinase B (PKB) is the immediate downstream mediator of PI3K and transduces signaling to activation of the pp70 ribosomal S6 kinase (p70S6K) as well as to inhibition of the glycogen synthase kinase (GSK). The former positively regulates the protein translation machinery, whereas the latter may interact with transcription factors, such as heat shock factor-1 and c-Jun, and negatively regulate gene expression (13–15).

The NMDA subtype of glutamate receptors is a ligand-gated, voltage-dependent Ca2+ channel in the central nervous system. NMDA receptors play an important role in brain development, neuronal differentiation, and plasticity (16, 17). Among three families of genes encoding the NMDA receptor subunits, genes in NR1 and NR2 families undergo significant up-regulation in the developing brain or differentiating neurons (18–21). We reported previously that NGF up-regulated the NR1 promoter in PC12 cells through actions on the tyrosine kinase receptor, TrkA (22). However, an analysis of the minimal promoter sequence responsive to NGF did not reveal any consensus elements for transcription factors previously known to be MAPK substrates. A GSG element is present and recognized by the NGFI/Egr family of transcription factors, whose expression is transiently induced by NGF. Two tandem GC box elements,

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1 The abbreviations used are: NGF, nerve growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; p70S6K, pp70 ribosomal S6 kinase; GSK, glycogen synthase kinase; NMDA, N-methyl-d-aspartate; NR1, NMDA receptor 1 subunit; EGF, epidermal growth factor; MEK1, MAPK/ERK kinase 1; EMSA, electrophoretic mobility shift assay; fpu, footprinting unit(s); PKA, protein kinase A; bp, base pair(s).
overlapping the GSG site, in the proximal region of the NR1 promoter were important for basal as well as NGF-regulated activities, suggesting that factors interacting with this region may be nuclear targets of NGF signaling (22).

The GC box is the binding motif for the Sp family of transcription factors, including Sp1, Sp3, and Sp4 (23–25). The prototype of this family, the Sp1 factor, is important for brain development and activation of many genes in the nervous system. Although it shows no tissue specificity, deletion of the Sp1 gene results in failure of brain development in animals (26). Sp1 protein undergoes post-translational glycosylation and phosphorylation. Sp1 phosphorylation by DNA-dependent kinase does not alter its binding affinity to DNA (27). However, phosphorylation by casein kinase II decreases Sp1 binding at the acetyl-CoA carboxylase promoter, whereas phosphorylation by a putative Sp1 kinase may increase Sp1-related promoter activity of the gastrin promoter and the dihydrofolate reductase promoter in response to epidermal growth factor (EGF) and serum, respectively (28). In terminally differentiated liver cells, casein kinase II phosphorylation was also found to cause a down-regulation of Sp1 activity (29). In contrast, inhibition of Sp1 dephosphorylation may underlie the mechanism of the human immunodeficiency virus gene expression (30). Interestingly, v-Raf-induced activation of the mdr1 and rep-35 gene promoters, which are serum-inducible, is Sp1-dependent (31). Thus, a Raf cascade activating MAPK is likely to be involved in regulating Sp1 activity. More recent studies of the apolipoprotein A-I (32) and gastrin promoters (33) in non-neuronal tissues suggest that EGF may activate a Ras/ERK cascade that in turn phosphorylates Sp1 and enhances promoter activities. Our previous studies also demonstrated that Sp1 protein interacts with the NR1 promoter directly and has a synergistic effect on promoter activity with an MEF2C factor (34). However, in view of differential effects between NGF and EGF on Ras/ERK activation in nervous cells (35) as well as the fact that a number of neuronal genes require GC-rich elements for NGF-induced expression (4, 36–38), it is still not known whether NGF-activated Ras/ERK will modify the Sp1 factor and regulate promoter activity in neuronal cells (36–38).

In the present study, we investigated the postreceptor cascades of NGF signaling leading to activation of the NR1 promoter, potential nuclear targets of the pathways, and possible molecular mechanisms of the phosphorylated target(s) acting on the promoter. We focused on both PI3K and Ras/ERK cascades and nuclear proteins interacting with the GSG/GC box elements.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—PC12 cells were cultured as described previously (39). All NR1 luciferase constructs and stable PC12 cell lines expressing the NR1 luciferase fusion genes have been described (22). PC12 cell transfectants were cultured at 0.5 × 10^6 cells/60-mm collagen-coated plate in medium containing serum. In some experiments, serum was removed from cells for 2 h before 100 ng/ml NGF was added for various times. Then, cells were harvested for luciferase assays (39). In some experiments, before NGF treatment, cells were pretreated for 15 min with 20 μM PD98059, 200 nM wortmannin, or 27 nM rapamycin (Calbiochem-Novabiochem International, Bad Paar, Germany) following the manufacturer’s instructions.

DNA-Protein Interactions—Electrophoretic mobility shift assays (EMSAs) were performed as described (39). Nuclear extracts were prepared from PC12 cells by a modified Dignam method (39). Recombinant human Sp1 protein was purified from HeLa cells overexpressing full-length human Sp1 cDNA in a recombinant vaccinia virus (Promega Corp., Madison, WI). Five footprinting units (fpu) of Sp1 protein or 45 μg of protein from PC12 nuclear extracts was reacted with 50 units of activated murine ERK2 (New England Biolabs, Beverly, MA), 5 units of murine protein kinase A (PKA) (New England Biolabs), or 400 units of λ-protein phosphatase (New England Biolabs) following the manufacturer’s instructions. For EMSA, 1 fpu of Sp1 or 2.5 and 4.5 fpu of protein from PC12 cell nuclear extracts were incubated with radiolabeled probes: a GC box consensus (Promega) and the 112-bp NR1 5′-flanking region (39). Protein Analysis—Phosphorylation of Sp1 protein in vitro was tested as follows. One fpu of recombinant Sp1 protein was incubated with 50 units of recombinant ERK2 in the presence or absence of 40 μg of [γ-32P]ATP (>6,000 Ci/mmol, PerkinElmer Life Sciences) in the buffer provided with ERK2 and supplemented with 0.2 mM ATP for 30 min at 37 °C. Then the reactions were run together with 0.25 fpu/lane Sp1 protein on a 6% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane. The membrane was exposed to X-film for 4 h at −80 °C and then probed by a polyclonal Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in immunoblot analysis with ECL for 5 s (Amersham Pharmacia Biotech). Immunoblotting experiments were performed as described previously (39). Nuclear extracts were fractionated on 4–20% SDS-gels and blotted onto polyvinylidene difluoride membranes. For ERK1 immunoprecipitation, immunoblot analysis was carried out with the above anti-ERK1 antibody (Santa Cruz Biotechnology) or 1.6 μg of a polyclonal anti-ERK1 antibody (Upstate Biotechnology, Lake Placid, NY) overnight at 4 °C. Immunocomplexes were precipitated with protein A/G-agarose, washed four times with ice-cold phosphate-buffered saline, and eluted with loading buffer at 95 °C. Eluted proteins were fractionated on 4–20% SDS-gels and blotted onto polyvinylidene difluoride membranes. For ERK1 immunoprecipitation, immunoblot analysis was carried out with the polyclonal Sp1 antibody first and, after stripping the membrane, with the ERK1 antibody; for Sp1 immunoprecipitation, the analysis was done with the above antibodies in a reverse order. In some experiments, the chemiluminescence signals associated with chosen bands(s) on the blots were quantified on a Kodak Image Station 440CF and analyzed by the 1D Image Analysis software following the manufacturer’s instructions (PerkinElmer Life Sciences). All experiments were repeated at least six times, and the DNA data analysis was done using Vector NTI suite software (Informax, North Bethesda, MD).

RESULTS

Effect of NGF and Serum on NR1 Promoter Activity—Our previous studies revealed that NGF is capable of up-regulating the NR1 promoter in the presence of serum (22). To clarify whether serum itself has a possible influence on the NR1 promoter, we studied the effects of serum and NGF on the promoter after serum deprivation. The experiments were done in two lines of stable transfectant PC12 cells: PC12/pNRL356/lacZ, expressing the 356-bp promoter-luciferase gene, and PC12/pNRL3029/neo expressing the 3,029-bp promoter-luciferase gene (22). As shown in Fig. 1, A and B, addition of serum to PC12/pNRL356/lacZ cells did not result in a robust increase of 13-fold within 2 h, which was higher than that before serum deprivation (Fig. 1B). In comparison, addi-
Cells with wortmannin blocked PI3K and inhibited GSK, as expected. However, pretreatment with rapamycin, which inhibits mTOR and blocks the PI3K cascade downstream of mTOR, showed no inhibitory effect (Fig. 2A). This suggests that PI3K may signal through multiple effectors including p70S6K.

As shown in Fig. 2A, pretreatment of PC12/pNRL356/neo cells with wortmannin blocked ~70% of the NGF effect on the NR1 promoter. These results indicate an involvement of the PI3K cascade in NGF-regulated NR1 promoter activity. Because PI3K may signal through multiple effectors including activation of p70S6K and inhibition of GSK, we tested these two kinases as possible downstream effectors. Interestingly, application of rapamycin to cells showed no significant effect on promoter activity (Fig. 2A). Then we examined whether GSK has an inhibitory effect on the promoter by transiently coexpressing GSKα and/or GSKβ with the 356-bp NR1 promoter/luciferase gene (pNRL356) in PC12 cells. Cotransfection showed no inhibitory effect; rather, there was a slight increase in promoter activity by 60% caused by GSKα (Fig. 2B). Therefore, the PI3K effect on the promoter probably is not transduced by p70S6K or by GSK.

Very interestingly, PD98059 pretreatment of PC12 cells suppressed about 70% of the NGF up-regulated activity (Fig. 2A). This blockade suggests that a Ras/ERK pathway is involved in the NGF effect. To test this possibility further, we examined the effects of components of the Ras/ERK cascade on pNRL356.
We performed cotransfection of the pNRL356 construct with cDNAs for the components in this pathway: Ras, Raf, or MEK1. As shown in Fig. 2C, all of these constitutively active factors robustly increased reporter activity up to 23-fold over that in cells transfected with pNRL356 plus vector pcDNA3. In comparison, cotransfection with the dominant-negative forms of Ras, Raf, or MEK1 had no effect or resulted in only a slight increase (1.9-fold) by dominant-negative Ras (N17Ras) (Fig. 2C). These results clearly indicate that the Ras/ERK cascade is important in the regulation of the NR1 promoter. Interestingly, no inhibition of the nonstimulated activity of this promoter was observed with dominant-negative components. This suggests that the Ras/ERK pathway is not involved in the nonstimulated, basal activity of the NR1 promoter. However, we did not test the effect of dominant-negative forms on NGF activity because transient cotransfection needs to be monitored for the transfection efficiency with a second reporter gene driven by a viral promoter that is often regulated by growth factors such as NGF (22).

**Involvement of the GC Box and Its Binding Proteins in the Ras/ERK Cascade**—Our previous studies revealed that a proximal promoter region containing a GSG and overlapping tandem GC boxes is important for the NGF response. In addition, sequence analysis of this region revealed no regulatory element for transcription factors known to be direct substrates of MAPK. Therefore, to examine the possibility that the GSG/GC boxes are important elements of Ras/ERK signaling, we transiently cotransfected the active MEK1 gene with wild type or mutated GC box pNRL356 constructs into PC12 cells. As shown in Fig. 3A, disruption of individual GC boxes dramatically reduced promoter activation by MEK1 to about 15% of full response of the wild type promoter, and a double mutation disrupted the individual GC boxes dramatically reduced promoter activation by MEK1 to about 15% of full response of the wild type promoter, and a double mutation disrupted the individual GC boxes dramatically reduced promoter activation by MEK1 to about 15% of full response of the wild type promoter.

It is known that the GC box in a double-stranded conformation interacts with three members of the Sp family, i.e., Sp1, Sp3, and Sp4 (23–25). To test their potential roles in responding to Ras/ERK for NR1 promoter activation, we cotransfected pNRL356 with expression constructs of Sp1, Sp3, and Sp4. As expected, overexpression of individual Sp proteins caused an increase in reporter activity of 3.0-fold by Sp1, 3.35-fold by Sp3, or 2.46-fold by Sp4 (Fig. 3B).

### Sp1 Factor Is a Substrate of ERK—Data shown in Fig. 3 strongly suggest that Sp proteins are potential effectors of ERK in mediating NGF signaling to the NR1 promoter. Searching for MAPK phosphorylation sites using a consensus of PXX/SP or XXT/SP (9) revealed 9 putative phosphorylation sites in human Sp1, 10 in mouse, and 10 in rat. The potential of each site was scored from 0 to 1 by using NetPhos 2.0 software (www.cbs.dtu.dk) (42) and is listed in Table I for those sites expected to be phosphorylated.

Figure 3. Effects of GSG/GC box and Sp-binding proteins on Ras/ERK-activated promoter activity. Panel A, inhibitory effect of GSG/GC box mutations on MEK1-induced activity in PC12 cells. Wild type or GC box (Sp1a and/or Sp1b) mutants of the pNRL356 construct were cotransfected into PC12 cells with an active MEK1 construct as described under “Experimental Procedures.” Luciferase activities were assayed for each construct without (basal) and with (induced) cotransfection of MEK1. The MEK1-induced activity was calculated over basal activity individually for wild type and each mutant promoter construct. MEK1-induced promoter activities of the mutants were then compared with wide type and expressed as a percentage of MEK1-induced wild type promoter activity. Panel B, effects of Sp proteins on the NR1 promoter. Expression constructs of Sp proteins (Sp1, Sp3, Sp4) were cotransfected individually with pNRL356 into PC12 cells, and relative luciferase activity was measured. Promoter activity as a fold increase was calculated as described in the Fig. 2B legend.
**TABLE I**

Putative MAPK sites in Sp1 protein

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<th>Score</th>
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<th>Score</th>
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<td>SRIESPNEN</td>
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<td>SGQCDTRQV</td>
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<tr>
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<td>IIRRTPTVVGOQ</td>
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<tr>
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<td>SGTATPSAL</td>
<td>0.726</td>
<td>738</td>
<td>SGTATPSAL</td>
</tr>
</tbody>
</table>

a Full-length amino acid sequence of the human Sp1 protein is derived from a combination of three sequences deposited in GenBank (AAA61154, AF67726, CAB75345).

b GenBank accession number BA02235.

c GenBank accession numbers AF022363 and NM013672.

d The position for the serine or threonine is given.

* MAPK phosphorylation motif of XXS/TP is used (9).

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**FIG. 4.** ERK2 interacts with and phosphorylates Sp1 protein. Panel A, coimmunoprecipitation of ERK1 with Sp1 and effects of NGF treatment in PC12 cells. PC12 cells were serum starved followed by NGF treatment. Whole cell lysates were immunoprecipitated by an ERK1-specific antibody (lanes 1–5) or an Sp1-specific antibody (lane 6). Eluted immunocomplexes were subjected to fractionation on a 4–20% SDS-polyacrylamide gel and underwent immunoblot analysis of Sp1 and ERK1. Cell treatments are as follows for each lane: lane 1, serum deprivation; lanes 2 and 6, NGF for 1 h; lane 3, NGF for 24 h; lane 4, transfected with Sp3; lane 5, 40 μg of proteins of whole cell lysates without immunoprecipitation. Panels B, MAPK phosphorylation of Sp1 protein. Recombinant human Sp1 protein was incubated with recombinant rat ERK2 in the presence of [γ-32P]ATP and fractionated on a SDS-polyacrylamide gel (lane 1). Untreated Sp1 protein was run in parallel (lane 2). After blotting, the polyvinylidene difluoride membrane was exposed to x-ray film and then subjected to Sp1 immunoblot analysis. The autoradiogram is shown in the right panel, and the immunoblot results are in the left panel. All other details are described under “Experimental Procedures.”

(Fig. 4B) In a subsequent immunoblot analysis with Sp1 antibody, a single band appeared from the reaction either in the presence or absence of [γ-32P]ATP and migrated to the same position as the radioactive band in the autoradiogram. These results indicate that ERK2 is capable of transferring 32P radioactivity (phosphate) to the Sp1 protein in vitro.

**Effect of Sp1 Phosphorylation on DNA Binding Capacity**—To explore the effect of ERK phosphorylation of Sp1, we tested the binding capacity of Sp1 factor modified by several enzymes: ERK2, PKA, and λ-phosphatase, which dephosphorylates all tyrosine, threonine, and serine residues. First, we treated nuclear extracts of PC12 cells with ERK2 in the presence of ATP and used EMSA to examine their binding activity on a GC box consensus probe (Promega) (Fig. 5A). Surprisingly, compared with untreated nuclear extracts, ERK2 treatment reduced formation of all shifted bands that include Sp1 factor as demonstrated in our previous studies (39). In contrast, λ-protein phosphatase-treated nuclear extracts showed no significant change. Furthermore, nuclear extracts from PC12 cells treated with NGF showed a time-dependent reduction in complex formation either with an NR1 promoter fragment (112 bp) encompassing the GSG/tandem GC box elements or with the GC box consensus probe (Fig. 5, B and C). Results from EMSA experiments with recombinant Sp1 also showed that ERK2 treatment deceased the binding to the GC box consensus compared with untreated Sp1, even though PKA or λ-protein phosphatase treatment showed no significant influence on the binding (Fig. 5D).

**DISCUSSION**

Neurotrophins play important roles in survival and differentiation of developing neurons. One of the molecular mechanisms of neurotrophin function is the transcriptional induction of neuronal genes through multiple signal cascades. In the developing nervous system, NMDA receptor genes undergo robust expression. Following our previous report that NGF up-regulates the NR1 promoter, we investigated signaling cascades used by NGF in this regulation and possible nuclear proteins that mediate the signaling at the promoter. Our results strongly suggest that NGF regulates the promoter by activating the PI3K and Ras/ERK cascades in PC12 cells. The Ras/ERK cascade may use Sp1 protein to mediate NGF signaling to the promoter.

Neurotrophin activity in regulating gene expression is mainly transduced by the Ras/ERK and PI3K cascades, although the Ras/ERK cascade may be involved more in differentiation and PI3K more in cell survival (1). Primarily, the PI3K cascade carries on the survival signaling and may activate Akt/PKB, which in turn acts on multiple effectors and regulates gene expression. In our studies, we applied wortmannin to inhibit PI3K directly and observed a 70% reduction in NGF effect on the NR1 promoter. In view of multiple effectors in the cascade, we searched for those that may transduce the signaling to the promoter. One function of Akt/PKB is to acti-
vate the p70S6K. Inhibition of p70S6K phosphorylation by rapamycin showed no interference with the NGF effect on the promoter. Therefore, it seems that p70S6K is not involved in NGF regulation of the NR1 promoter. Then, we tested the possible role of GSK in transducing PI3K signaling. We did not observe any inhibitory effect of overexpressed GSKs on the NR1 promoter (Fig. 2B) and thus believe that release of a GSK inhibitory effect on the promoter may not be a mechanism underlying NGF-induced promoter activation. Activated PKB may also inhibit functions of several proteins, such as BAD (43) and caspase 9 (44), in rescuing cells from stress or death. However, in view of the nature of these factors, it is unlikely that they participate in regulation of the NR1 promoter. Interestingly, a recent cloning study revealed that PKB is able to phosphorylate a novel nuclear p70S6K-related kinase directly (45), which may be a nuclear mediator of the PI3K signaling. Another potential mediator may be the forkhead family of transcription factors. Studies of their roles in neuronal differentiation are just beginning, and recently it was shown that insulin-like growth factor I induces phosphorylation of FKHRL1 forkhead protein via PKB in PC12 cells (46). Nevertheless, how the PI3K signaling regulates the NR1 promoter still remains unsolved.

The Ras/ERK cascade in neurotrophin signaling has been well studied. Activated tyrosine kinases on the membrane recruit Grb-2 and SOS to activate Ras, a GTP-binding protein. Ras then activates Raf, MEK1, and ERK1/2 sequentially (1–3). A number of studies have reported that NGF activates this cascade through TrkA and regulates several neuronal genes at the promoter level. By blocking MEK1 with a specific inhibitor PD98059, we were able to inhibit NGF-induced activity of an NR1 promoter-reporter gene that has been integrated into chromosomes in the nucleus. In a transient transfection system, we observed that coexpressing active components of this cascade with the promoter-reporter gene robustly up-regulated the promoter to a high level. Our results support the notion that the NGF-activated Ras/ERK cascade is important in the regulation of the NR1 promoter. Interestingly, NGF regulation of promoters of neuronal genes utilizes several combinations of different signal cascades. For example, the zif268 promoter is regulated by NGF through a combination of Ras/ERK, PI3K, and JNK cascades (47), whereas the promoters of the chromogranin A gene, the Bel-2 gene, and the calcitonin gene-related peptide gene are targeted solely by Ras/ERK signaling (48, 49). Another similar case is seen in NGF regulation of the promoter of the neuronal β4 nicotinic acetylcholine receptor gene (50). The present studies suggest that both PI3K and Ras/ERK cascades are involved in the NR1 promoter activation. These examples may present several mechanisms underlying the diversity of NGF effects in regulating various neuronal genes.

One possible mechanism underlying the divergence of the NGF signaling in regulating transcription is that each promoter has distinct regulatory sequences and thus interacts with various transcription factors that can be modified by alternate NGF signaling. Our efforts in searching for crucial elements in the NR1 promoter revealed that the GSK/tandem GC box region responded to both NGF and Ras/ERK signaling. In the zif268 promoter, a GSK element is also considered one of candidates targeted by NGF/Ras/ERK signaling. Furthermore, we found no other putative sites for transcription factors known as ERK substrates. Therefore, transcription factors interacting with this GSK/GC box region could be the nuclear target(s) of the Ras/ERK cascade. In our previous studies, we showed that Sp1, Egr-1, and single-stranded DNA-binding proteins may interact with this region (22, 39, 51). ERK regulation of gene transcription can be mediated by two pathways: phosphorylated ERKs enter into the nucleus and modify transcription factors directly (4, 5), or ERKs may modify cytoplasmic ribosomal S6 kinase that translocates to the nucleus and regulates transcription (6). Therefore, those factors that respond...
to the Ras/ERK signaling may be the substrates of either ERKs or Rsk. Sp1 protein retains 6 potential sites of 9 putative motifs for MAPK and is able to bind to ERK1 and to be phosphorylated by ERK2. More importantly, NGF treatment enhanced this interaction. Therefore, we believe that the ERKs activated by NGF may act directly on the Sp1 transcription factor and regulate gene expression in neurons. This notion may help to explain the NGF effect on other genes. For example, NGF can up-regulate the promoters of the p21 WAF1/CIP1 and cyclin D1 genes (38) and the β4 nicotinic acetylcholine receptor gene (50), where the Ras/ERK is activated and the Sp1 factor is involved.

In a recent report, EGF was found to trigger a Ras/ERK cascade that may modify the Sp1 factor and up-regulate the apolipoprotein A-I promoter in a human hepatoma cell line (32). However, EGF may generate different signaling from NGF even in activating the Ras/ERK cascade in neuronal tissues. EGF activates Ras/ERK transiently, but neurotrophins trigger a fast and long term activation (35). Functionally, EGF supports cell survival and growth, whereas neurotrophins stimulate both cell survival and differentiation (52–54). Therefore, the genes induced by neurotrophins may include those related to neuronal differentiation, such as the NR1 gene.

To explain the functional relevance of reduced Sp binding activity after ERK2 treatment, one may propose that phosphorylation of Sp1 protein caused a reduction in its DNA binding activity but enhanced its capability for protein-protein interactions. Protein-protein interactions are thought to be important in the transactivation of gene expression, particularly for non-DNA-binding proteins in transcriptional regulation (55). In fact, MAPK phosphorylation of TCF/EkI-1 enhances the formation of ternary complexes in EMSAs (56). It was also reported that NGF is able to increase the interaction between the Sp1 and p300 factor, suggesting a role of protein phosphorylation (57). With regard to the NR1 gene, the Sp1 factor interacts with the MEF2C transcription factor and synergistically regulates the promoter (34). However, using both an NR1 promoter 112-bp probe and an Sp1 consensus probe in EMSAs (Fig. 5), we did not observe the formation of any larger complexes from either recombinant, phosphorylated Sp1 protein or extracts from NGF-treated PC12 cells. Similar results were also observed in PC12 cell extracts treated with ERK2 in vitro (Fig. 5A). Our studies suggest another hypothesis, that the phosphorylation of Sp1 protein by ERK causes Sp1 complex to drop off the DNA and expose the binding element for other transcription factors. Candidates for such transcription factors may include nuclear factor-xB and Egr-1, which can be activated by NGF (58, 59) and compete for the same sites in double-stranded DNA conformation as Sp1 (60–62). In particular, a GSG element for Erg-1 binding overlaps the GC boxes in the NR1 promoter. However, a double-stranded probe of this region used in EMSAs did not reveal any new complex (Fig. 5B), and therefore factors binding to double-stranded DNA are unlikely to replace Sp1 from the NR1 promoter. Other potential candidates may be single-stranded DNA-binding proteins that can also be activated by NGF and bind to the same promoter region as revealed by our previous studies (51). In that report, we observed that single-stranded DNA-binding complex 2 interacted with a T(G)A sequence overlapping the GC boxes, but in a single-stranded DNA conformation only, and NGF treatment of PC12 cells for 30 min increased this interaction significantly. Very interestingly, a T(G)A sequence is also located upstream of a GC-rich element in the Egr-1 promoter that was found to be up-regulated by NGF (59, 63). Molecular cloning of these putative single-stranded DNA-binding factors is currently under way to directly test this hypothesis.

We also noticed that in nonstimulated PC12 cells, overexpression of Sp proteins moderately increased NR1 promoter activity, suggesting a stronger interaction of Sp protein with the promoter. However, it has been reported by several laboratories that MAPK remains mostly inactive in the cytosol of nonstimulated PC12 cells (64–67). In addition, our studies showed that dominant-negative Ras, Raf, or MEK did not suppress the basal activity of the promoter in nonstimulated cells (Fig. 2C). Taken together with results obtained from studies of Sp1 phosphorylation by ERKs, we believe that Sp1 protein may participate in both basal regulation and NGF induction of the NR1 promoter but by different molecular mechanisms.

In this study, we observed that the PI3K and Ras/ERK cascades participate in transducing the NGF signal to the NR1 promoter. Interactions of factors at the GSG tandem GC boxes in the proximal region of this promoter may play an important role in the response to the Ras/ERK cascade as well as in the control of basal promoter activity. Sp1 protein, a general transcription factor, might be a substrate of NGF-activated MAP kinase, and a reduction of Sp1 DNA binding affinity may be involved in the regulatory mechanism. We expect to uncover factor(s) that may replace the Sp1 binding after MAPK-induced phosphorylation.

REFERENCES
