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Differential Regulation of the Transcriptional Activity of the Orphan Nuclear Receptor NGFI-B by Membrane Depolarization and Nerve Growth Factor*

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The immediate-early gene NGFI-B (also called *nur77*) encodes an orphan nuclear receptor that activates transcription through a unique response element (NBRE). NGFI-B is rapidly induced and modified via phosphorylation by a variety of stimuli that induce cells to differentiate or to proliferate. We have shown that the *in vitro* phosphorylation of Ser^{350} located within the "A-box," a motif necessary for DNA binding by NGFI-B, results in a decrease in the binding of NGFI-B to its response element (Hirata, Y., Kiuchi, K., Chen, H.-C., Milbrandt, J., and Guroff, G. (1993) *J. Biol. Chem.* 268, 24808–24812). We show here that nerve growth factor (NGF)-induced changes in the *in vivo* phosphorylation of Ser^{350} accompany transcriptional deactivation of NGFI-B in PC12 cells, that membrane depolarization and NGF treatment cause differential phosphorylation of NGFI-B, and that the transcriptional activation caused by exogenous expression of NGFI-B or membrane depolarization can be inhibited by NGF treatment. In addition, the mutation of Ser^{350} to Ala abolished the inhibitory effect of NGF on the transcriptional activation of NGFI-B in PC12 cells. These data could provide new insights into the regulation of transcriptional activity required for some neurons to switch from activity-dependent survival to neurotrophin-dependent survival during development.

Neurons are constantly exposed to extracellular stimuli that can cause death, support survival, induce or maintain differentiation, and alter cell morphology and synaptic connectivity. Clearly, these stimuli change as the neuron develops and change profoundly when the neuron connects with a target cell through synapse formation and is exposed to target cell-derived growth factors. In response to growth factors and other stimulatory signals, specific neuronal genes are expressed or repressed, and this, in turn, stimulates or inhibits the production of proteins that determine the structure and function of the cell.

Activation of a set of genes termed the immediate-early genes is pivotal to this response. NGFI-B, also called *nur77*, is one of the immediate-early genes originally identified by virtue of its rapid activation by nerve growth factor (NGF)[1] in PC12 pheochromocytoma cells (1) and by serum in fibroblasts (2). The PC12 cell line is a good model for the study of NGF action and responds to NGF by differentiating into a postmitotic cell type with neuronal characteristics (3). PC12 cells also provide a good system for studying the effects of electrical signals on neuronal gene expression because they have excitable membranes that can be depolarized by specific neurotransmitters or by elevated levels of KCl (3).

The NGFI-B gene encodes a member of the steroid-thyroid hormone receptor superfamily, a class of ligand-dependent transcriptional modulator proteins (4). NGFI-B is rapidly synthesized in PC12 cells in response to a variety of growth factors, to phorbol ester, and to treatments resulting in calcium influx (5). The protein is rapidly modified via phosphorylation, and the extent of phosphorylation is dependent on the stimulus (5, 6). Using a genetic selection procedure, NGFI-B was found to recognize a specific nucleotide sequence (NBRE) (7), and a region outside the zinc finger domain (A-box) was shown to play a role in DNA binding specificity (8). Although no specific ligand for NGFI-B has been identified, cotransfection experiments using a reporter gene coupled to the NBRE demonstrate that NGFI-B is a strong transcriptional activator in the cells examined (9–11). However, the exact function(s) of NGFI-B in neuronal cells remains to be elucidated.

We have demonstrated previously that a recombinant DNA-binding domain of NGFI-B (amino acids 244–352) expressed in bacteria binds specifically to the NBRE and that the *in vitro* phosphorylation of Ser^{350} in the A-box of NGFI-B reduces its ability to bind to the DNA (12). Furthermore, we have identified a kinase (NGFI-B kinase I) that is induced by NGF and that phosphorylates Ser^{350} (13). To test the hypothesis that phosphorylation of NGFI-B at Ser^{350} in PC12 cells by NGF treatment results in the failure of DNA binding, we used recombinant DNA-binding domain (rDBD) expressed in PC12 cells as a probe and examined whether its phosphorylation state regulates the activity of NGFI-B. We show here that NGF-induced changes in the phosphorylation of Ser^{350} accompany transcriptional deactivation of NGFI-B, that membrane depolarization and NGF treatment cause differential phosphorylation of NGFI-B, that the transcriptional activation caused by either exogenous expression of NGFI-B or membrane depolarization can be inhibited by NGF treatment, and that this effect of NGF is abolished if Ser^{350} is not available for phosphorylation.

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[1] The abbreviations used are: NGF, nerve growth factor; NBRE, NGFI-B response element; rDBD, recombinant DNA-binding domain; EGF, epidermal growth factor; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; CREB, cAMP response element-binding protein.

EXPERIMENTAL PROCEDURES

Reagents—NGF was prepared by the method of Bocchini and Angeletti (14). EGF was obtained from Collaborative Biomedical Research. A monoclonal antibody against NGFI-B (2E1) (5), the cDNA of NGFI-B (1) cloned into pBluescript (Stratagene), and NBRE-luc (7), which contains eight copies of the NBRE upstream of a minimal prolactin promoter driving the firefly luciferase gene, were prepared as described. Anti-c-Myc epitope-tagged antibody (9E10) was purchased from Cambridge Research Biochemicals (Cheshire, United Kingdom). Anti-Nur77 antibody was from Santa Cruz Biotechnology. A polyclonal antibody specific for phosphorylated Ser^{350} of NGFI-B was raised in rabbits against the peptide GRRGRLPS(P)KPKC (amino acids 343–353) coupled to keyhole limpet hemocyanin (Sigma) through a C-terminal cysteine. The antibody against the unphosphorylated peptide (anti-350 antibody) was adsorbed with the unphosphorylated peptide coupled to CNBr-activated Sepharose 4B. The antibody specific for the phosphorylated peptide (anti-350P antibody) was further purified with the phosphopeptide resin. Protein phosphatase types 1 and 2A were from Upstate Biotechnology, Inc., and okadaic acid was obtained from Alexis Corp.

DNA Construction—pMKITneo, a mammalian expression vector containing the SRα promoter, was a gift from Dr. K. Maruyama (Tokyo Medical and Dental University, Tokyo, Japan). The cDNA of NGFI-B was cloned into the *Eco*RI and *Mlu*I sites of pMKITneo (pMKIT-NGFI-B). A mutation of Ser^{350} to Ala was introduced into pMKIT-NGFI-B with a Quickchange site-directed mutagenesis kit (Stratagene). The DNA-binding domain of NGFI-B (amino acids 247–362) attached to the SV40 large T-antigen nuclear localization signal (PKKKRKV) at the N terminus and to the c-Myc epitope tag (EQKLISEEDLN) at the C terminus was amplified by polymerase chain reaction using the upstream primer 5′-TTGAATTCAT GCCCAAGAAG AAGCGCAAGG TGGGCGGCGG CGCACCCGTA ACCTCCACCA AG-3′ and the downstream primer 5′-ATAAGCTTAC AGGTCCTCCT CAGAGATCAG CTTCTGCTCA TTGGTAGGGG AGGCATCTGG GGG-3′. Three glycine residues were introduced downstream of the nuclear localization signal as a spacer to ensure exposure of the nuclear localization signal in the folded molecule. The polymerase chain reaction product was digested with *Eco*RI and *Hin*dIII and then subcloned into pBluescript KS II(+) (pBSKS-ZINC). The sequence of the insert was verified by DNA sequencing. pBSKS-ZINC was digested with *Eco*RI and *Xho*I and then subcloned into pMKITneo (pMKIT-ZINC) for transient expression and into pLXSN (15) (pLXSN-ZINC), a retroviral expression vector, for stable expression. The DNA-binding domains of NGFI-B, corresponding to amino acids 244–352, with a mutation (Ser to Ala) at position 340 (NGFI-B327(S340A)) or at position 350 (NGFI-B327(S350A)) were expressed in *Escherichia coli*, purified, and phosphorylated *in vitro* with protein kinase A (Promega) as described previously (12, 13).

Cell Lines and Tissue Culture—PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum, 7% horse serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. A human embryonal kidney cell line 293 and retroviral packaging cell lines (ΨCRE and PA317) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cell lines expressing the zinc finger domain of NGFI-B were generated by amphotropic retroviral infection of PC12 cells in the presence of 8 μg/ml Polybrene (Aldrich). Amphotropic retrovirus was produced by transient transfection of pLXSN-ZINC into the ΨCRE ecotropic packaging cell line, followed by infection into the PA317 amphotropic packaging cell line. Infected PC12 cells were selected in 600 μg/ml G418 (Life Technologies, Inc.).

Immunoblotting—PC12 cells were treated with NGF (50 ng/ml), EGF (100 ng/ml), or different concentrations of KCl for various periods of time. The cells were then harvested and washed twice with saline. The washed cells (1×10^7) were treated with 10% trichloroacetic acid for 30 min at 4 °C. The precipitate was collected by centrifugation, and the pellet was solubilized in 80 μl of 9 M urea, 2% Nonidet P-40, and 1% DTT and sonicated. Twenty μl of 10% lauryl sulfate was added, and the pH was adjusted to neutral with 1 M Tris. Samples were resolved on an SDS-polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane (Millipore Corp.). The blots were incubated with 5% nonfat dry milk in phosphate-buffer saline, pH 7.4, containing 0.1% Tween 20 for 1 h and then incubated with 2E1 (1:200 dilution of culture supernatant), 9E10 (10 μg/ml), anti-350 antibody (2 μg/ml), or anti-350P antibody (2 μg/ml) for 1 h. Bound antibodies were detected by sheep anti-mouse Ig or donkey anti-rabbit Ig antibody conjugated with horseradish peroxidase (Amersham Corp.) and analyzed with the ECL detection system (Amersham Corp.). For analyses of NGFI-B expressed exogenously, PC12 cells were transfected with pMKIT-NGFI-B. Forty-eight h after transfection, the cells were lysed with lysis buffer (10 mM phosphate buffer, 0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.2% SDS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.3 mg/ml leupeptin, and 1 mM Na_3VO_4, pH 7.4). Immunoprecipitation was performed with polyclonal anti-Nur77 antibody as described previously (16). Immunoprecipitates were resolved by SDS-PAGE, followed by immunoblotting as described above.

In Vitro Translation—The plasmid used to express NGFI-B *in vitro* was created by deletion of the 5′-noncoding region (*Xba*I-*Nco*I fragment) from pBSKS-NGFI-B, followed by treatment with Klenow enzyme and T4 DNA ligase (pBSKS-NGFI-BΔ). pBSKS-NGFI-BΔ and pBSKS-ZINC were linearized by *Mlu*I and *Hin*dIII digestion, respectively, for transcription *in vitro*. Transcription reactions contained 5 μg of linearized DNA, 40 mM Tris, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 0.5 mM ^{7}mGpppG, 1000 units/ml RNasin, 800 units/ml T7 RNA polymerase, and 0.5 mM each ATP, CTP, GTP, and UTP. Incubation was carried out at 37 °C for 1 h, followed by DNase digestion, phenol/$CHCl_3$ extraction, and ethanol precipitation. Generation of proteins was accomplished with a rabbit reticulocyte lysate system (Promega) according to the manufacturer's protocol. In a typical reaction, 100 ng of mRNA was used in a final volume of 50 μl. Five μl of the translation mixture was used in each gel retardation assay.

Gel Retardation Assay—Nuclear protein extracts of PC12 cells and transfectants were prepared according to Staal *et al.* (17). Briefly, 1×10^6 cells, appropriately stimulated, were resuspended in 0.4 ml of buffer A (10 mM Hepes, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.3 mg/ml leupeptin, and 1 mM Na_3VO_4, pH 7.8) and incubated on ice for 15 min. Then 25 μl of a 10% Nonidet P-40 solution was added, and the cells were vigorously mixed for 15 s and centrifuged. Pelleted nuclei were resuspended in 50 μl of buffer B (50 mM Hepes, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.3 mg/ml leupeptin, and 1 mM Na_3VO_4, pH 7.8). Following gentle mixing at 4 °C for 20 min, the tubes were centrifuged, and the supernatants containing the nuclear proteins were collected. Gel retardation assays were performed using the NBRE-containing B1a oligonucleotide as described (12, 13), with 4 μg of poly(dI-dC)·poly(dI-dC) as nonspecific competitor and 3–10 μg of crude nuclear extract. The effects of antibodies on the gel mobility shift interactions were examined by addition of 2E1 (2 μl of culture supernatant) or 9E10 (0.2 μg) to the reaction mixture. For phosphatase treatment, Na_3VO_4 was eliminated from buffers A and B in the preparation of nuclear proteins. Nuclear proteins were treated with both protein phosphatase types 1 and 2A (0.2 units/reaction) in buffer (50 mM Hepes, 50 mM KCl, 300 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, and 10% glycerol, pH 7.8) at 30 °C for 30 min. Okadaic acid (500 nM) was used to inactivate the phosphatases. The DNA binding activity was examined as described above.

Reporter Gene Assay—PC12 cells cultured in six-well plates (Nunc) were transfected using LipofectAMINE (Life Technologies, Inc.) with 1 μg of NBRE-luc and 0.05 μg of the internal control pRL-TK (Promega), which contains *Renilla* luciferase downstream of the herpes simplex virus thymidine kinase promoter. Eighteen h after transfection, the cells were treated with NGF (50 ng/ml) or with KCl for various periods. Cell lysates were prepared with the Dual-Luciferase Reporter assay system (Promega), and firefly and *Renilla* luciferase activities were measured in Lumat LB9507 (Berthold, Wildbad, Germany). Transfection efficiency was normalized with the *Renilla* luciferase activity. Data are expressed as mean ± S.E. In some experiments, pMKIT-NGFI-B was cotransfected into PC12 cells. In inhibition experiments, 293 cells cultured in six-well plates were transfected with 1 μg of NBRE-luc, 0.1 μg of pMKIT-NGFI-B, various amounts (0–0.9 μg) of pMKIT-ZINC or pMKITneo, and 0.05 μg of pRL-TK. Cells were harvested 24 h after transfection, and the luciferase activity was measured as described above. In some experiments, PC12 cells were transfected with 1 μg of NBRE-luc, 0.05 μg of pRL-TK, and 1 μg of pMKIT-NGFI-B. One h after transfection, NGF (50 ng/ml) was added to the culture. The cells were harvested 9 h after transfection, and the luciferase activity was measured as described above. In other studies, PC12 cells were transfected with 1 μg of NBRE-luc and 0.05 μg of pRL-TK. Eighteen h after transfection, the cells were stimulated with various concentrations of KCl. Sixty min after KCl stimulation, the KCl was removed, and the cells were cultured for 1.5 h in the presence or absence of NGF (50 ng/ml). The cells were harvested, and the luciferase activity was measured as described above.

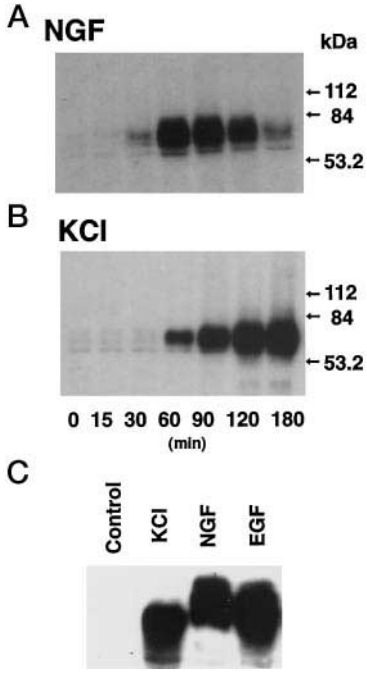

FIG. 1. **Post-translational modification of NGFI-B in stimulated PC12 cells.** *A* and *B*, time course of NGFI-B induction in PC12 cells by NGF (50 ng/ml) and KCl (50 mM), respectively. Cells were harvested at the indicated times, and cell lysates were prepared as described under "Experimental Procedures." NGFI-B was analyzed by immunoblotting with 2E1, a monoclonal anti-NGFI-B antibody. *C*, differential phosphorylation patterns of NGFI-B induced by NGF, EGF, and KCl. PC12 cells were treated with NGF (50 ng/ml) for 60 min, EGF (100 ng/ml) for 60 min, and KCl (50 mM) for 180 min. NGFI-B was detected by immunoblotting.

RESULTS

Differential Phosphorylation Patterns of NGFI-B Are Induced by NGF and KCl in PC12 Cells—The NGFI-B gene is rapidly activated by a variety of stimuli that induce cells to differentiate or proliferate (5, 6). We selected NGF, EGF, and membrane depolarization as stimuli since the signal transduction pathways mediating their actions have been well characterized (18–20). NGFI-B protein was induced in PC12 cells by both NGF and KCl. The expression levels rose more rapidly after NGF treatment, with peak levels occurring 60–90 min after NGF addition, than after KCl treatment, with peak levels occurring after 180 min (Fig. 1, *A* and *B*). The time course of NGFI-B induction by EGF was similar to that by NGF (data not shown) (6). NGFI-B induced by NGF, EGF, or KCl migrated as a diffuse band. The fact that alkaline phosphatase treatment of NGFI-B immunoprecipitated from stimulated PC12 cells resulted in a single predominant band on SDS-PAGE suggests that phosphorylation is the major form of post-translational modification of the molecule (data not shown) (5, 6). Slowly migrating NGFI-B species were detected in cells stimulated with either NGF or EGF and, to a lesser degree, with KCl (Fig. 1*C*), an observation consistent with previous reports (5, 6). This indicates that there is stimulation-specific phosphorylation of NGFI-B upon membrane depolarization and NGF or EGF treatment.

Differential Transcriptional Activities of NGFI-B Are Induced by NGF, EGF, and KCl in PC12 Cells—NGFI-B is a transcriptionally active member of the nuclear receptor family. To examine the possible effects of differential phosphorylation on the transcriptional activity of NGFI-B, reporter gene assays were performed by transfecting the NBRE-luc reporter plasmid into PC12 cells and monitoring the luciferase activity (Fig. 2). When transfected PC12 cells were treated with KCl, the luciferase activity was detected after 2 h, and the peak of the activity was between 6 and 8 h (Fig. 2*A*). This delayed appearance of the luciferase activity seems reasonable when compared with the time course of NGFI-B induction with KCl (Fig. 1*B*). In contrast, very little or no activity of luciferase was observed when the cells were treated with NGF or EGF even though NGFI-B induction itself was detected on immunoblots (Fig. 1). When NGFI-B was expressed exogenously in PC12 cells by cotransfection of pMKIT-NGFI-B, the luciferase activity was extremely high (Fig. 2*B*) even though the protein expression level was much lower than that endogenously induced by NGF (data not shown). These results demonstrate that NGFI-B endogenously induced by either NGF or EGF is unable to function as a transcription factor, whereas NGFI-B induced by membrane depolarization does have transcriptional activity. It should be noted that both NGF and EGF induce NGFI-B kinase I (13), which phosphorylates Ser^{350} of the DNA-binding domain of NGFI-B. As shown below, KCl treatment does not lead to the phosphorylation of this residue.

rDBD of NGFI-B Recognizes the NBRE Sequence—We have shown that *in vitro* phosphorylation of the DNA-binding domain of NGFI-B expressed in *E. coli* abolishes its ability to bind to the NBRE (12). To further investigate the effect of phosphorylation, we generated rDBD of NGFI-B, shown schematically in Fig. 3*A*. A gel retardation assay using *in vitro* translation products (NGFI-B and rDBD) demonstrated that rDBD retains the same DNA binding ability as that of wild-type NGFI-B (Fig. 3*B*). rDBD present in the protein-DNA complex was also identified by supershift analysis with 9E10, a monoclonal anti-c-Myc epitope-tagged antibody. When 293 cells were transiently cotransfected with pMKIT-ZINC (rDBD), pMKIT-NGFI-B, and an NBRE-luc reporter plasmid, the luciferase activity induced by NGFI-B was inhibited in a dose-dependent manner (Fig. 3*C*). Taken together, these data show that rDBD is able to recognize the same DNA sequence as NGFI-B (12) and, accordingly, has the ability to inhibit the transcriptional activity of NGFI-B by competitive binding to the NBRE.

DNA Binding of NGFI-B and rDBD Is Regulated by Membrane Depolarization and NGF Treatment—To study the DNA binding activity of rDBD in PC12 cells, transfectants expressing rDBD (PC12zinc) were established by the retrovirus gene transfer method (15). Nuclear extracts were prepared from parental PC12 and PC12zinc cells stimulated with NGF or KCl and subjected to gel retardation assays (Fig. 4*A*). rDBD derived from the transfected cells was able to bind to the NBRE, which is consistent with the result using the *in vitro* translation product. When transfectants were treated with KCl (30 min), the ability of rDBD to bind was not affected. However, rDBD from NGF-treated cells (30 min) failed to bind to the NBRE. The fact that phosphatase treatment of rDBD restored its ability to bind to the NBRE sequence (Fig. 4*B*) indicates the importance of the phosphorylation of the DNA-binding domain of NGFI-B. Wild-type NGFI-B induced in PC12 cells appeared to behave in a similar way. While NGFI-B induced by KCl (3 h) did bind to the NBRE, the molecule induced and phosphorylated by NGF (1 h) did not bind. These results, taken together with the reporter gene assay (Fig. 2), strongly suggest the presence of mechanisms regulating the binding of NGFI-B to the NBRE in PC12 and PC12zinc cells.

NGF Treatment Induces the Phosphorylation of NGFI-B Ser^{350} in PC12 Cells—We have shown the possible involvement of Ser^{350} phosphorylation *in vitro* in the binding of NGFI-B to its response element (12). To detect the phosphorylation of NGFI-B at Ser^{350} in intact PC12 cells upon NGF treatment, we prepared a polyclonal antibody specific for phosphorylated Ser^{350} (anti-350P). Fig. 5 demonstrates the specificity of this

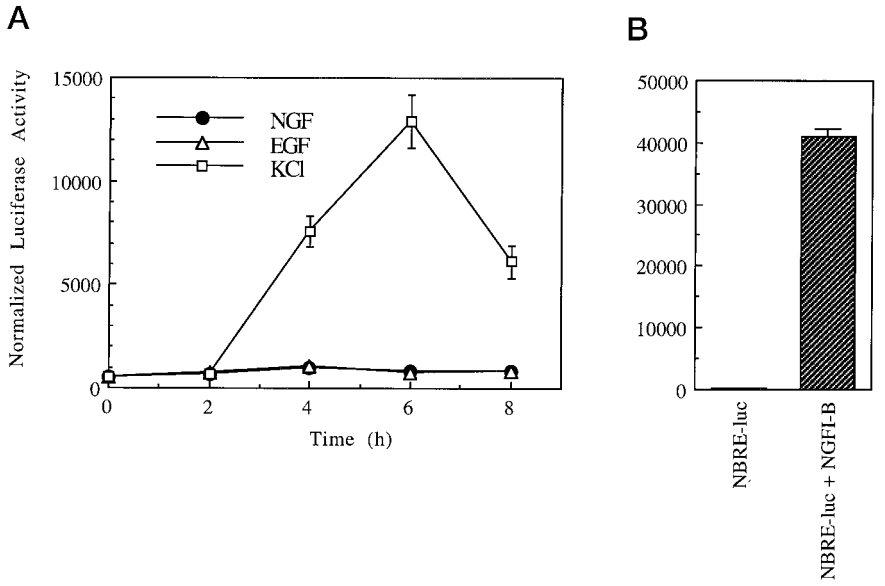

FIG. 2. **Transcriptional activity of NGFI-B in PC12 cells.** *A*, PC12 cells were transfected with 1 μg of NBRE-luc and 0.05 μg of the internal control pRL-TK. Eighteen h after transfection, cells were treated with NGF (50 ng/ml), EGF (100 ng/ml), or KCl (50 mM) for the indicated time periods before the preparation of cell lysates with the Dual-Luciferase Reporter assay system. *B*, PC12 cells were cotransfected with 1 μg of pMKIT-NGFI-B, 1 μg of NBRE-luc, and 0.05 μg of the internal control pRL-TK. Cell lysates were prepared 24 h after transfection. The luciferase activity was measured, and all data were normalized with the *Renilla* luciferase activity.

antibody. *E. coli* fragments, NGFI-B327(S350A) and NGFI-B327(S340A), were prepared and treated with protein kinase A in the presence or absence of ATP. When fully phosphorylated in the presence of ATP, the proteins detected with anti-350 antibody migrated more slowly on SDS-PAGE (Fig. 5*A*, *lanes 2* and *4*). Anti-350P antibody reacted with NGFI-B327(S340A) only when it was phosphorylated (Fig. 5*B*, *lane 2*), suggesting the specific recognition of phosphorylated Ser^{350} by the antibody.

Phosphorylation of rDBD in cells upon stimulation was examined using this antibody. rDBD was constitutively expressed in PC12zinc cells, and the residue corresponding to Ser^{350} of wild-type NGFI-B was phosphorylated when the cells were treated with NGF, but not with KCl (Fig. 6, *C* and *D*). Shorter periods of treatment were utilized to prevent endogenous NGFI-B induction in these transfectants. In PC12 cells (Fig. 6, *A* and *B*), NGFI-B induction was observed with both NGF and KCl; however, phosphorylation of Ser^{350} was detected only in response to NGF treatment, an observation consistent with what is seen in these PC12zinc transfectants. Furthermore, phosphorylation for Ser^{350} was not observed when NGFI-B was exogenously expressed in PC12 cells by DNA transfection (Fig. 6*E*). These data, along with the gel retardation assay (Fig. 4), confirm that NGF-induced changes in the phosphorylation state of Ser^{350} accompany transcriptional deactivation of NGFI-B.

Treatment with NGF after Exogenous Expression of NGFI-B or Membrane Depolarization Reduces the Transcriptional Activity of NGFI-B in PC12 Cells—Since the induction of NGFI-B by NGF results in minimal transcriptional activation, we asked whether NGF treatment would inhibit the transcriptional activity of NGFI-B introduced into the cells by other methods. Accordingly, we examined whether the transcriptional activity of NGFI-B induced either exogenously or endogenously in PC12 cells is reduced by NGF treatment. PC12 cells transiently transfected with pMKIT-NGFI-B and the NBRE-luc reporter plasmid were treated with NGF. Since the activation of NGFI-B kinase I was maximal within 5 min and remained at a lower level for several hours (13), we used shorter periods of NGF stimulation in these experiments. Fig. 7*A* shows that NGF treatment reduced the transcriptional activity of NGFI-B introduced exogenously by DNA transfection. Furthermore, NGF had no inhibitory effect on NGFI-B(S350A)-induced transcriptional activity, indicating that Ser^{350} is critical for the regulation of NGFI-B by NGF. Similar results were obtained when NGFI-B induced endogenously by KCl was examined. PC12 cells transfected with the NBRE-luc reporter plasmid were sequentially treated with KCl for 60 min, washed to remove KCl, and then treated with NGF or medium. NGF treatment reduced the luciferase activity induced by KCl (Fig. 7*B*). Immunoblot data obtained under these conditions revealed specific phosphorylation of NGFI-B at Ser^{350} in these PC12 cells (Fig. 7*C*). These data indicate that NGF treatment inhibits the transcriptional activation of NGFI-B.

DISCUSSION

Many immediate-early genes, including c-*fos* and c-*jun*, and zinc finger proteins, such as NGFI-B, encode transcriptional regulatory proteins. These gene products are thought to be important in regulating the cell's response to environmental changes and are probably the nuclear messengers of this response. Transcription factors, including members of the steroid-thyroid receptor family, are commonly modified by phosphorylation. Alterations in the phosphorylation of these receptors effect changes in their activity, stability, or subcellular location. In this report, we present several lines of evidence that indicate that NGF regulates the transcriptional activity of NGFI-B in PC12 cells and that this regulation is due, at least in part, to a specific phosphorylation of NGFI-B. First, NGFI-B induced by NGF does not show significant transcriptional activity, whereas NGFI-B induced by KCl does. This is associated with a diminished DNA binding of the protein induced by NGF. Second, NGFI-B synthesized upon induction by NGF or KCl is differentially phosphorylated. Phosphorylation of Ser^{350} located in the A-box is induced when the cells are exposed to NGF, but not to KCl. Third, phosphorylation of Ser^{350} both *in vitro* and *in vivo* abolishes the DNA binding activity of rDBD, indicating that NGF-induced changes in the phosphorylation state of Ser^{350} could contribute to transcriptional deactivation of NGFI-B. Finally, the transcriptional activity induced by wild-type NGFI-B DNA transfection is reduced by NGF treatment, whereas the activity induced by NGFI-B(S350A) is not, suggesting that Ser^{350} phosphorylation is necessary for the regulation by NGF. It is unlikely, however, that phosphorylation of Ser^{350} is solely responsible for these regulatory changes since we have observed Ser^{350} phosphorylation under conditions where transcription is not decreased (data not shown). We postulate that Ser^{350} phosphorylation is necessary, but not sufficient, for the regulation of transcriptional activation of NGFI-B. Indeed, it is known that NGFI-B can be phosphoryl-

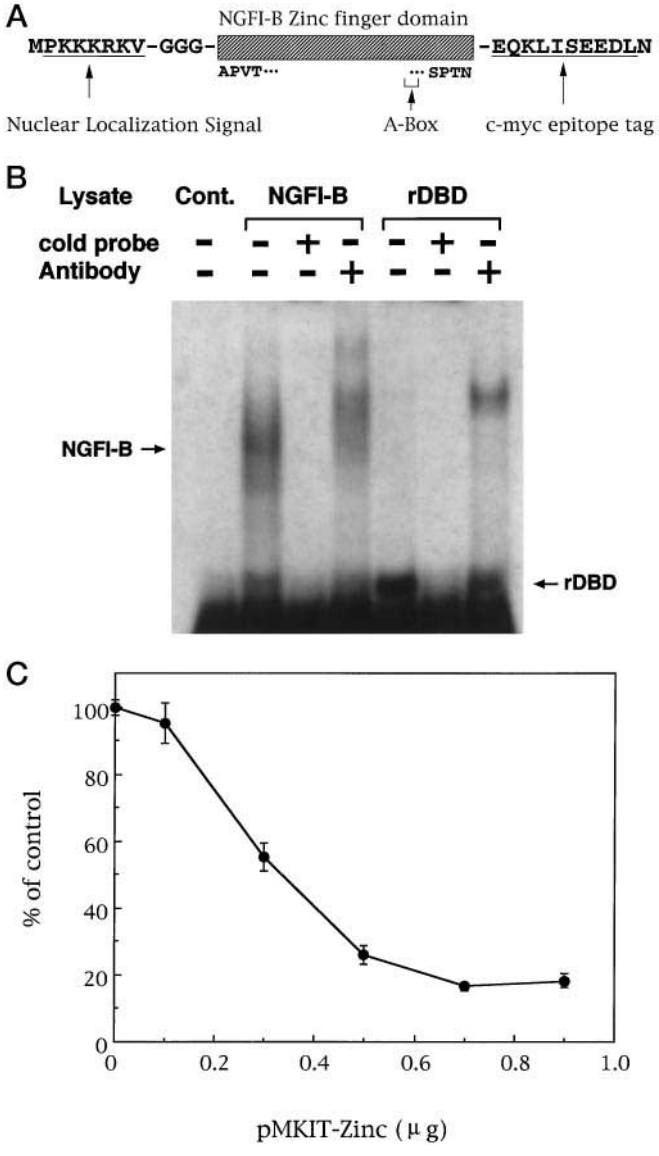

FIG. 3. **Structure of rDBD and its binding to the NBRE.** *A*, schematic structure of rDBD. rDBD includes an SV40 large T-antigen nuclear localization signal at the N terminus and a c-Myc epitope tag at the C terminus. Three glycine residues were introduced downstream of the nuclear localization signal as a spacer to ensure exposure of the nuclear signal from the folded molecule. *B*, binding of rDBD to the NBRE. NGFI-B (*second* through *fourth lanes*) and rDBD (*fifth* through *seventh lanes*) were generated with rabbit reticulocyte lysates, and gel retardation assays were performed with labeled double-stranded B1a oligonucleotide. Lysates were incubated with unlabeled B1a oligonucleotide in a 100-fold molar excess (*third* and *sixth lanes*). NGFI-B and rDBD present in the protein-DNA complexes were identified by supershift analyses with monoclonal antibodies 2E1 (*fourth lane*) and 9E10 (*seventh lane*). The *first lane* shows the *in vitro* translation system incubated without an RNA source (control (*Cont.*)). *C*, 293 cells transfected with 1 μg of NBRE-luc, 0.1 μg of pMKIT-NGFI-B, various amounts (0–0.9 μg) of pMKIT-ZINC or pMKITneo, and 0.05 μg of pRL-TK. Cells were harvested 24 h after transfection, and the luciferase activity was measured. Transfection efficiency was normalized with *Renilla* luciferase activity.

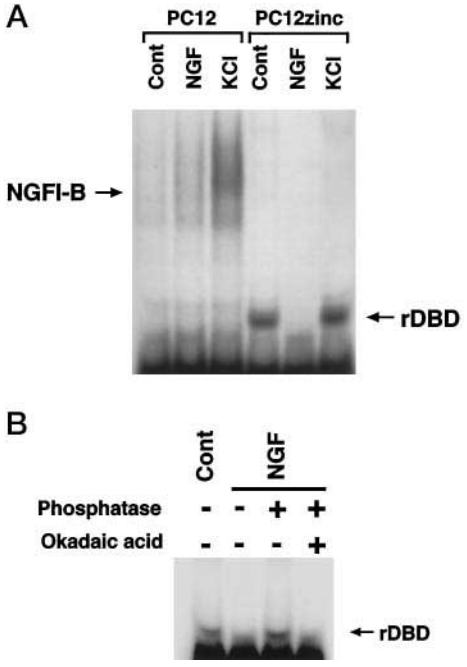

FIG. 4. **Regulation of DNA binding of NGFI-B and rDBD by NGF and KCl treatment.** *A*, nuclear extracts were prepared from PC12 cells treated with NGF (50 ng/ml, 60 min) or KCl (50 mM, 3 h) and from PC12zinc cells treated with NGF (50 ng/ml, 30 min) or KCl (50 mM, 30 min). Shorter periods of treatment were utilized to prevent endogenous NGFI-B induction in PC12zinc transfectants. The gel retardation assay was performed with the B1a oligonucleotide. *B*, nuclear extracts from NGF-treated PC12zinc cells were incubated with both protein phosphatase types 1 and 2A (0.2 units each) at 30 °C for 30 min in the presence or absence of okadaic acid (500 nM). The samples were subjected to the gel retardation assay. *Cont*, control.

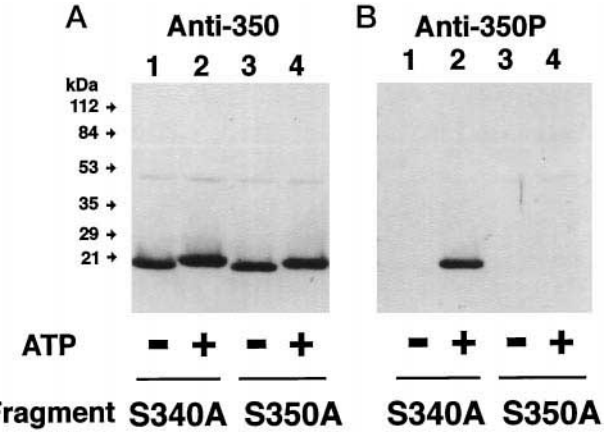

FIG. 5. **Specificity of anti-350P antibody.** NGFI-B327(S350A) and NGFI-B327(S340A) were expressed in *E. coli*, purified, and treated with protein kinase A in the presence or absence of ATP. After SDS-PAGE and transfer to membrane, immunoblot analysis was performed with anti-350 (*A*) or anti-350P (*B*) antibody. *Lanes 1* and *2*, NGFI-B327(S340A); *lanes 3* and *4*, NGFI-B327(S350A). *Lanes 1* and *3*, proteins treated in the absence of ATP; *lanes 2* and *4*, proteins treated in the presence of ATP.

ated at multiple sites, and cellular localization studies have demonstrated that NGFI-B is found in approximately equal amounts in the cytoplasm and the nucleus of NGF-stimulated PC12 cells and that the highly phosphorylated species are predominantly cytoplasmic (5, 6), indicating that phosphorylation events might be involved in the cellular localization of NGFI-B in PC12 cells. Alternatively, it is possible that phosphorylation of Ser^{350}, while decreasing the ability of NGFI-B to bind to the NBRE, enhances its ability to bind to other, as yet unidentified sites.

The role of phosphorylation in modulating the activity of transcription factors is well documented (21, 22). One of the best examples is the transcription factor cAMP response element-binding protein (CREB). CREB, which was originally identified as a mediator of gene expression that occurs in response to increased concentrations of cAMP, regulates the cellular response to growth factors. Growth factors enhance the

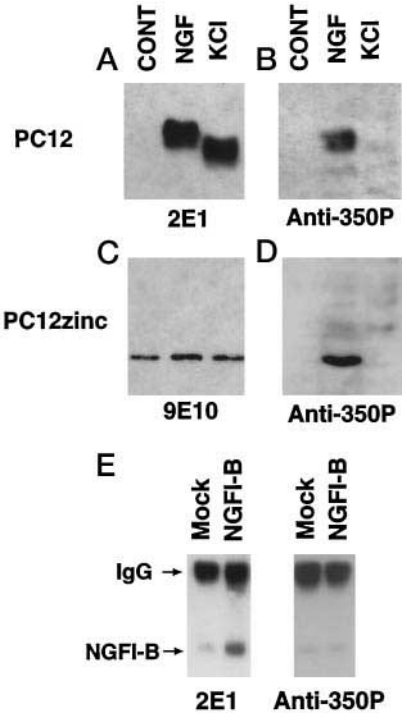

FIG. 6. **Phosphorylation of Ser^{350} upon NGF treatment in PC12 cells.** PC12 cells were treated with NGF (50 ng/ml) for 60 min and KCl (50 mM) for 180 min. PC12zinc transfectants were treated with NGF (50 ng/ml) and KCl (50 mM) for 30 min. Shorter periods of treatment were utilized to prevent endogenous NGFI-B induction in PC12zinc transfectants. Cell lysates were prepared as described under "Experimental Procedures." Samples were resolved by SDS-PAGE and immunoblotting. *A* and *B*, samples from PC12 cells; *C* and *D*, samples from PC12zinc cells. *A*, stained with 2E1; *B* and *D*, stained with anti-350P antibody; *C*, stained with 9E10. *CONT*, control. *E*, lysates from mock- and NGFI-B-transfected PC12 cells immunoprecipitated with polyclonal anti-Nur77 antibody. The immunoprecipitates were resolved by SDS-PAGE, and immunoblot analysis was performed with 2E1 and anti-350P antibody.

transcriptional potential of CREB by stimulating CREB phosphorylation at a specific amino acid, Ser^{133} (22). Phosphorylation of CREB at Ser^{133} is critical for NGF induction of c-*fos* transcription in PC12 cells (22). Recently, CREB kinase was identified as a member of the pp90^{rsk} family, RSK2 (23). It is of interest that the NGF-inducible kinase, NGFI-B kinase I, appears to be very similar to CREB kinase (13).

We found that the transcriptional activity of NGFI-B introduced by DNA transfection is reduced by NGF treatment. We also found that sequential treatment of PC12 cells with KCl and NGF reduces the transcriptional activity of NGFI-B compared with that seen with KCl treatment alone. Finally, although NGF itself induces NGFI-B, the protein so induced has little or no transcriptional activity. Thus, we suggest that the induction of NGFI-B kinase(s) by NGF regulates the transcriptional activity of NGFI-B, whether induced by NGF or membrane depolarization.

It is of interest to consider the biological meaning of the NGF-induced decrease in the transcriptional activity of NGFI-B. It is known that spontaneous electrical activity in prenatal neurons, which develops before the establishment of synapses, is important and that electrical impulses can regulate the gene expression necessary for neuronal differentiation. For example, Itoh *et al.* (24) have reported the down-regulation of the neural cell adhesion molecule L1 on murine dorsal root ganglion neurons by specific patterns of neural impulses. Furthermore, the establishment of synapses and the switch of survival requirements for some neurons from activity-depend-

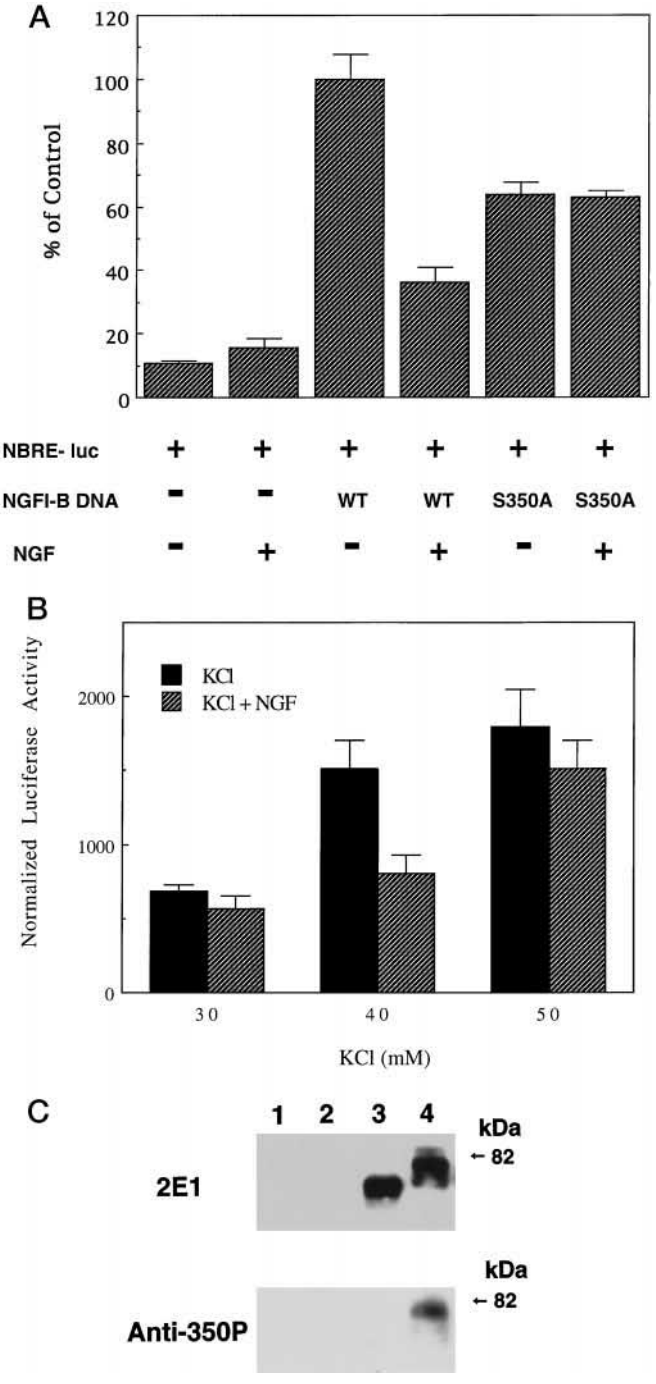

FIG. 7. **Reduction of the transcriptional activity of NGFI-B by NGF in PC12 cells.** *A*, PC12 cells were transfected with 1 μg of pMKIT-NGFI-B or pMKIT-NGFI-B(S350A), 1 μg of NBRE-luc, and 0.05 μg of pRL-TK. One h after transfection, the solution was removed, and culture medium with or without NGF (50 ng/ml) was added. The cells were harvested 9 h after transfection, and the luciferase activity was measured. *WT*, wild type. *B*, PC12 cells were transfected with 1 μg of NBRE-luc and 0.05 μg of pRL-TK. Eighteen h after transfection, cells were stimulated with various concentrations of KCl. Sixty min after stimulation, KCl was removed. Then NGF (50 ng/ml) or culture medium alone was added, and the cells were cultured for another 1.5 h. Cells were harvested, and the luciferase activity was measured. *C*, PC12 cells were treated with KCl (40 mM) or with medium for 60 min, and the medium was removed. Then NGF (50 ng/ml) or culture medium alone was added, and the cells were cultured for another 30 min. Cell lysates were prepared and analyzed by immunoblotting with 2E1 and anti-350P antibody. *Lane 1*, no treatment; *lane 2*, medium and then NGF treatment; *lane 3*, KCl treatment and then medium; *lane 4*, KCl treatment and then NGF treatment.

ent stimuli to neurotrophin-dependent survival appear to alter the requirements for the expression of certain genes. Thus, Itoh *et al.* (24) further demonstrated that NGF abolished the down-regulation imposed by electrical impulses. It is also known that the expression of specific gene products, such as the subunits of the acetylcholine receptor, changes upon synapse formation and the associated availability of trophic factors (25). Although specific genes containing the NBRE motif whose expression is altered by NGF are not known so far, our findings could provide an insight into the changes in gene expression that occur upon synapse formation, and they could eventually reveal a new set of transcriptional events that are required for neuronal development before synapses are formed, but that are suppressed when neurotrophins become available through synapse formation.

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