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In pheochromocytoma (PC12) cells nerve growth factor (NGF) and epidermal growth factor (EGF) activate similar receptor tyrosine kinase signaling pathways but evoke strikingly different biological outcomes: NGF induces differentiation and EGF acts as a mitogen. A novel approach was developed for identifying transcription factor activities associated with NGF-activated, but not EGF-activated, signaling, using random oligonucleotide clones from a DNA recognition library to isolate specific DNA binding proteins from PC12 nuclear extracts. A protein complex from NGF-treated, but not EGF-treated, cells was identified that exhibits increased mobility and DNA binding activity in gel mobility shift assays. The binding complex was identified in supershift assays as Fra-2/JunD. The clones used as probes contain either AP-1 or cAMP response element binding (CREB) recognition elements. Time course experiments revealed further differences in NGF and EGF signaling in PC12 cells. NGF elicits a more delayed and sustained ERK phosphorylation than EGF, consistent with previous reports. Both growth factors transiently induce c-fos, but NGF evokes a greater response than EGF. NGF specifically increases Fra-1 and Fra-2 levels at 4 and 24 hr. The latter is represented in Western blots by bands in the 40–46 kDa range. NGF, but not EGF, enhances the upper bands, corresponding to phosphorylated Fra-2. These findings suggest that prolonged alterations in Fra-2 and subsequent increases in Fra-2/JunD binding to AP-1 and CREB response elements common among many gene promoters could serve to trigger broadly an NGF-specific program of gene expression.

Key words: nerve growth factor; epidermal growth factor; differentiation; transcription factor; Fra-2; PC-12

Growth factors are critical mediators of cellular survival, proliferation, and differentiation. The rat pheochromocytoma (PC12) cell line has been used extensively to study both the biological outcomes induced by growth factors and their associated intracellular signaling cascades. In these cells nerve growth factor (NGF) triggers neuronal differentiation (Greene and Tischler, 1976), but epidermal growth factor (EGF) elicits a proliferative response (for review, see Wells, 1999). Both NGF and EGF activate receptor tyrosine kinases, resulting in receptor autophosphorylation as well as phosphorylation of a targeted pool of downstream molecules exhibiting Src homology 2 (SH2) domains. The SH2 domain-containing molecules recruited by the EGF and NGF receptors are virtually identical. They include Shc and Grb2, which trigger the well known Ras/Raf/MAP kinase pathway, as well as phosphoinositide-3 kinase (PI-3 kinase) and phospholipase C3 (PLCγ), which activate additional signaling cascades (for review, see Chao, 1992; Greene and Kaplan, 1995; Marshall, 1995; Whitmarsh and Davis, 1996; Wells, 1999). Given the close similarities in early signaling events initiated by NGF and EGF, the question arises as to how they induce such different biological programs in the same cells.

Much research in this area has focused on identifying early points of divergence in the EGF and NGF signaling pathways (for review, see Marshall, 1995). NGF produces a more prolonged pattern of tyrosine phosphorylation of PLCγ and PI-3 kinase-associated proteins (Blumberg et al., 1995) and a more sustained activation of MAP kinase (also known as extracellular-related kinase; ERK) (Qiu and Green, 1992; Nguyen et al., 1993) than does EGF. Sustained ERK activation is sufficient for neuritogenesis in PC12 cells (Traverse et al., 1994; Fukuda et al., 1995; Yamada et al., 1996). Sustained, but not transient, activation of ERK permits its translocation to the nucleus (Chen et al., 1992; Nguyen et al., 1993; Traverse et al., 1994), where it may modulate gene expression via the phosphorylation of transcription factors.

The present study was designed to detect downstream changes in transcription factor binding activity associated with NGF, but not EGF, signaling. The experimental approach is based on studies in which single transcription factors, such as MyoD, were incubated with random oligonucleotides to identify consensus DNA recognition elements (Blackwell and Weintraub, 1990; Sun and Baltimore, 1991; Gogos et al., 1992). In the present experiments complex nuclear extracts were used in place of single purified transcription factors. Random oligonucleotides were cloned into a DNA recognition element library (DREL); then individual clones were used to probe nuclear extracts from NGF-versus EGF-treated PC12 cells and identify NGF-specific protein–DNA binding interactions. This strategy revealed that NGF, but not EGF, mediates a long-term increase in the binding of an AP-1 complex composed of Fra-2 and JunD to both AP-1 and cAMP response element binding (CREB) consensus sites. Such elements are found in a wide variety of gene promoters, and long-lasting differences in AP-1 activity could facilitate a diver-
gence between the differentiative response evoked by NGF and the mitogenic response induced by EGF.

**MATERIALS AND METHODS**

**Cell culture and nuclear extraction**

PC12 cells were a gift from Dr. H. C. Palfrey (University of Chicago, IL), subcloned from a parental PC12 line (Dr. Eric Shooter, Stanford University, CA) for maximal neuritogenesis in response to NGF. Cells were grown on plain or collagen-coated plates in DMEM with 10% fetal bovine serum, 200 μM glutamine, and 50 μg/ml penicillin–50 μg/ml streptomycin (Complete medium). Cells either were untreated or were incubated in the presence of 2.5S NGF (50–100 ng/ml; Alomone Labs, Jerusalem, Israel) or 30–50 ng/ml EGF (Promega, Madison, WI) for various times as listed below. Nuclear extracts from PC12 cells were prepared in early experiments via a modification (Abmayr and Workman, 1989) of the protocol of Dignam et al. (1983). In later experiments a different protocol based on the method of Shapiro et al. (1988) was used because of its higher yield. In this procedure growth factor-treated or untreated cells from four 150 mm plates at 40–70% confluence were rinsed quickly with room temperature PBS, scraped into 10 ml/plate of ice-cold PBS, pooled, and pelleted at 2000 × g in a 4°C centrifuge. Pellets were resuspended in 5 × packed cell volume (PCV) of hypotonic buffer [containing in mM] 20 HEPES-KOH, pH 7.9, 100 KCl, 0.75 spermidine, 0.15 spermine, 0.1 EDTA, 0.1 EGTA, 1.0 dithiothreitol, and 0.2 PMSF plus 1 complete protein-pretase inhibitor tablet (Roche, Indianapolis, IN) per 10 ml] and swelled on ice for 10 min. Cells were spun at 2000 × g for 10 min, and the pellet was resuspended in 400–600 μl of a solution containing 3.3 g NH4SO4/ml, 15% glycerol, and (in mM) 4 HEPES-NaOH, pH 7.9, 10 Tris-HCl, pH 7.9, 50–150 KCl, 1 EDTA, 1 dithiothreitol, and 0.2 PMSF in a final volume of 20 μl. The reaction proceeded for 30 min at 37°C. In the first round of screening 100 ng of the initial radiolabeled double-stranded R65 oligonucleotide pool was added; in subsequent rounds the input oligonucleotide probes were derived by PCR amplification (see below). After a 20 min binding reaction at 30°C the samples were electrophoresed with a 4% Tris-glycine gel (4% acrylamide, 0.05% bis-acrylamide, 2.5% glyc erol, 50 mM Tris base, 380 mM glycine, and 2 mM EDTA). The autoradiogram derived from the gel was used to identify and excise the appropriate lanes. For both NGF and control extracts there were three lanes representing binding reactions containing 50, 100, and 150 mM KCl, respectively. Each lane, excluding the lower region containing unbound oligonucleotide, was cut into six segments, which were electroluted at 200 V for 2 hr. Using gel segments, as opposed to a single gel slab containing all of the “bound” oligonucleotides, resulted in higher yields of oligonucleotides after elution and concentration as described below as well as a greater efficiency for the subsequent PCR reactions. Tracking of the radiolabeled indicated yields of 50–90%.

To remove PCR inhibitors, we concentrated the R65 oligonucleotide subpool from each gel segment in 0.5× SSC, using a centrifugal concentrator (Amicon, Beverly, MA), and then incubated it for 45 min with streptavidin–paramagnetic particles (SA-SMP; Promega) to form R65–SA-SMP complexes. The complexes were constrained magnetically and washed with 0.5× SSC and then treated with 200 mM NaOH at 60°C to release the radiolabeled single R65 strand from the remainder of the complex. The radiolabeled strand was neutralized with 3 mM sodium acetate, pH 7, and then transferred to a centrifugal concentrator to reduce the volume and change the diluent to H2O. The final yield of each selected single-stranded radiolabeled R65 oligonucleotide subpool was >80%.

The population of oligonucleotides isolated from each gel segment was assessed in a separate PCR reaction. The forward primer R1 was identical to the first 20 bases of the upper strand of R65 and was 5′-radiolabeled as described above. The biotinylated oligonucleotide br2 (see above) was used as the reverse primer. Each reaction contained 5 μl of purified R65 eluate; 50 pmol of each primer; 200 μM each of dATP, dCTP, dGTP, and dTTP; 1.25 U of Pfu DNA polymerase (Stratagene, La Jolla, CA); and 5 μl of Pfu 10× buffer in a final reaction volume of 50 μl. The entire volume of each eluted R65 subpool was amplified. Amplification conditions were 95°C for a single 2 min cycle; then 95°C for 30 sec, followed by 60°C for 30 sec for 10 cycles; and finally 60°C for a single 7 min cycle. The total number of cycles was kept low to prevent preferential amplification of individual clones. After amplification the reaction volumes for all PCR reactions were combined to include oligonucleotides selected by both NGF-treated and control extracts. They were quantified spectrophotometrically and divided into 100 ng aliquots. These combined oligonucleotide subpools were used as probes for subsequent rounds of EMSA selection. After the fourth round of binding, selection, amplification, and combination, the enriched oligonucleotide populations were ligated into pBluescript (Stratagene).

**Cloning of selected R65 oligonucleotides and screening of the DREL by EMSA**

Each enriched oligonucleotide population was digested with EcoRI and XhoI, ligated into an EcoRI/XhoI double-digested pBluescript plasmid, and maintained in Escherichia coli XL1-Blue (Stratagene). Plasmids were prepared from single colonies, and the inserts were sequenced by dyeode chain termination, using a commercial kit and protocol (Amersham, Arlington Heights, IL). To PCR-amplify in-
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Analysis of selected oligonucleotides

Individual R65 clones were identified that showed reproducibly different gel shift patterns for NGF- versus EGF-treated PC12 cells. To characterize two of these clones further, we used shorter versions (35 bp) of the A9 clone (A9WT) and the C35 probe (C35WT), which contained the respective N25 core sequences. In addition, five oligonucleotides designated A9m1–A9m5, generated by mutating sequential 5 bp sites in the A9WT probe, were used in EMSAs as competitors for the labeled A9WT probe. Oligonucleotides for these experiments were purchased from Operon Technologies (Alameda, CA). For EMSAs that used the shorter probes, the polyacrylamide concentration was increased to 6%; the binding reaction was optimized for each probe by adding salmon sperm DNA (1 µg per binding reaction) and varying the concentration of poly(dI/dC)poly(dI/dC).

EMSA supershift assays were performed as described above, except that nuclear extracts were preincubated with 2 µl of water (control) or antibody for 10 min at room temperature; then probe was added for the binding reaction (37°C for 30 min). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), except for the antibody recognizing CREB, which was a gift from Dr. Jeremy Boss (Emory University, Atlanta, GA).

Western blot analysis of nuclear extracts and whole-cell extracts

Nuclear extracts were prepared as described above for EMSA. Protein concentrations were determined by a kit from Bio-Rad (Richmond, CA). Nuclear proteins (10–20 µg/lane) were diluted with H2O to a volume of
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RESULTS

A novel approach was developed for the identification of transcription factors in PC12 cells that are associated with NGF-activated, but not EGF-activated, signaling, using oligonucleotide clones from a DREL as affinity tags to isolate specific DNA binding proteins (Fig. 1). PC12 cells were incubated for 1–3 d in the presence and absence of NGF (100 ng/ml) Nuclear extracts prepared from these cells were incubated with a pool of labeled double-stranded R65 oligonucleotides containing random 25 bp core sequences. Oligonucleotide subpools that bound to the extracts were selected by EMSA, amplified by PCR, and then combined for use as the input oligonucleotide pool for a subsequent round of the procedure. After four rounds of binding, selection, amplification, and combination, the initially random oligonucleotide pools were enriched for oligonucleotides containing sequences recognized by DNA binding proteins in the PC12 nuclear extracts. The enriched heterogeneous oligonucleotide population was cloned into pBluescript to form a DREL consisting of ~3200 clones. DNA sequencing of 100 of these individual clones revealed that their random core sequences were unique, indicating that the library was not composed of merely a few highly amplified clones. A few of the clones, such as C35 (see below), contained core sequences of only 24 bp in length, probably owing to PCR error.

Three of the 100 clones that were tested displayed similar gel shift patterns that were reproducibly different for NGF- versus control or EGF-treated cells. These clones were designated as A9, C9, and C35, and their N25 core sequences are listed in Figure 2A. The proteins for which the DNA binding activities were regulated by NGF were designated as nerve growth factor-regulated DNA binding activities (NERDs). As the gel shift
Figure 5. NERD1 binding to C35WT and A9WT probes is supershifted by antibodies that specifically recognize Fra-2 and JunD in EMSAs. A, B, NERD1 binding for untreated PC12 cells (−) or cells treated for 24 hr with 30 ng/ml EGF (E) or 100 ng/ml NGF (N) is supershifted by antibodies specific for Fra-2 and JunD, but not by antibodies recognizing CREB, c-Jun, or other members of the AP-1 family. A. The C35WT probe detects a single strong band in PC12 nuclear extracts (arrow), which is supershifted partially by antibodies specifically recognizing Fra-2 or JunD. B. The NERD1 band (arrow) also is supershifted by Fra-2 and JunD antibodies when extracts are probed with A9WT, CREB- and ATF-2-specific antibodies supershift other complexes but have no effect on NERD1. This figure is representative of four independent experiments.

patterns in Figure 2B illustrate, NGF, but not EGF, upregulated binding and increased the mobility of nuclear protein complexes binding to clones A9, C35, and C9 (NERDs 1, 2, and 3, respectively). In contrast, the majority of the tested clones did not bind extracts differentially that were treated with either growth factor, as clone C50 typifies (Fig. 2B). In addition, two clones bound NERDs that apparently were downregulated by NGF as compared with EGF (data not shown).

To facilitate the identification of NGF-upregulated NERDs and their specific binding sites, we prepared 35 bp truncated versions (C35WT and A9WT) of the respective C35 and A9 probes (full length = 65 bp) containing the 25 bp core sequences. The truncated versions of these probes generally elicited fewer bands on EMSAs than the full-length probes (compare Figs. 2, 3). This difference is attributable to nuclear proteins binding to the flanking sequences of the full-length probes, because the core sequences are identical. NGF elicited an increase in both the mobility and intensity of the NERD band in EMSAs with either C35WT or A9WT probes. The truncated oligonucleotides mutually competed for NERD binding (Fig. 3). The A9, C9, and C35 long oligonucleotides also were able to compete away NERD binding to either of the truncated probes (data not shown). These data indicate that NERDs 1, 2, and 3 are the same entity (referred to herein as NERD1). A9WT contains two known protein recognition elements (Fig. 4A): a CREB-responsive element (CRE; Comb et al., 1986; Delegeane et al., 1987) and an Ets site (for review, see Wasylyk et al., 1993). To identify which of the sites in A9WT is recognized by NERD1, we applied a series of five mutant oligonucleotides, A9m1–A9m5, in excess as unlabeled competitors to block interaction between the A9WT probe and nuclear extracts from NGF-treated PC12 cells. As Figure 4B shows, the NERD1/A9WT binding was disrupted by excess unlabeled A9WT as well as by mutants A9m4 and A9m5. In contrast, mutants A9m1–A9m3 failed to inhibit the A9WT–NERD1 interaction. These data indicate that the CRE spanned by the A9m1–A9m3 mutants is necessary for NERD1 binding to the A9WT probe. A search of the TRANSFAC database (Wingender et al., 2000) determined that C35WT contains an AP-1 consensus site, TGAGTCA (Angel et al., 1987; Lee et al., 1987), but no other known recognition elements, suggesting that NERD1 binds to AP-1 recognition sites as well as to CRE. The C9 core sequence also contains a CREB/AP-1-like binding site, ACGTCA, identified by TRANSFAC search.

NERD1 was identified in supershift assays as an AP-1 complex. EMSAs were performed in the presence and absence of antibodies specific for CREB or various members of the AP-1 family. Only antibodies recognizing JunD and Fra-2 supershifted the NERD1 band in EMSAs with either C35WT (Fig. 5A) or A9WT (Fig. 5B). These antibodies did not produce similar bands in EMSAs in the absence of nuclear extracts (data not shown). Antibodies specific for ATF-2 and CREB supershifted other complexes probed with A9WT but had no effect on NERD1 (Fig. 5B). These data indicate that NERD1 is composed of Fra-2 and JunD and concur with previous evidence that AP-1 complexes bind to both CRE and AP-1 binding elements (Sassone-Corsi et al., 1990). Western blots were performed to identify NGF-specific changes in expression or size of the Fra-2 or JunD proteins. In agreement with previous reports, Fra-2 was represented by two or more bands ranging from 40–46 kDa, with the larger bands representing phosphorylated forms of the protein (Yoshida et al., 1991; Gruda et al., 1994; Suzuki et al., 1994). The exact number

Figure 6. NGF, but not EGF, increases the expression of Fra-2 but has no effect on JunD in PC12 cells. A, An antibody that specifically recognizes Fra-2 reveals a series of bands in the 40–46 kDa range in Western blots of PC12 nuclear extracts. All lanes were loaded with 10 μg of protein. NGF (N; 100 ng/ml, 24 hr) induces a large increase in the size of upper band(s), producing a broad smear in the 43–46 kDa range. EGF (E; 30 ng/ml, 24 hr) has no effect on Fra-2 expression. B, An antibody specific for JunD p39 recognizes a doublet in the 37–40 kDa range. Neither EGF (E) nor NGF (N) affects expression of JunD in PC12 cells. This figure is representative of three independent experiments.
of bands varied among experiments, although there was always a faint band or smear at ~40 kDa and at least one other band at 43–46 kDa. NGF, but not EGF, markedly increased the size of the upper band (Figs. 6A, 7E). In contrast, neither EGF nor NGF induced changes in JunD (Fig. 6B).

Time course experiments were performed to examine the relationship between NGF and EGF receptor activation, the duration of the associated ERK response, and the expression of AP-1 proteins in PC12 cells. As Figure 7 illustrates, EGF elicited a very transient increase in phosphorylated ERK (not detectable after 30 min) and a transient increase in c-fos that reached a maximum at ~1 hr. EGF had no effect on Fra-2 and did not induce Fra-1 expression. NGF elicited a much longer increase in ERK phosphorylation (phospho-ERK was still detectable at 4 hr) and a more robust c-fos response than that induced by EGF, although the time course for the c-fos response was similar for both growth factors. In contrast to EGF, NGF induced a delayed Fra-2 response after 4 hr. Delayed Fra-1 expression also was induced by NGF. The possibility was tested that the high dose of EGF used in these experiments was suboptimal for eliciting changes in Fra-1 and Fra-2 responses (Greenberg et al., 1985). EGF consistently induced rapid phosphorylation of ERK yet failed to increase Fra-1 (data not shown) or Fra-2 expression at 4 hr at any dose. An example of the NGF-induced Fra-2 response at 4 hr is included in the same blot for comparison. All lanes were loaded with 10 μg of protein. This illustration is representative of three independent experiments.

**DISCUSSION**

This study used a novel experimental approach to identify a long-term increase in DNA binding activity of a Fra-2/JunD AP-1 complex associated with the differentiative growth factor NGF, but not with the mitogenic factor EGF. AP-1 activity generally is associated with cell proliferation and transformation (for review, see Angel and Karin, 1991). However, the effects of AP-1 on transcription are highly dependent on the composition of the AP-1 complex (Suzuki et al., 1991). AP-1 is a heterodimeric or homodimeric protein complex containing products of the c-jun and c-fos proto-oncogene family. Fos proteins include c-fos, FosB, ΔFosB, Fra-1, and Fra-2. The Jun family is composed of c-Jun...
and its homologs v-Jun, JunB, and JunD. Members of the Fos and Jun family contain leucine zipper motifs and can form heterodimers; the Jun family can homodimerize also. In addition, these proteins can interact with non-AP-1 proteins containing leucine zipper motifs (for review, see Foletta, 1996). Although c-fos and c-Jun both are associated strongly with cell proliferation induced by a variety of mitogens such as serum, EGF, and other mitogenic growth factors, evidence suggests that Fra-2 may be an important signaling component in development and differentiation (for review, see Angel and Karin, 1991; Foletta, 1996). For example, in myeloma cell lines, 8Br-CAMP triggers increased Fra-2 levels and concomitant enhancement of the DNA binding activity of a Fra-2/JunD complex, together with cell cycle arrest (Rezzonico et al., 1995). In osteoblasts, Fra-2 levels increase with differentiation, and inhibition of Fra-2 function inhibits differentiation (McCabe et al., 1996). Paradoxically, Fra-2 also has been implicated in cellular transformation and proliferation in chick embryo fibroblasts (Nishina et al., 1990; Foletta et al., 1994). However, Fra-2 is nontransforming in rodent fibroblasts. Evidence suggests that a C-terminal transactivation domain that is present in some members of the AP-1 family, but not Fra-2, is required for transformation in rodent cells, but not avian cells (Wisdom and Verma, 1993). Thus, species-specific factors may determine the function of Fra-2 as a proliferative versus differentiative agent. An additional determinant of Fra-2 function could be its Jun family partner. c-Jun cooperates with ras to transform cells, but JunD suppresses ras-induced transformation (Pfarr et al., 1994). In chick embryo fibroblasts Fra-2/c-Jun diminishes transcription, but Fra-2/JunD enhances transcriptional activity (Suzuki et al., 1991). The Fra-2/c-Jun complex is crucial to transformation in these cells (Nishina et al., 1990; Suzuki et al., 1994). In other cells, Fra-1, but not Fra-2, complexes with c-Jun to mediate transformation (Mchta et al., 1997). Thus, transcriptional regulation by complexes of Fra-1 or Fra-2 with c-Jun may be a key to transformation. In contrast, the present findings, together with those of other investigators (Rezzonico et al., 1995; McCabe et al., 1996), suggest that the Fra-2/JunD complex is associated with conditions in which the cell cycle is arrested.

In PC12 cells NGF, but not EGF, increases the expression of a 45–46 kDa form of Fra-2 and concomitantly alters its DNA binding activity. Both changes in expression level and post-translational modifications, such as phosphorylation, alter the function of AP-1 proteins. Many stimuli, including phorbol esters, serum, and growth factors, increase both the expression levels and phosphorylation of AP-1 proteins, including Fra-2 (Yoshida et al., 1991; Gruda et al., 1994; Murakami et al., 1997, 1999). Serum or v-src-induced transformation elicits phosphorylation of Fra-2 in chick embryo fibroblasts (Yoshida et al., 1991; Suzuki et al., 1994; Murakami et al., 1999) and Swiss 3T3 cells (Gruda et al., 1994). Phosphorylation changes the size of Fra-2 from 40 kDa to multiple larger forms ranging 43 and 48 kDa (Yoshida et al., 1991; Gruda et al., 1994; Murakami et al., 1997, 1999; Treinies et al., 1999). The size of the Fra-2 protein that is induced by NGF in PC12 cells is consistent with a phosphorylated form of the protein. Phosphorylation is associated with increased mobility of Fra-2-containing complexes in EMSAs and with increased DNA binding activity (Gruda et al., 1994), similar to that observed in NGF-treated PC12 cells. It is likely that NGF-specific phosphorylation of Fra-2, together with its increased expression, is responsible for the increased DNA binding activity of Fra-2/JunD complexes to AP-1 and CREB-responsive elements in PC12 cells. Evidence suggests that ERK signaling is essential for the phosphorylation and increased expression of Fra-2 (Gruda et al., 1994; Murakami et al., 1997, 1999; Cook et al., 1999; Treinies et al., 1999). Cook et al. (1999) demonstrated that both transient and sustained ERK activation (as detected by its phosphorylation) can trigger c-fos expression, whereas sustained ERK signaling is necessary for the induction of Fra-1 and Fra-2. The present findings are in agreement with their report. EGF elicits a rapid brief ERK phosphorylation, together with c-fos expression, but no Fra-1 or Fra-2 expression. In contrast, NGF elicits a more sustained ERK phosphorylation and induces expression of c-fos, Fra-1, and Fra-2. The induction of the Fra-1 and Fra-2 is delayed, suggesting that they occur downstream of ERK activation. Several studies indicate that ERK signaling is responsible for the phosphorylation of Fra-1 and Fra-2. ERK is a substrate for MEK, and the MEK inhibitor PD098059 (Pang et al., 1995) blocks phosphorylation of Fra-1 and Fra-2 (Treinies et al., 1999). In addition, constitutively active MEK induces phosphorylation of Fra-2 (Murakami et al., 1999). Furthermore, there is evidence that ERK itself phosphorylates Fra-1 and Fra-2 (Gruda et al., 1994; Murakami et al., 1997).

Not only is ERK activity involved in phosphorylation of these protein, it also is necessary for their increased expression (Murakami et al., 1999; Treinies et al., 1999). The Fra-2 promoter is regulated via two AP-1 sites and a CRE (Yoshida et al., 1993; Sonobe et al., 1995), and these sites are responsible for Fra-2 upregulation in response to ERK signaling (Murakami et al., 1997, 1999). In fibroblasts during response to serum, the AP-1 consensus sites in the Fra-2 promoter are occupied successively by different AP-1 combinations. A complex of c-fos/c-Jun binds from 1–2 hr after stimulation, followed by Fra-2/c-Jun binding (Sonobe et al., 1995). A similar pattern of transactivation could occur in NGF-treated PC12 cells. After NGF or EGF treatment c-fos is upregulated. However, the c-fos response is much greater for NGF than for EGF and potentially could produce a greater transactivation of the Fra-2 promoter. In addition, Fra-2 auto-regulates its own promoter activity. ERK phosphorylation of Fra-2 converts it to a more efficient transcriptional activator, permitting positive autoregulation of the Fra-2 promoter (Murakami et al., 1997, 1999). A positive autoregulatory loop would permit a continuous long-term enhancement of Fra-2 expression such as that observed in PC12 cells after NGF treatment.

The present work demonstrates that NGF, but not EGF, induces a sustained upregulation and phosphorylation of the Fra-2 protein, together with an increase in DNA binding activity of the protein, which are dependent on the activation of the ERK signaling cascade. Thus, the differentiation of PC12 cells induced by NGF is mediated through the phosphorylation of Fra-2, which in turn activates a complex of Fra-2/JunD. This complex, in turn, activates the expression of downstream genes, including c-fos and c-Jun. This process is dependent on the activation of ERK, which is stimulated by NGF but not EGF. Therefore, the present findings further support the role of ERK in the regulation of AP-1 and CREB-responsive elements in PC12 cells, and provide a molecular mechanism for the differentiation of PC12 cells induced by NGF.

Table 1. NGF-sensitive late-response gene products, transcriptionally regulated in PC12 cells via AP-1- or CREB-binding sites

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Regulation</th>
<th>Source</th>
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<tbody>
<tr>
<td>Latexinα</td>
<td>(1)</td>
<td></td>
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<tr>
<td>IL-1αβ</td>
<td>(2)</td>
<td></td>
</tr>
<tr>
<td>Choline acetyltransferase</td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td>Neurofilament, light (NF-L)α</td>
<td>(4)</td>
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<tr>
<td>Neuropeptide Y (NP-Y)α</td>
<td>(5)</td>
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<tr>
<td>VGFβ</td>
<td>(6)</td>
<td></td>
</tr>
<tr>
<td>Dopamine β-hydroxylaseα</td>
<td>(7)</td>
<td></td>
</tr>
<tr>
<td>Tyrosine hydroxylaseα</td>
<td>(8)</td>
<td></td>
</tr>
</tbody>
</table>

*Shown are proteins for which expression levels or mRNA levels are upregulated by NGF in PC12 cells and for which AP-1 proteins and/or CREB-binding elements in respective gene promoter regions are implicated in controlling transcription. All of the proteins listed are products of late-response genes PC12 cells. |
Fra-2/JunD complex in PC12 cells. These data support a hypothesis that growth factors coupled to sustained ERK activity upregulate protein expression at the transcriptional level via Fra-2/JunD transactivation of AP-1 and CREB sites. Such sites are common in a variety of NGF-regulated late-response genes in PC12 cells, such as those listed in Table 1. For some of these genes, such as interleukin-1α (IL-1α; Alheim et al., 1996), neurotrophin Y (NP-Y; Holliday et al., 1995), and VGF (Salton et al., 1991), and rat light neurofilament (NF-L; Reeben et al., 1995) are upregulated differentially by NGF over EGF. N-type Ca2+ channels also are upregulated selectively in PC12 cells by NGF (Colston et al., 1998). Although the rat promoter sequence is not available, the human promoter for these channel subunits contains two AP-1 binding sites (Kim et al., 1997). Thus, the Fra-2/JunD complex could act as a broad regulatory control switch that regulates transcription and promotes differentiation in PC12 cells.

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