Identification of a nerve growth factor- and epidermal growth factor-regulated protein kinase that phosphorylates the protooncogene product c-Fos

(transcriptional regulation/transrepression/PC12 cells)

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ABSTRACT  Nerve growth factor (NGF) treatment of rat pheochromocytoma (PC12) cells induces the synthesis of the transcription factor c-Fos, which becomes highly phosphorylated relative to that produced as a result of depolarization of the cell. A peptide derived from the carboxyl terminus of c-Fos (residues 359–370, RKGSSNPESSD) containing putative phosphorylation sites was used to detect a NGF-stimulated Fos kinase. NGF treatment of PC12 cells resulted in a rapid activation of a protein kinase which phosphorylated both the c-Fos peptide and authentic c-Fos at its carboxyl terminus. The kinase was selectively activated by NGF and epidermal growth factor but was not induced by depolarization or other agents. The c-Fos peptide was phosphorylated at a serine corresponding to Ser362, a site critically implicated in the capacity of c-Fos to exhibit transrepressive activity (Offer, R., Dwaraki, V. J., Rashid, D. & Verma, I. M. (1990) Nature (London) 348, 80–82]). The NGF-stimulated Fos kinase may play an important role in regulating the expression and transforming potential of c-Fos.

The protooncogene c-fos is one member of a class of immediate early genes that are rapidly and transiently induced upon treatment of rat pheochromocytoma (PC12) cells with nerve growth factor (NGF) or a number of other agents (1). The c-fos gene product, c-Fos, is a transcriptional regulator, mediating transactivation by interacting with AP-1 sites as a heterodimer with c-Jun (2–7). Two amino-terminal domains are necessary for transactivation: a basic DNA-binding domain and a leucine zipper domain through which c-Fos associates with c-Jun (8–10). In addition to its role as a transactivator, c-Fos can repress transcription from its own promoter, as well as from those of other immediate early genes (4, 11–13). This transrepressive function of c-Fos is independent of its basic region and leucine zipper and is mediated by the carboxyl terminus of this protein (11, 12). A c-Fos mutant lacking the carboxyl-terminal 27 amino acids (residues 355–381) cannot repress transcription (11). v-Fos, the oncogenic viral cognate of c-Fos, contains both the leucine zipper and DNA-binding domains and maintains the ability to transactivate over AP-1 sites. However, v-Fos differs from c-Fos due to a deletion-induced frameshift resulting in 48 unrelated amino acids at the carboxyl terminus (13, 14). Like c-Fos mutants lacking the carboxyl terminus, the v-Fos protein does not exhibit transrepressive activity (15). c-Fos contains potential phosphorylation sites at its carboxyl terminus that are absent in v-Fos, one of which is phosphorylated in vivo (16, 17). Phosphorylation of c-Fos at sites in the carboxyl-terminal region is increased upon serum stimulation of rat 208F cells (16).

NGF treatment of PC12 cells results in the stimulation of the Trk protein-tyrosine kinase (18, 19), initiating a signal transduction cascade and the serial activation of a number of protein kinases (20–23). As a result, many proteins show increased levels of phosphorylation, including c-Fos (24). Curran and Morgan (24) reported that NGF treatment of PC12 cells resulted in the hyperphosphorylation of newly synthesized c-Fos protein relative to cells in which Fos synthesis was stimulated by membrane depolarization, suggesting the existence of a NGF-regulated Fos kinase. This observation provoked us to search for a NGF-regulated protein kinase that phosphorylates c-Fos. We describe here a rapidly activated, NGF-sensitive protein kinase that phosphorylates the carboxyl terminus of c-Fos, a locus shown to be involved in the transrepressive activity of this molecule (25). We have identified the phosphorylated residue within the peptide substrate as that corresponding to Ser362.

EXPERIMENTAL PROCEDURES

Materials. NGF was prepared by the method of Smith et al. (26). Radiolabeled ATP was synthesized by using Gamma Prep A (Promega). The c-Fos peptide, comprising residues 359–370 (RKGSSNPESSD), was synthesized at facilities at the Medical University of South Carolina (Charleston) and by Coast Scientific (La Jolla, CA). c-Fos and Fos ΔF proteins, a gift of Tom Curran (Hoffmann–La Roche), were expressed in Escherichia coli as a His, fusion protein as described by Abate et al. (27). Fos Kinase Isolation. PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum and 5% fetal bovine serum in an atmosphere of 10% CO2. The cells were collected, by trituration in phosphate-buffered saline containing bovine serum albumin (1 mg/ml) and glucose (1 mg/ml), resuspended at a concentration of 2 × 106 cells per ml, and treated as described for the indicated period of time. After collection by centrifugation, the cells were lysed by sonication in 20 mM Tris, pH 7.4/1 mM EGTA, pH 7.4/100 μM sodium orthovanadate (lysate buffer) containing 10 mM p-nitrophenyl phosphate, essentially as described by Smith et al. (28). The cellular lysate was centrifuged at 100,000 × g and the supernatant was collected. The soluble cell lysate was applied to a Mono Q HR 5/5 column (Pharmacia) in lysate buffer and developed with a 0–0.4 M NaCl gradient. The peak of activity eluted at 0.18 M NaCl was collected, pooled, and dialyzed in 20 mM Hepes, pH 6.8/1

Abbreviations: NGF, nerve growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SRE, serum response element; SRF, serum response factor.

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mM EGTA/100 μM sodium orthovanadate. The sample was then applied to a Mono S HR 5/5 column equilibrated in the same buffer and developed with a 0—0.4 M NaCl gradient.

**Kinase Assays.** Kinase reactions were performed in triplicate using 1 μg of protein per sample, or 40 μl of column fractions, in lysis buffer containing 0.5 mM c-Fos peptide, 1.2 mM dithiothreitol, 20 μM [γ-32P]ATP (6.6 cpm/fmol), and 10 mM MgCl2 at 22°C for 30 min. Reactions were stopped by addition of trichloroacetic acid (3.7%, wt/vol) and 10 μg of bovine serum albumin. The samples were cooled to 4°C for 15 min and centrifuged for 5 min. The supernatants were applied to Whatman P81 paper in triplicate and washed four times for 10 min in 75 mM phosphoric acid, and incorporated radioactivity was measured (28). Fos protein phosphorylation was evaluated by incubation as described above, except that [32P]ATP specific activity was 13.2 cpm/fmol and reactions were stopped by the addition of 3× Laemmli sample buffer. Products were resolved by SDS/10% PAGE and visualized by autoradiography.

**Sequencing of Radiolabeled c-Fos Peptide.** The c-Fos peptide (25 nmol) was incubated with Fos kinase under the same reaction conditions as described above, except that [32P]ATP specific activity was 528 cpm/fmol. After 1 hr, the reaction mixture was diluted to 2 ml of 0.05% trifluoroacetic acid and loaded onto a Pharmacia Super-Pac Pep-S HPLC column. The column was washed with 5 column volumes of 0.05% trifluoroacetic acid and the peptide was eluted in 20% acetonitrile containing 0.05% trifluoroacetic acid. The radiolabeled peptide was covalently attached to alylamine-derivatized poly(vinylidene difluoride) membranes (Sequelon-AA; Millipore) according to the manufacturer’s instructions, using water-soluble carbodiimide. The membranes were analyzed by sequencing in a model 473A automatic protein sequencer (Applied Biosystems). Cycles were modified to extract the aminothiazolinone derivatives with liquid trifluoroacetic acid followed by n-butyl chloride/ethyl acetate (1:1, vol/vol) containing 0.01% (vol/vol) phosphoric acid. The amino acid derivatives at each cycle were collected directly into scintillation vials, mixed with 10 ml of liquid scintillant (National Diagnostics, Manville, NJ), and measured in a scintillation counter.

**RESULTS**

NGF treatment of PC12 cells resulted in the activation of a protein kinase that phosphorylated a synthetic peptide corresponding to the carboxyl terminus of c-Fos (residues 359—370). NGF maximally activated the enzyme within 5 min and the activity remained elevated at least 2-fold up to 2 hr later (Fig. 1). Fos kinase activity was elevated throughout the period during which c-Fos is actively synthesized (24), consistent with phosphorylation of the nascent protein by this enzyme.

The Fos kinase was activated in response to NGF and epidermal growth factor to approximately the same levels (Fig. 2). Basic fibroblast growth factor, dibutyryl cAMP, phorbol 12-myristate 13-acetate, or depolarization of the cells with KCl had little or no effect upon kinase activation. The finding that Fos kinase activity was not stimulated following depolarization is consistent with the observation of Curran and Morgan (24) that the c-Fos protein produced in response to depolarization was hypophosphorylated relative to that generated following NGF treatment.

A major concern was that the Fos kinase represented another known protein kinase that exhibited activity toward the Kemptide peptide substrate. We have attempted to rule out the involvement of other protein kinases based on assays for their activities or immunological cross-reactivity (L.K.T. and G.E.L., unpublished work). Fos kinase was partially purified by ion-exchange chromatography on a Mono Q column. The

**Fig. 1.** Time course of NGF activation of Fos kinase. PC12 cells were treated with NGF (50 ng/ml) for the indicated time. Fos kinase activity was measured following partial purification by Mono Q chromatography. Data are expressed as cpm of [32P] incorporated into c-Fos peptide (mean ± SD, measured in triplicate).

NGF-stimulated Fos kinase was eluted at 0.18 M NaCl and was well resolved from p42 mitogen-activated protein kinase (MAPK, also known as extracellular signal-regulated kinase 1, ERK1) and p44 MAPK (ERK2), which were eluted at 0.15 and 0.22 M NaCl, respectively. Similarly, Fos kinase was not coeluted with the major peak of Kemptide kinase activity. Immunoblot analysis using anti-p85rsk antibodies demonstrated that this enzyme was eluted from the column in a peak prior to Fos kinase. Chromatography on the Mono S column allowed clear resolution of Fos kinase from kinases phosphorylating the S6 peptide substrate, as well as contaminating Kemptide kinase activity. These enzymes were of concern due to sequence similarity between these peptide substrates and the c-Fos peptide. We estimate a 2000-fold purification of the Fos kinase following Mono S chromatography.

The partially purified, NGF-stimulated, Fos kinase phosphorylated authentic c-Fos protein. c-Fos was phosphorylated when combined with Fos kinase obtained from NGF-

**Fig. 2.** Ligand specificity of Fos kinase. PC12 cells were treated with NGF (50 ng/ml), epidermal growth factor (EGF, 5 ng/ml), basic fibroblast growth factor (FGF, 30 ng/ml), dibutyryl cAMP (1 mM), KCl (50 mM), or phorbol 12 myristate 13-acetate ([12-tetradecanoylphorbol 13-acetate] (TPA), 50 ng/ml) for 5 min. Fos kinase activity was assayed following Mono Q chromatography. Data are expressed as cpm incorporated into c-Fos peptide (mean ± SD of triplicate measurements).
Fig. 3. Phosphorylation of c-Fos. Fos kinase (~6 ng) from control or NGF-stimulated cells was incubated alone (lanes a), with 1.8 pmol of c-Fos (lanes b), or with 1.8 pmol of Fos ΔF (lanes c) and the phosphorylation reaction was carried out for 60 min. Proteins were fractionated by SDS/10% PAGE and visualized by autoradiography. Positions of the molecular size (kDa) standards are shown.

Fig. 4. Cation-exchange chromatography of Fos kinase. NGF-stimulated Fos kinase activity obtained following chromatography on a Mono Q column was applied to a Mono S column and developed with a 0–0.4 M NaCl gradient. Column fractions were then assayed alone (lanes a) or in the presence of 1.8 pmol of Fos (lanes b) or 1.8 pmol of Fos ΔF (lanes c). Proteins were separated by SDS/10% PAGE. Fos peptide kinase assays were performed in parallel with fractions obtained from control (c) or NGF-treated (e) cells. Data are expressed as the mean ± SD of triplicate determinations.

Fig. 5. Sequence analysis of the phosphorylated c-Fos peptide. Fos kinase isolated by Mono Q and Mono S chromatography was incubated with c-Fos peptide, and the phosphorylated peptide was subjected to microsequencing. Data are expressed as cpm released from the peptide per cycle. Amino acids identified in cycles 1–12 (left to right) are represented by standard one-letter symbols. Most likely due to incomplete peptide bond cleavage in the previous cycle.

DISCUSSION

An increase in c-fos transcription is a common response in a number of cell types to a variety of stimuli. The c-fos gene product can participate in both the activation and repression of transcription. The ability of c-Fos to differentially regulate gene expression is central to its capacity to affect cell growth, differentiation, and transformation. It is not presently clear how these multiple effects of c-Fos are achieved; however, phosphorylation of Fos and other transcription factors is likely to be a prevalent mechanism through which their activities are regulated. The protein kinases responsible for these regulatory phosphorylations are important in the hormonal control of cellular phenotype.

We have identified a NGF-regulated Fos kinase in PC12 cells. Fos kinase was distinct from previously described NGF-stimulated kinases. Fos kinase was not cAMP-dependent kinase, as it was not inhibited by IP20 (at concentrations up to 1 μM), a specific peptide inhibitor of this enzyme, and cAMP-dependent kinase displays different chromatographic behavior (30, 31). Although c-Fos is phosphorylated in vitro at its carboxyl terminus by cAMP-dependent kinase (31), dibutyryl cAMP treatment of PC12 cells did not result in increased Fos kinase activity toward the peptide substrate. As previously indicated, Fos kinase was activated separately from MAPKs (ERKs) on ion-exchange chromatography and did not phosphorylate their substrates, myelin basic protein and microtubule-associated protein 2. The PSSD motif in the Fos peptide has been identified as a potential casein kinase II site due to its similarity to the casein kinase II phosphorylation site in serum response factor (SRF; ref. 32). However, Fos kinase was not inhibited by heparin up to 1 μM, indicating that Fos kinase was not casein kinase II. This is consistent with the observations of Abate et al. (31) showing that casein kinase II does not phosphorylate c-Fos in vitro. Fos kinase was distinct from protein kinase C, as it was not stimulated by phorbol ester and had no requirement for Ca²⁺. Similarly, the absence of a dependence upon Ca²⁺ also indicated that calmodulin kinases were not responsible for this activity. Antibodies to the S6II kinase family failed to precipitate Fos kinase activity (L.K.T. and G.E.L., unpublished).
lished work). Moreover, the activation kinetics and chromatographic behavior of Fos kinase were inconsistent with previously reported S6 kinases in PC12 cells (20). While Fos kinase is implicated in c-Fos phosphorylation, given its rapid activation, it is also likely to phosphorylate other substrates.

The carboxyl terminus of c-Fos is essential for the transcriptional repression exhibited by this protein (18, 26). Ofir et al. (25) provided evidence that phosphorylation of serine residues contained within the peptide employed in the present study are necessary for transrepression. Substitution of alanine residues within the GSSS sequence (residues 361–364) inhibited the transrepressive activity of c-Fos, which was restored upon introduction of negatively charged residues into this motif (25). The phosphorylation site at the carboxyl terminus of c-Fos has been identified as Ser<sup>262</sup> (17), corresponding to the phosphorylation site within the c-Fos peptide substrate of the NGF-activated Fos kinase described here. The requirement for the presence of this phosphorylation site for expression of transrepressive activity strongly suggests that phosphorylation of c-Fos by a kinase with the specificity of Fos kinase regulates this event. The potential functional importance of phosphorylation at this locus is demonstrated by the enhanced transforming capacity of Fos mutants lacking serine residues 362–364 (17).

The mechanism through which c-Fos is phosphorylated at its carboxyl terminus may be both cell type- and ligand-specific. It has been suggested that cAMP-dependent protein kinase mediates the phosphorylation of Fos within this motif; based on the ability of this kinase to phosphorylate this site in vitro and the observation that c-Fos becomes phosphorylated following forskolin treatment of Jeg3 cells (17, 31). However, dibutyryl cAMP treatment of CHO cells had no effect on c-Fos phosphorylation (16). NGF does not cause an increase in cAMP levels in PC12 cells (33), suggesting cAMP-dependent kinase is unlikely to be responsible for the resulting phosphorylation of c-Fos. It is likely that multiple, alternative mechanisms exist that activate the transrepressive function of this transcription factor.

The mechanism(s) through which c-Fos exerts its transrepressive activity is presently unclear. The CarG [CC(A+T-rich)<sub>6</sub>GG sequence motif] elements of the serum response element (SRE) in the Fos promoter are required for transrepression. However, c-Fos does not directly interact with this DNA motif (11). A protein complex of SRF and p62<sub>TCF</sub> binds to the SRE and is responsible for increased c-fos transcription following growth factor or serum stimulation (34–37). It is hypothesized that a protein–protein interaction between newly synthesized c-Fos and the SRF/p62<sub>TCF</sub> complex downregulates c-fos transcription. The phosphorylation of c-Fos at its carboxyl terminus by Fos kinase is thought to render it able to participate in such an interaction. Consistent with involvement of newly synthesized c-Fos protein, ongoing protein synthesis is required for the repression of c-fos transcription following growth factor stimulation (38). Depolarization results in the transient transcription of c-Fos through the cAMP/Ca<sup>2+</sup> response element, but the period of transcriptional activation is considerably longer (39). This pathway is mechanistically distinct from growth factor-induced transcriptional events mediated by the SRE (40), suggesting that depolarization-induced c-Fos transcription may be downregulated by alternative means. Importantly, cellular stimuli that act through the cAMP/Ca<sup>2+</sup> response element to modulate transcription fail to activate Fos kinase.

Identification of a hormonally stimulated Fos kinase provides an additional link in the chain of events through which the cell is able to rapidly alter the pattern of gene transcription and subsequently modulate its response to signals impinging upon it. The Fos kinase identified here may have a wider role in transcriptional regulatory events and in the cascade of protein kinase activation following NGF receptor occupancy.

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