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Calcium Influx via the NMDA Receptor Induces Immediate Early Gene Transcription by a MAP Kinase/ERK-Dependent Mechanism

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The regulation of gene expression by neurotransmitters is likely to play a key role in neuroplasticity both during development and in the adult animal. Therefore, it is important to determine the mechanisms of neuronal gene regulation to understand fully the mechanisms of learning, memory, and other long-term adaptive changes in neurons. The neurotransmitter glutamate stimulates rapid and transient induction of many genes, including the c-*fos* proto-oncogene. The c-*fos* promoter contains several critical regulatory elements, including the serum response element (SRE), that mediate glutamate-induced transcription in neurons; however, the mechanism by which the SRE functions in neurons has not been defined. In this study, we sought to identify transcription factors that mediate glutamate induction of transcription through the SRE in cortical neurons and to elucidate the mechanism(s) of transcriptional activation by these factors. To facilitate this analysis, we developed an improved calcium phosphate coprecipitation procedure to transiently introduce DNA into primary neurons, both efficiently and consistently. Using this protocol, we demonstrate that the transcription factors serum response factor (SRF) and Elk-1 can mediate glutamate induction of transcription through the SRE in cortical neurons. There are at least two distinct pathways by which glutamate signals through the SRE: an SRF-dependent pathway that can operate in the absence of Elk and an Elk-dependent pathway. Activation of the Elk-dependent pathway of transcription seems to require phosphorylation of Elk-1 by extracellular signal-regulated kinases (ERKs), providing evidence for a physiological function of ERKs in glutamate signaling in neurons. Taken together, these findings suggest that SRF, Elk, and ERKs may have important roles in neuroplasticity.

*Key words: transfection; neurons; transcription, c-*fos*; glutamate; MAP kinase; ERK; SRF; Elk*

Glutamate is the major excitatory neurotransmitter in the mammalian brain and regulates activity-dependent changes in neuronal function both during development and in the mature nervous system. Two classes of glutamate receptors are expressed on the neuronal cell surface: the ionotropic receptors, which mediate ion influxes, and the G-protein-coupled metabotropic receptors (Nakanishi, 1992; Seeburg, 1993). NMDA receptors are ionotropic glutamate receptors that, when activated, mediate calcium influx. There is considerable evidence that NMDA receptors are critical mediators of activity-dependent synaptic changes (Kleinschmidt et al., 1987; Fox et al., 1991; Madison et al., 1991; Rabacchi et al., 1992). A well characterized example of this is NMDA-dependent long-term potentiation (LTP) in the neocortex (Kirkwood et al., 1993) and hippocampus (Jahr and Lester, 1992; Bliss and Collingridge, 1993; Malenka, 1994).

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LTP is an activity-dependent enhancement of synaptic efficacy that can last for hours or days in intact animals (Bliss and Lømo, 1973; Jahr and Lester, 1992; Bliss and Collingridge, 1993; Bear and Malenka, 1994; Malenka, 1994). Classical LTP in the CA1 region of the hippocampus (E-LTP) persists for only a few hours and is independent of new protein synthesis. In contrast, long-lasting LTP in the CA1 (L-LTP), which is produced by multiple trains of high-frequency stimuli, lasts for hours or even days and requires *de novo* transcription and protein synthesis (Grecksch and Matthies, 1980; Mathies et al., 1990; Huang et al., 1994; Nguyen et al., 1994). LTP may be a useful model for studying molecular mechanisms that contribute to learning and memory and to experience-dependent synaptic plasticity in the visual cortex (Grant et al., 1992; Silva et al., 1992; Kirkwood et al., 1995; Wu et al., 1995). Like LTP, learning has two distinct components: a short-term form, which is independent of mRNA synthesis, and a long-lasting form, which is mRNA- and protein synthesis-dependent (Grecksch and Matthies, 1980; Montarolo et al., 1986; Yin et al., 1994). Given the importance of new gene expression for both LTP and learning, it is critical to elucidate the mechanisms by which glutamate regulates transcription in neurons.

Several studies have identified genes that are induced by LTP paradigms as well as by glutamate. These genes include c-*fos* and zif268 (also termed NGFIA/Egr-1/Krox24) (Cole et al., 1989; Dragunow et al., 1989; Jeffery et al., 1990; Bading et al., 1993; Lerea and McNamara, 1993; Worley et al., 1993; Williams et al., 1995); however, the mechanisms by which glutamate regulates these genes in neurons are largely undefined. In other cell culture systems, the c-*fos* gene has proved to be a useful model for

defining the signaling pathways by which extracellular stimuli trigger changes in gene expression (Sheng and Greenberg, 1990; Ginty et al., 1992; Ghosh and Greenberg, 1995; Treisman, 1995). Within the promoter of the c-*fos* gene, several regulatory elements have been defined that are important for the c-*fos* transcriptional response. These include the serum response element (SRE) and the calcium/cyclic AMP response element (CRE/CaRE) that are located approximately 310 nucleotides (SRE) and 60 nucleotides (CRE) 5′ of the initiation site of c-*fos* mRNA synthesis (Deschamps et al., 1985; Treisman, 1985; Gilman et al., 1986; Sheng et al., 1988; Rivera and Greenberg, 1990). *In vitro* binding studies have shown that the SRE can interact with many transcription factors, including the serum response factor (SRF), which binds to the SRE as a homodimer (Gilman et al., 1986; Prywes and Roeder, 1986; Treisman, 1986; Greenberg et al., 1987; Norman et al., 1988), the ternary complex factor (TCF) (Treisman, 1994), NF-IL 6, YY-1, and Phox1 (Treisman, 1992). TCFs are a family of Ets-domain containing transcription factors that include Elk-1, Sap-1a, Sap-1b, and Sap-2/Net/ERP (Rao et al., 1989; Shaw et al., 1989; Hipskind et al., 1991; Dalton and Treisman, 1992; Giovane et al., 1994). TCFs interact with the c-*fos* SRE only when SRF is already bound, thereby forming a ternary complex composed of an SRF dimer and a single molecule of TCF (Mueller and Nordheim, 1991; Shaw, 1992; Treisman, 1992). Studies using fibroblasts and PC12 cells have revealed that phosphorylation of the TCF Elk-1 at its C terminus is critical for its ability to stimulate transcription in response to serum or growth factor addition (Gille et al., 1992; Janknecht et al., 1993; Marais et al., 1993; Hipskind et al., 1994; Gille et al., 1995; Miranti et al., 1995; Price et al., 1995). The phosphorylation of Elk-1 that occurs on growth factor stimulation seems to be catalyzed by members of the microtubule-associated protein (MAP) kinase family, the extracellular signal-regulated kinases (ERKs).

Recent studies have shown that the SRE can mediate glutamate induction of c-*fos* transcription in hippocampal neurons (Bading et al., 1993); however, the mechanisms by which glutamate regulates SRE-mediated transcription in neurons had not been characterized. It was not known which of the many transcription factors that can bind to the SRE *in vitro* are involved in glutamate regulation of c-*fos* expression. For example, are SRF or TCFs critical for the c-*fos* response? If TCFs are found to be important, it will be useful to know which of the TCF family members are involved. In addition, it will be worthwhile to define the signal transduction pathways that lead to the activation of the SRE bound transcription factors. Although the ERKs are activated by glutamate treatment in cultured hippocampal and cortical neurons (Bading and Greenberg, 1991; Fiore et al., 1993a,b) (L. Rosen, S. Finkbeiner, and M. E. Greenberg, unpublished observations), whether these enzymes play a role in transmitting the glutamate signal to the nucleus is unknown. On the basis of the results of studies of c-*fos* transcription in non-neuronal cells, we hypothesized that glutamate stimulation leads to ERK activation and that ERKs then catalyze the phosphorylation of Elk-1 and the activation of c-*fos* transcription. In the present study, we provide evidence in support of this hypothesis.

MATERIALS AND METHODS

Plasmids. The expression vectors for SRF (pMLV.SRF.M2), Elk-1 (pMLV.NL.Elk-1), and Elk-1 mutant pMLV.NL.Elk-1(383/389), and the reporter plasmid pF4, were obtained from Dr. R. Treisman (Treisman, 1985; Hill et al., 1993). The expression vector for the MAP kinase phosphatase pSG5.MKP-1 was obtained from Drs. H. Sun and N. K. Tonks (Sun et al., 1993). The following constructs have been described previously: pSVα1 (Shyu et al., 1989), pAF42.SRE.WT and pAF42.SRE.mut2 (Rivera et al., 1990), pAF42.SRE.pm1 and pAF42.SRE.mut6 (Misra et al., 1994), pAF42.SRE.M2, pAF42.SRE.LM2 (Hill et al., 1993; Miranti et al., 1995), and pON260 (Cherrington and Mocarski, 1989).

Cell culture. Neurons were grown in several different media, all of which were compatible with calcium phosphate transfection. Hippocampal neurons shown in Figure 2 were cultured from newborn (P0) rats as described previously (Bading et al., 1993). Cortical neurons from P0 rats were grown in growth media based on either DMEM (Life Technologies, Gaithersburg, MD) or basal medium Eagle (BME) (Sigma, St. Louis, MO). Cortical neurons from embryonic day 17/18 (E17/E18) rats were grown in BME growth medium. The DMEM growth medium was composed of DMEM supplemented with 1 mM glutamine (Sigma), 5% rat serum (Harlan, Indianapolis, IN), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma). The BME growth medium was composed of (per 100 ml) 91 ml of BME, 5 ml of calf serum (Hyclone, Logan, UT), 1.4 ml of 2.5 M glucose (35 mM final), 0.5 ml of 0.2 M L-glutamine (1 mM final), 0.5 ml of penicillin/streptomycin (Sigma), 0.45 ml of Stable Vitamin Mix (3 mg/ml L-proline, 3 mg/ml L-cystine, 1 mg/ml *p*-aminobenzoic acid, 0.4 mg/ml vitamin B-12, 2 mg/ml *myo*-inositol, 2 mg/ml choline chloride, 5 mg/ml fumaric acid, 80 μg/ml coenzyme A, 0.4 μg/ml D-biotin, 0.1 mg/ml DL-6,8,-thioctic acid), 50 μl of ITS (5 mg/ml insulin, 5 mg/ml human transferrin, 5 μg/ml sodium selenite; Sigma), 0.5 ml of 1.6 mg/ml putrescine (Sigma), 0.5 ml of 5 mg/ml transferrin (Sigma), and 12 μl of 1 mM progesterone. The cells were seeded into 24-well plates on glass coverslips (Bellco) at a density of 2×10^5 cells/well (for Fig. 1*B–F*) or 3×10^6 cells/60-mm-diameter dish, and maintained in a humidified incubator with 5% CO_2 at 37°C. Plates and coverslips were coated with polylysine and laminin (Collaborative Biomedical Product, Bedford, MA). Cytosine-β-D-arabinofuranoside (Ara-C, 2.5 μM) was added to P0 cultures on the second day after seeding (2 DIV).

Calcium phosphate transfection of neurons. We took a number of steps to minimize neurotoxicity and maximize transfection efficiency. Potential toxicity was assessed by examining cells for morphological degeneration or nuclear fragmentation (using the DNA dye Hoechst 33258; Sigma). Transfection efficiency was assessed by determining either the percentage of cells expressing β-galactosidase (after staining cells with the substrate X-gal, 5-bromo-4-chloro-3-indolyl-β-gal) or the amount of α-globin RNA transcribed (using RNase protection analysis). One step taken to reduce toxicity was the inclusion in the transfection medium of kynurenate and $MgCl_2$, inhibitors of ionotropic glutamate receptors (both NMDA and non-NMDA types) and NMDA receptors, respectively. The addition of these inhibitors decreased neurotoxicity when cortical neurons from P0 rats were transfected 3 d after seeding (3 DIV) or later. For the transfections shown in Figure 1, D(−)-2-amino-5-phosphonovaleric acid (APV), a specific NMDA receptor antagonist, was added to the culture medium after transfection to reduce toxicity. This should be useful for studies not involving signaling through NMDA receptors. We found that a critical variable for successful transfection is the amount of calcium phosphate/DNA precipitate added per plate and the duration of exposure of the neurons to the precipitate. The optimum volume of the calcium phosphate/DNA precipitate was 120 μl/60-mm-diameter dish; larger volumes increased toxicity. The duration of the incubation with DNA/calcium phosphate precipitate was dictated by how fast the precipitate was formed on the plate. Typically, 30–45 min were required for the layer of precipitate to form on the plates, and the incubation was stopped 20–25 min later. Longer exposures to DNA/calcium phosphate precipitate caused toxicity. Another important factor for improved transfection efficiency was a 2% DMSO or 5% glycerol "osmotic shock" at the end of the incubation with the calcium phosphate/DNA precipitate. The 2% DMSO shock did not increase transfection efficiency, but it reduced variability between transfections without increasing toxicity. The 5% glycerol shock consistently increased transfection efficiency, but caused some toxicity if P0 cortical neurons were transfected after 5 DIV. In addition to these factors, the pH of the HEPES buffered saline (HBS) was critical. The optimum pH for the 2 × HBS was 7.07, although it is advisable to check several different pH values.

Using these optimized conditions, cortical or hippocampal neurons from P0 rats were transfected on 3 DIV (third day after seeding). The conditioned culture media were removed and saved. The cells were incubated in 3–5% CO_2 for 1 hr with 3 ml of fresh DMEM (per 60-mm-diameter plate) supplemented with 1 mM sodium kynurenate/10 mM $MgCl_2$ in 5 mM HEPES, pH 7.5. During this time, the DNA/calcium phosphate precipitate was prepared by mixing one volume of DNA in 250 mM $CaCl_2$ with an equal volume of 2 × HBS (274 mM NaCl, 10 mM KCl,

1.4 mM Na_2HPO_4, 15 mM D-glucose, 42 mM HEPES, pH 7.07). The precipitate was allowed to form for 25–30 min at room temperature before addition to the cultures. Six to eight micrograms of total plasmid DNA were used for each 60-mm-diameter plate. In a typical experiment, the following amounts of plasmid were used: 1 μg reporter plasmid, 200–300 ng of expression vectors for SRF or Elk-1, 3 μg of the SV40 promoter-driven human α-globin gene, 4 μg of the expression vector for the MAP kinase phosphatase MKP-1 or its empty cloning vector pSG5, and carrier plasmid DNA, e.g., pUC19, whenever necessary to maintain a constant amount of DNA per dish. One hundred and twenty microliters of the DNA/calcium phosphate precipitate were added drop-wise to each 60-mm-diameter dish and mixed gently. Plates were then returned to the 3–5% CO_2 incubator. The incubation was stopped 20–25 min after the layer of precipitate first formed on the plates by "shocking" the cells for 1–2 min with 1 × HBS/1 mM sodium kynurenate and 10 mM $MgCl_2$ in 5 mM HEPES, pH 7.5/2% DMSO or 5% glycerol. Cells were then washed three times with 3 ml of DMEM. The saved conditioned medium was added back to each plate, and the cells were returned to the 5% CO_2 incubator at 37°C.

A similar transfection protocol was followed for E17/18 cortical neurons growing in 24-well plates (Fig. 1*B,C*), with the following modifications: cultures were transfected on 3 or 4 DIV using 20 μl of DNA/calcium phosphate precipitate containing 1–2 μg plasmid DNA per well. Twenty to twenty-five min after the layer of precipitate first formed on the plates, cells were washed two times with 0.5 ml DMEM containing 1 mM sodium kynurenate and 10 mM $MgCl_2$ without the "osmotic shock." The conditioned culture medium supplemented with 100 μM APV was added back, and the cells were returned to the 5% CO_2 incubator at 37°C.

Cell staining. Cells were stained on 6 DIV or 2–3 d after transfection. Expression of β-galactosidase was detected either with the substrate X-gal or by immunostaining with a mouse monoclonal antibody to β-galactosidase (1:300 dilution; Promega, Madison, WI) and fluorescein-conjugated goat antibody to mouse immunoglobulin (IgG). To visualize the nuclei of transfected cells, we included the DNA dye Hoechst 33258 (2.5 μg/ml; Sigma) in the wash after the secondary antibody incubation. To identify neurons in culture, the expression of the neuronal marker protein MAP-2 was detected by immunostaining with a rabbit polyclonal antibody to MAP-2 (1:2000 dilution; a gift from Dr. R. Vallee) and Texas Red-conjugated goat antibody to rabbit IgG (Fig. 1*B*), or biotinylated goat antibody to rabbit IgG and the avidin/biotin/peroxidase detection system (Vector Laboratories, Burlingame, CA) (Fig. 3*A*). Immunocytochemistry with antibodies to cyclic AMP regulatory element binding protein (CREB) (anti-N-CREB) or CREB phosphorylated at serine residue 133 (anti-P-CREB) was performed as described (Ginty et al., 1993).

Cell stimulation. Cultures were stimulated as described but with the following modifications (Bading et al., 1993). Cells were treated with glutamate, NMDA, or membrane depolarization on 6 DIV for cortical neurons, or on 5 or 8 DIV for hippocampal neurons. Tetrodotoxin (1 μM) and CNQX (40 μM) were added to all plates the night before stimulation to reduce endogenous synaptic activity. Control stimulated cultures were pretreated for 30 min with both 100 μM APV and 5 μM nimodipine. Cells stimulated with glutamate or NMDA were pretreated for 30 min with 5 μM nimodipine. Cells exposed to a membrane-depolarizing concentration of KCl (55 mM) were pretreated for 30 min with 100 μM APV. For Western blotting analysis, cells were then stimulated for 10 min with 10 μM glutamate, and cell extracts were prepared in boiling SDS sample buffer. For RNase protection analysis, cells were stimulated with 10 μM glutamate, 100 μM NMDA, or 55 mM KCl, and total RNA was collected 50 min after stimulation. To prevent excitotoxic cell death, 1 mM sodium kynurenate and 10 mM $MgCl_2$ were added to the medium 10 min after the addition of glutamate, NMDA, or KCl.

RNA isolation, RNase protection analysis, and quantitation. RNA was isolated and RNase protections were performed as described (Chomczynski and Sacchi, 1987; Sheng et al., 1988). Because there is little or no expression of the transfected fos^h reporter constructs in unstimulated cells, the fold induction of transcription after glutamate treatment cannot be calculated. Therefore, the expression level of the fos^h mRNA relative to that of the globin mRNA was used for quantitation. The level of transcription from each reporter construct was determined by dividing the signal intensity of the fos^h band, obtained using a PhosphorImager, over that of the globin band (fos^h/globin). The relative levels of transcription between different reporter constructs after glutamate treatment were determined by this ratio (fos^h/globin).

RESULTS

Transfection of primary neurons using a modified calcium phosphate method

Studies of the mechanisms controlling gene expression in neurons have been limited because of difficulties encountered when attempting to transfect postmitotic neurons. To explore mechanisms by which glutamate regulates transcription in neurons, it was necessary to develop a method for reliably and efficiently introducing DNA into neurons with minimal toxicity. Although there have been a few studies describing transfection of cultured neurons by the calcium phosphate method (Korner et al., 1989; Werner et al., 1990; Gabellini et al., 1992; Blochl and Thoenen, 1995), this method has not been used extensively because of its toxicity for neurons. We have developed a modified calcium phosphate transfection method applicable for the transient transfection of CNS neurons in primary culture. We have accomplished nontoxic transfection of neurons at relatively high efficiency by optimizing several experimental parameters. These include the amount of calcium phosphate DNA precipitate added per plate, the duration of exposure of neurons to the calcium phosphate/DNA precipitate, the inclusion of inhibitors of glutamate receptors, and an osmotic shock of the neurons at the end of the incubation with the calcium phosphate/DNA precipitate (for details, see Materials and Methods).

Using this method, rat cortical neurons were transfected with a plasmid encoding β-galactosidase, pON260, and 2 d later cells were stained with X-Gal, a β-galactosidase substrate. Numerous transfected cells were readily detectable (Fig. 1*A*); the transfection efficiency in these experiments was ~2%. To confirm that the transfected cells were neurons, we performed double-immunofluorescence staining for β-galactosidase and the neuron-specific marker protein MAP-2 (Fig. 1*B*). More than 90% of the transfected cells stained positive for MAP-2 (Fig. 1*B*), consistent with the low abundance of non-neuronal cells in these cultures (see also Fig. 3). The morphology of the transfected neurons, including the presence of phase-bright cell bodies and long processes, indicated that they remained healthy after transfection. In addition, staining of transfected neurons with the DNA dye Hoechst 33258 revealed that ~90% of the transfected cells showed an evenly stained, nonfragmented, and noncondensed nuclear morphology (Fig. 1*C*), suggesting that these transfected cells were healthy. Thus, this transfection method allows efficient and nontoxic DNA transfection of cultured neurons.

It was important to determine whether genes introduced into neurons by this method are regulated correctly in response to extracellular stimuli. To address this question, plasmid pF4, which contains a 5.4 kb fragment of the intact human c-*fos* gene including 750 bp of 5′ regulatory sequence (Treisman, 1985), was transfected into cortical neurons. A plasmid that directs the constitutive expression of human α-globin gene was cotransfected with pF4 and serves as an internal control for variations in transfection efficiency and RNA recovery between samples. The transfected cells were stimulated by exposure to either 55 mM KCl, which depolarizes the membrane and stimulates Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (VSCCs) (Tsien et al., 1988; Bean, 1989), or 10 μM glutamate, which stimulates Ca^{2+} influx through NMDA receptors. When cells were stimulated with 55 mM KCl, APV (an NMDA-receptor antagonist) and CNQX (an inhibitor of non-NMDA ionotropic glutamate receptors) were included in the stimulation media to minimize the activation of glutamate receptors. To minimize activation of non-NMDA iono-

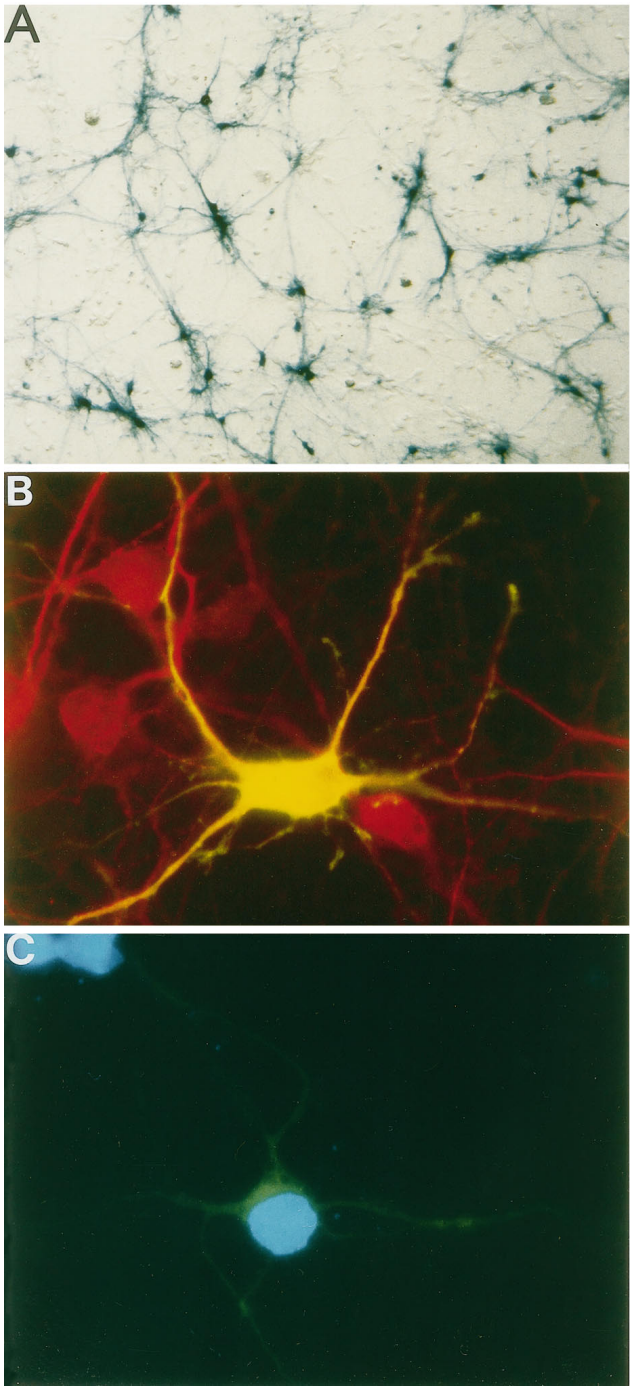

Figure 1. Transient transfection of the β-galactosidase gene into primary cortical neurons using a modified calcium phosphate procedure. *A*, X-Gal staining (*blue*) of cortical neurons (P0) transfected at 3 DIV with an expression vector encoding β-galactosidase. *B*, A representative immunofluorescence photomicrograph of a neuron transfected with β-galactosidase. Transfected cortical neurons cultured from E17/18 rats were detected by immunostaining with a monoclonal antibody to β-galactosidase, visualized by fluorescein-conjugated goat antibody to mouse IgG (*green*). The cells were coimmunostained for the neuronal marker protein MAP-2, which was visualized using a Texas Red-conjugated goat antibody to rabbit IgG (*red*). The transfected neuron appears *yellow* because of the colocalization of *green staining* from anti-β-galactosidase and *red staining* from anti-MAP-2. *C*, A representative immunofluorescence photomicrograph of a healthy neuron expressing β-galactosidase. A transfected E17/18 rat cortical neuron was detected as in *B*. The nucleus was visualized using the DNA dye Hoechst 33258 (*blue*). The evenly stained, round morphology is typical of a healthy (nonapoptotic) nucleus.

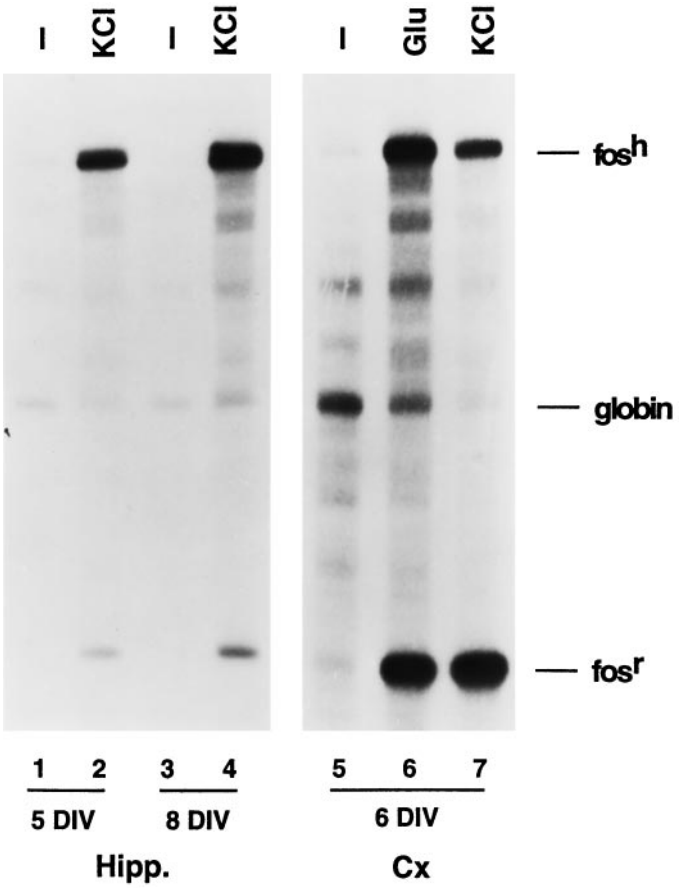

Figure 2. Genes transfected into neurons are correctly regulated in response to extracellular stimuli. The plasmid pF4, which contains the intact human c-*fos* gene including 750 bp of 5′ regulatory sequence, was transfected 3 d after plating (3 DIV) into hippocampal (*Hipp.*, *lanes 1–4*) or cortical neurons (*Cx*, *lanes 5–7*). The plasmid pSVα1, which encodes the human α-globin gene under the control of the SV40 promoter, was cotransfected as an internal control for transfection efficiency and RNA recovery (2 μg/plate). For hippocampal neurons, 0.9×10^6 cells were plated onto each 60-mm-diameter plate, and 5 μg of pF4 plasmid DNA was used for transfection. For cortical neurons, 3×10^6 cells were plated onto each 60-mm-diameter plate, and 4 μg of pF4 plasmid DNA was used for transfection. Transfected cells were either left untreated (−) or stimulated with 55 mM KCl or 10 μM glutamate (*Glu*) on *5 DIV* (*lanes 1* and *2*), *6 DIV* (*lanes 5–7*), or *8 DIV* (*lanes 3* and *4*). RNase protection analysis was used to measure the expression of the transfected human c-*fos* gene (fos^h), the human α-globin gene, and the endogenous rat c-*fos* gene (fos^r).

tropic glutamate receptors and the opening of VSCCs, CNQX and nimodipine (an antagonist of VSCCs) were included in the media when cells were stimulated with glutamate. In each experiment, the levels of expression of the transfected human c-*fos* gene (fos^h), the endogenous rat c-*fos* gene (fos^r), and the transfected α-globin gene were determined using an RNase protection assay (Sheng et al., 1988). The correctly initiated and spliced human c-*fos* gene transcript protects a 296-nucleotide fragment of the c-*fos* probe, whereas the endogenous rat c-*fos* mRNA protects a 65-nucleotide fragment. The correctly initiated human α-globin gene transcript protects a 133-nucleotide fragment of the globin probe. In addition to these three major bands, another band is observed between fos^h and globin. This 190-nucleotide band is present when RNase protection assays are performed using mRNA from cells transfected with the α-globin gene in the

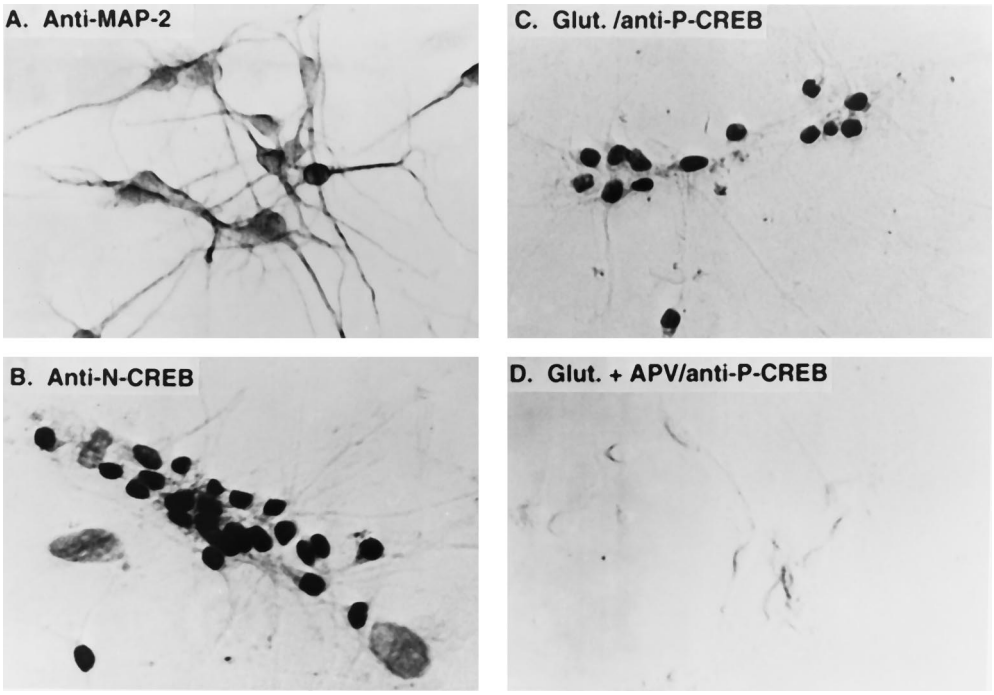

Figure 3. Glutamate responsiveness of cultured cortical neurons. Cortical neurons were immunostained with an antibody to the neuronal marker protein MAP-2 (*A*), an antibody to the N terminus of CREB (*B*), or an antibody specific for CREB that is phosphorylated at serine[133] (*C, D*). Cells were unstimulated (*A, B*), or stimulated for 10 min with 10 μM glutamate in the absence (*C*) or presence (*D*) of 100 μM APV. Glutamate induced CREB phosphorylation only in cells with neuronal morphology.

absence of the human c-*fos* gene (S. Finkbeiner and M. E. Greenberg, unpublished data). Therefore, the band may be derived from α-globin mRNA expressed from the transfected human globin gene, and most likely reflects either partial digestion of the globin probe or incorrect initiation or splicing of the human globin gene transcript in these neurons.

The correctly initiated pF4 message, like the endogenous c-*fos* mRNA, was found to be induced by either KCl or glutamate treatment of transfected hippocampal or cortical neurons (Fig. 2 and data not shown). A consistently high level of c-fos^h mRNA was synthesized on stimulation from the exogenously introduced c-*fos* gene, even when the time period between the initial transfection and the stimulation was varied between 1 and 5 d. Taken together, these data suggest that the c-*fos* gene transfected into neurons by the calcium phosphate precipitation method is regulated correctly in response to extracellular stimuli in different cell types and at a range of times after transfection.

Intact binding sites for SRF and TCFs within the SRE are required for a full transcriptional response to glutamate

An objective of the present study was to identify specific transcription factors that mediate glutamate induction of c-*fos* transcription in neurons. To address this issue, we have used primary cortical neurons cultured from P0 rats. More than 90% of the cells in our culture preparation were neurons, as indicated by positive staining with an antibody that recognizes MAP-2 (Fig. 3*A*). The MAP-2-positive neurons have small round cell bodies and an extensive array of neurites, whereas the MAP-2-negative nonneuronal cells have large flat cell bodies and few processes. To confirm that the transcriptional response to glutamate in these cultures occurs in neurons, we determined whether the cell types that were responsive to glutamate stimulation were neurons or glia. This was achieved by monitoring the phosphorylation of a transcription factor, CREB, before and 10 min after glutamate treatment. We have previously generated an antibody that specifically recognizes CREB when it is phosphorylated at serine[133] (anti-P-CREB), and demonstrated that CREB undergoes specific phosphorylation at amino acid residue serine[133] when hippocampal or cortical neurons are exposed to glutamate (Ginty et al., 1993). Immunohistochemical analysis using the anti-P-CREB antibody demonstrated that glutamate treatment induced phosphorylation of CREB at serine[133] in >90% of the cells in our cortical cultures within 10 min (Fig. 3*C*). By contrast, in unstimulated cultures, very few cells stained positive with the anti-P-CREB antibody (data not shown). The P-CREB-positive cells in the glutamate-stimulated cultures displayed a neuronal morphology (Fig. 3*C*). Glutamate induction of CREB phosphorylation required the activation of NMDA receptors, because the phosphorylated form of CREB was not detected when cells were pretreated with APV before glutamate addition (Fig. 3*D*). The failure to detect significant amounts of serine[133]-phosphorylated CREB in non-neuronal cells was not attributable to the absence of CREB in these cells, because all of the cells in the culture stained positive when an antibody that recognizes CREB regardless of its phosphorylation state (anti-N-CREB) was used (Fig. 3*B*). Taken together, these results suggest that in primary cortical cultures, it is the neurons, not the glia, that are directly responsive to glutamate stimulation.

To begin to identify the specific transcription factors that mediate glutamate induction of c-*fos* transcription, we introduced various mutations into the SRE that are known to interfere with the binding of specific transcription factors. Human c-*fos* reporter genes bearing these mutations were introduced into cortical neurons, and the effects of the mutations on glutamate-stimulated c-*fos* mRNA expression were analyzed. The plasmid pAF42.SRE.wt contains a single copy of the wild-type c-*fos* SRE inserted 42 bp 5′ of the transcription initiation site in the plasmid pAF42. pAF42 contains a copy of the human c-*fos* gene in which the promoter has been deleted of all known transcription regulatory sequences 5′ of the TATAA box (Rivera et al., 1990). Three days after cortical cultures were transfected with pAF42.SRE.wt, they were stimulated with glutamate in the presence of nimodipine and CNQX.

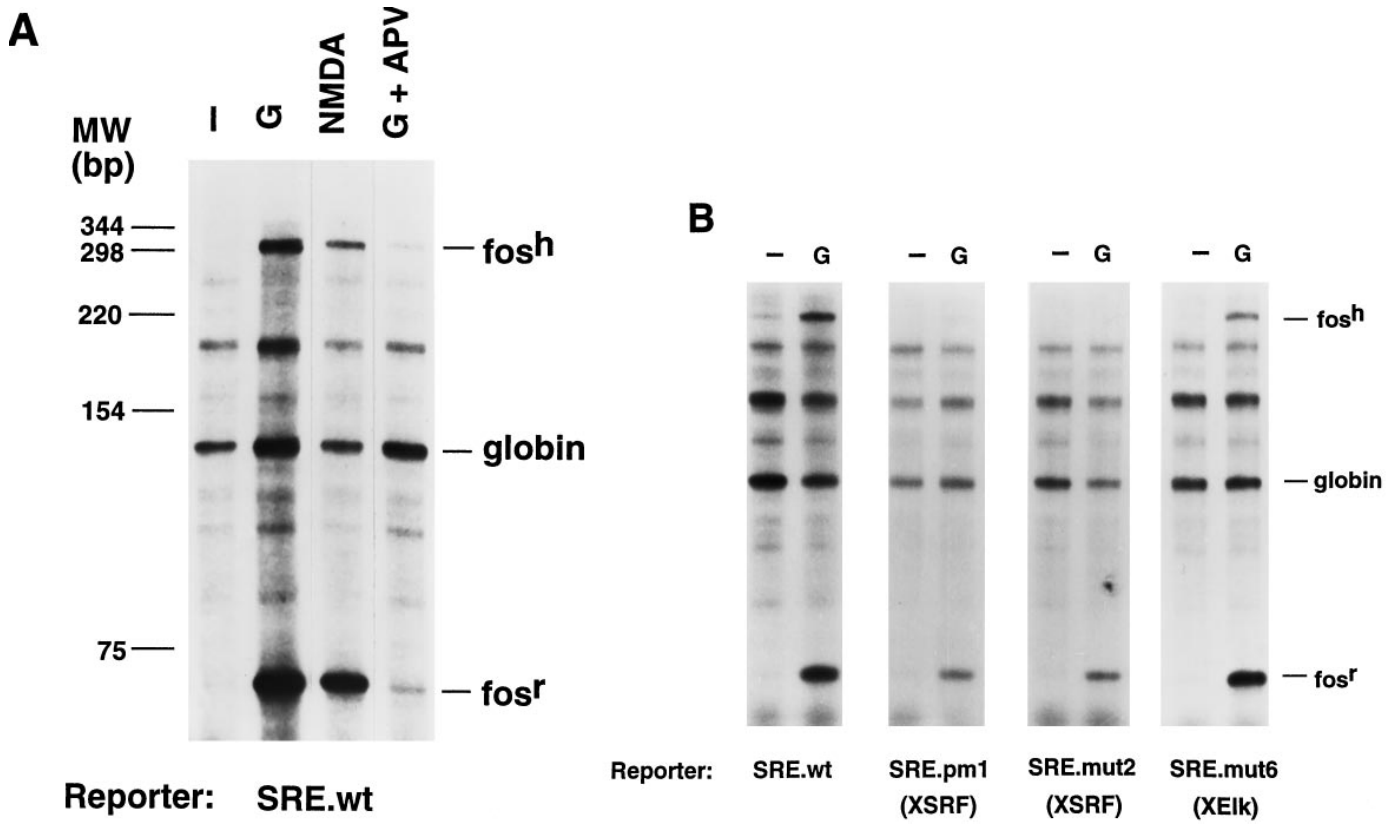

Figure 4. Contribution of the SRF and TCF binding sites for glutamate stimulation of SRE-mediated transcription. *A*, Glutamate stimulation of SRE-mediated transcription is dependent on the activation of NMDA receptors. Cortical neurons (3×10^6 cells/plate) were transfected with the wild-type c-*fos* SRE-containing construct pAF42. SRE.wt (1 μg/plate) at 3 DIV. Three days later, cells were left untreated (−), treated with 10 μM glutamate in the absence or presence of 100 μM APV, or treated with 100 μM NMDA. RNase protection analysis was used to measure the expression of the transfected human c-*fos* gene (fos^h), the human α-globin gene, and the endogenous rat c-*fos* gene (fos^r). *B*, Effects of mutations within the SRE that interfere with the binding of SRF or TCFs on SRE-mediated transcription. Cortical neurons (3×10^6 cells/plate) were transfected with 1 μg of pAF42.SRE.wt, or constructs with mutations in the SRE that disrupt SRF binding (pAF42.SRE.pm1 or pAF42.SRE.mut2), or TCF binding (pAF42.SRE.mut6). Three days later, the transfected cells were either left untreated (−) or stimulated with 10 μM glutamate. Similar results were obtained from three independent experiments. The relative levels of transcription between different reporter constructs after glutamate treatment were determined by the ratio of fos^h/globin.

pAF42 (which lacks the SRE) was unresponsive to glutamate (data not shown). Transcription from the pAF42.SRE.wt reporter was efficiently induced by glutamate, however, and this transcriptional response was abolished by APV treatment (Fig. 4*A*). NMDA, an agonist specific for the NMDA receptor, also induced transcription from the pAF42.SRE.wt reporter but not pAF42. Together, these data suggest that NMDA-receptor activation can trigger SRE-dependent transcription in cortical neurons. In contrast to the wild-type SRE reporter, mutations that disrupt the binding of SRF to the SRE (SRE.pm1 or SRE.mut2) (Rivera et al., 1990; Misra et al., 1994) abolished glutamate induction of transcription through the SRE (Fig. 4*B*). When the binding site for TCFs was mutated (SRE.mut6) (Misra et al., 1994), the induction of transcription was 36% of that of the wild-type SRE after correcting for variations in transfection efficiency (Fig. 4*B*); similar results were seen in three independent experiments. Mutation of the TCF binding site has been shown previously not to interfere with SRF binding to the SRE (Misra et al., 1994). Taken together, these findings suggest that the binding site for SRF or an SRF-like factor is obligatory for SRE-mediated transcription and that the binding site for TCFs is required for a full transcriptional response to glutamate.

Expression of recombinant SRF and Elk-1 can activate SRE-mediated transcription on glutamate stimulation

The results described above suggest that the binding sites for SRF and TCFs are required for full SRE-mediated transcriptional activation in response to glutamate. To identify the specific transcription factors that can mediate glutamate induction of SRE-dependent transcription, we examined the possible roles of SRF and one of the TCF family members, Elk-1, in this process. We used an altered binding-specificity assay that allows the evaluation of the function of transfected SRF and Elk-1 without interference from their endogenous counterparts (Hill et al., 1993; Miranti et al., 1995). The pAF42.SRE.wt reporter construct was mutagenized to generate pAF42.SRE.M_2, a construct in which the DNA binding site for SRF in the SRE (the CaRG box) was replaced with that of the yeast SRF homolog MCM-1 (Fig. 5). Endogenous wild-type SRF does not recognize the altered SRE (SRE.M_2). A cotransfected mutant SRF (SRF.M2) can bind to SRE.M_2, however, because it contains the DNA binding domain of MCM-1 in place of the DNA binding domain of the wild-type SRF. To evaluate the role of Elk-1 in SRE-dependent transcription, a mutation was introduced into pAF42.SRE.M_2 to generate pAF42.SRE.LM_2. In pAF42.SRE.LM_2, the DNA binding site for

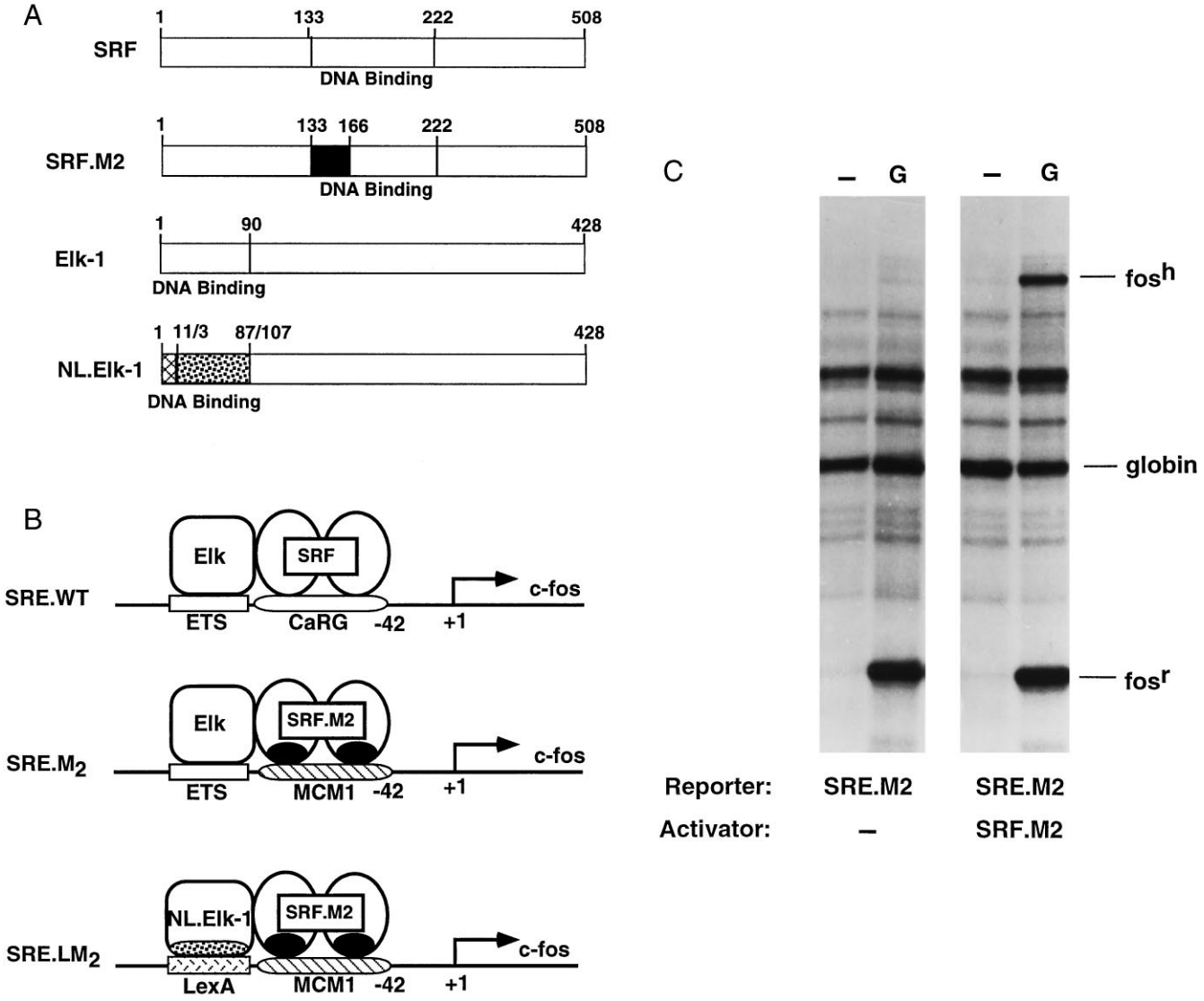

Figure 5. Ectopically expressed SRF can mediate SRE-dependent transcription stimulated by glutamate. *A*, Expression vectors used in the altered binding specificity assay (Hill et al., 1993). Amino acids 133–166 of the DNA binding domain of SRF were replaced with 33 amino acids from the DNA binding domain of MCM1 (*black box*) to create SRF.M2. Amino acids 3–87 of the LexA DNA binding domain (*dotted box*) were fused in-frame in front of amino acid 107 of Elk-1, which eliminates the Elk-1 DNA binding domain, to generate LexA-Elk. Ten additional amino acids encoding a nuclear localization signal (*crossed box*) were added to the N terminus of LexA-Elk to ensure its nuclear localization (NL.Elk-1). *B*, Reporter constructs used in the altered binding specificity assay (Hill et al., 1993; Miranti et al., 1995). Endogenous SRF binds as a homodimer to the CArG box of the wild-type SRE (pAF42.SRE.wt). TCFs, e.g., Elk-1, bind as a monomer to an ETS sequence adjacent to the CArG box only when SRF is already bound to the SRE. The CArG site was replaced by an MCM1 promoter sequence to generate pAF42.SRE.M_2, which can bind SRF.M2 but not wild-type endogenous SRF. Endogenous Elk-like proteins can still bind to the ETS site in the SRE.M2. Additional substitution of the ETS site with a LexA site generated pAF42.SRE.LM_2, which contains mutations in both the SRF and Elk binding sites and is therefore incapable of binding endogenous SRF or Elk. Elk binding activity can be restored to the SRE.LM_2 promoter element by NL.Elk-1 when SRF.M2 is present. Reporters with two adjacent copies of the mutated SREs were used. *C*, SRF can mediate SRE-dependent transcription in response to glutamate. Cortical neurons were transfected with the reporter construct pAF42.SRE.M2 either alone or together with an expression vector encoding SRF.M2. Three days later, cells were either left untreated (−) or treated with 10 μM glutamate. The level of mRNA transcribed from the reporter plasmid was assessed as described in the legend to Figure 4. Similar results were obtained from three independent experiments.

TCFs (the ETS site) was substituted with a site that binds the bacterial transcription factor LexA. Therefore, pAF42.SRE.LM_2 contains mutations in both the SRF and TCF binding sites and is incapable of binding to endogenous SRF or TCFs. A cotransfected modified version of Elk-1 (NL.Elk-1), in which the DNA binding domain of Elk-1 is replaced with that of LexA, can interact with SRE.LM_2 but only when SRF.M2 is also bound.

When transfected into cortical neurons, pAF42.SRE.M_2 was completely unresponsive to glutamate stimulation (Fig. 5*C*), consistent with the inability of endogenous SRF to bind to SRE.M_2. Glutamate induction of transcription from pAF42.SRE.M_2 was restored when the expression vector SRF.M2 was cointroduced into neurons. The reporter plasmid pAF42.SRE.LM_2, which can bind neither the endogenous SRF nor TCFs, was also unresponsive to glutamate stimulation (Fig. 6). Expression of the SRF.M2 protein, however, allowed glutamate induction of transcription from the pAF42.SRE.LM_2. Coexpression of NL.Elk-1 reproducibly increased the level of transcription induced by glutamate by

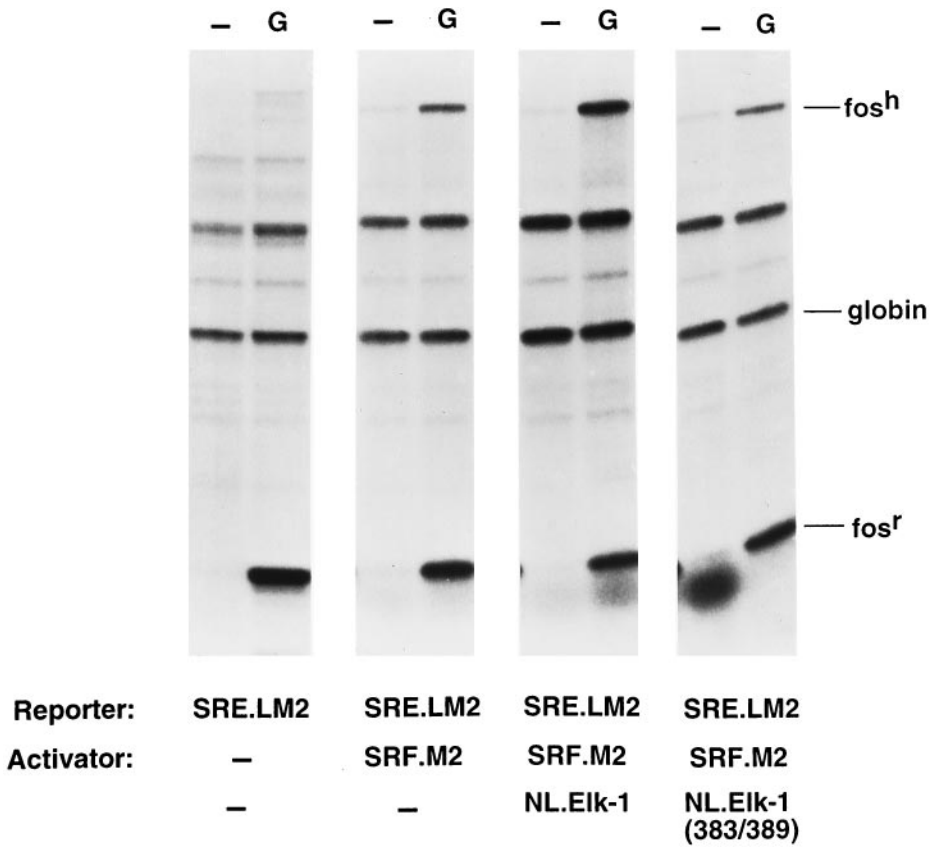

Figure 6. Glutamate stimulates SRE-mediated transcription through an Elk-dependent pathway. Cortical neurons (3×10^6 cells/plate) were transfected with the c-*fos* reporter pAF42.SRE.LM_2 (1 μg) together with 200 ng of various expression plasmids as indicated: SRF.M2, NL.Elk-1, or NL.Elk-1(383/389). NL.Elk-1(383/389) contains alanine substitutions at Ser^{383} and Ser^{389}, two of the MAP kinase phosphorylation sites in the C terminus of Elk-1. Three days after transfection, cells were either left untreated (−) or treated with 10 μM glutamate. The level of mRNA transcribed from the reporter plasmid was assessed and quantitated as described in the legend for Figure 4. Relative levels of transcription from the reporter pAF42.SRE.LM_2 after glutamate stimulation are SRF.M2, 100%; SRF.M2 + NL.Elk-1, 154 ± 5% (SEM, $n = 5$); SRF.M2 + NL.Elk-1(383/389), 104 ± 12% (SEM, $n = 3$).

50%. These data suggest that both SRF and Elk-1 can contribute to SRE-dependent transcription in response to glutamate. There are apparently two pathways by which glutamate can activate transcription through the SRE: an SRF-dependent but Elk-independent pathway, because expression of SRF.M2 alone can mediate a significant level of transcription from the pAF42.SRE.LM_2 reporter, and an Elk-dependent pathway, because addition of NL.Elk-1 further enhances transcription.

Activation of ERKs mediates glutamate signaling to the SRE

Phosphorylation of Elk-1 at residues Ser^{383} and Ser^{389} is crucial for Elk-1 to activate transcription (Hill et al., 1993; Marais et al., 1993; Janknecht et al., 1994; Miranti et al., 1995). Previous studies have shown that ERKs can directly phosphorylate Elk-1 at these residues *in vitro*. Furthermore, studies in fibroblasts and PC12 cells have demonstrated a close correlation between activation of the ERKs and the subsequent phosphorylation of Elk-1. These studies have suggested that activation of ERKs plays a critical role in regulating Elk-mediated gene expression in these cells. Although glutamate stimulation also activates ERKs in hippocampal (Bading and Greenberg, 1991) and cortical neurons (Fiore et al., 1993a,b) (L. Rosen, S. Finkbeiner, and M. E. Greenberg, unpublished observations), the function of ERKs in glutamate signaling in neurons has not been known. To examine whether phosphorylation of Elk-1 in response to glutamate regulates the ability of Elk to activate transcription, we tested whether a mutant Elk-1 in which the two regulatory serine residues Ser^{383} and Ser^{389} were replaced by alanines [NL.Elk-1(383/389)] enhanced transcription from pAF42.SRE.LM_2. In contrast to results obtained using the wild-type protein NL.Elk-1, coexpression of NL.Elk-1(383/389) failed to further enhance the level of SRF.M2-mediated transcription initiated from pAF42.SRE.LM_2 (Fig. 6). This suggests that full transcriptional activation by glutamate through the SRE involves activation of a kinase, most likely an ERK, which catalyzes the phosphorylation of Elk-1 at serine residues Ser^{383} and Ser^{389}.

To test further whether glutamate-stimulated ERKs regulate SRE-dependent transcription, we blocked ERK activation by introducing into cortical neurons a MAP kinase phosphatase, MKP-1, which is known to dephosphorylate and inactivate ERKs (Alessi et al., 1993; Sun et al., 1993, 1994). The ERKs are activated through a kinase cascade in which the kinase Raf phosphorylates and activates the dual specificity Thr–Tyr protein kinase MEK, which then phosphorylates and activates the ERKs (Cobb et al., 1991; Campbell et al., 1995). Constitutive expression of MKP-1 has been shown previously to lead to dephosphorylation of the ERKs, so that even in the presence of extracellular stimuli the ERKs are not activated effectively. Therefore, we tested the effect on glutamate induction of SRE-mediated transcription of cotransfecting an expression vector for MKP-1 along with expression vectors for SRF and Elk-1. As shown in Figure 7, compared to cotransfection with the empty vector pSG5 (*top*), coexpression of MKP-1 prevented NL.Elk-1 from further enhancing transcription initiated by SRF.M2 through the pAF42.SRE.LM_2 (*bottom*). This result suggests that the ERKs, or a closely related kinase (Whitmarsh et al., 1995; Raingeaud et al., 1996), may play an important role in mediating Elk-dependent transcription in response to glutamate.

DISCUSSION

The objectives of this study were to identify transcription factors that activate transcription through the SRE in response to glutamate in neurons, and to characterize the signaling pathways that

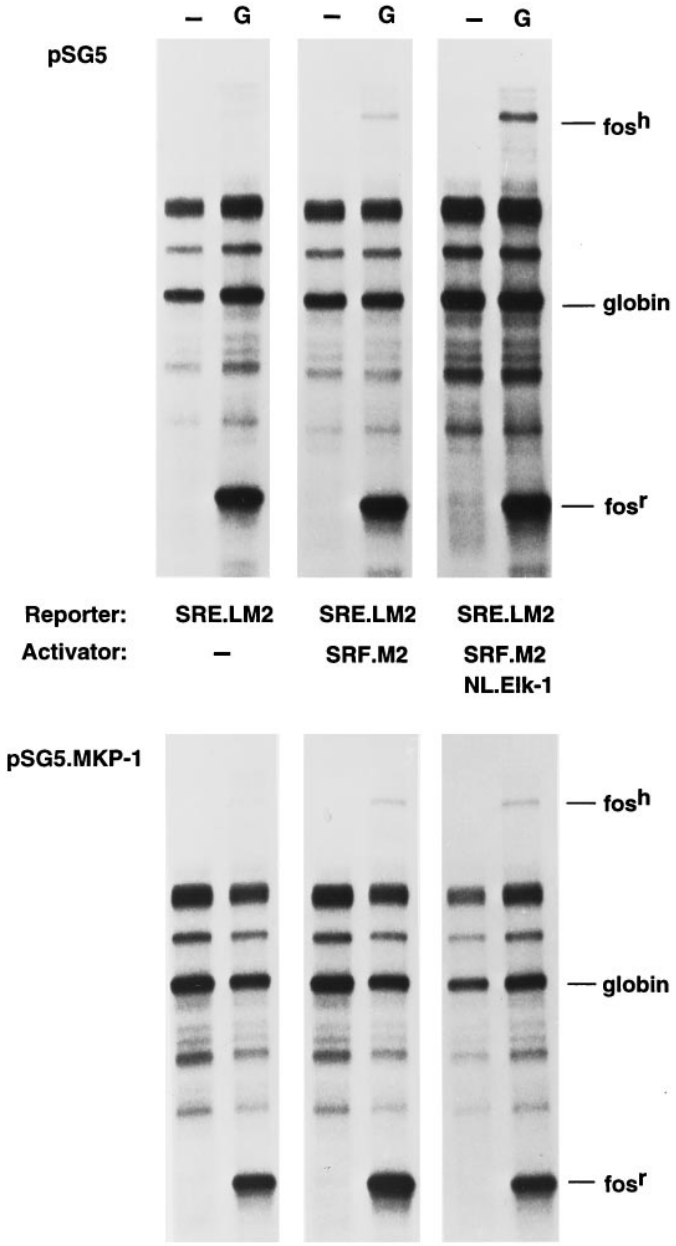

Figure 7. Coexpression of a MAP kinase phosphatase MKP-1 blocks Elk-dependent transcription. Cortical neurons were transfected with the c-*fos* reporter pAF42.SRE.LM_2 alone or together with expression plasmids encoding SRF.M2 or NL.Elk-1, as indicated. Cells were also cotransfected with either the empty cloning vector pSG5 (*top*) or the expression vector pSG5.MKP-1 (*bottom*). Three days later, cells were either left untreated (−) or treated with 10 μM glutamate. The level of mRNA transcribed from the reporter plasmid was determined as described in the legend to Figure 4. In cells expressing MKP-1 (*bottom*), coexpression of Elk-1 failed to enhance transcription over that obtained with SRF alone, in comparison to cells transfected with vector alone (*top*). Similar results were obtained from two independent experiments.

lead to the activation of these transcription factors. To achieve these goals, we developed a reliable protocol for introducing DNA into neurons. We have used this method to transfect neurons cultured from hippocampus, cortex, striatum, spinal cord, and cerebellum (this study and data not shown). In addition to facilitating the study of gene regulation in neurons, this transfection protocol should also be useful for studies of many other aspects of neuronal function. For example, it should be of use in characterizing the mechanisms controlling intracellular protein targeting and electrophysiological responses in neurons. We have recently applied this transfection method to identify at the single cell level signal transduction pathways involved in neuronal differentiation, survival, and death. For example, transfection of an expression vector encoding the apoptosis-inducing protein interleukin-1-β-converting enzyme caused 85% of transfected cortical neurons to undergo apoptosis (H. Dudek and M. E. Greenberg, unpublished observations).

In this report, we have demonstrated that glutamate induces c-*fos* transcription by an SRE-dependent mechanism in cortical neurons. Glutamate induction of SRE-dependent transcription seems to be mediated by the activation of NMDA receptors, because it is blocked by the NMDA-receptor antagonist APV. There are two distinct pathways by which glutamate activates SRE-mediated transcription. One pathway is SRF-dependent but Elk-independent, because the SRF binding site within the SRE is required for SRE-mediated transcription, and expression of SRF alone, in the absence of Elk, is sufficient to induce significant levels of transcription in response to glutamate. The second pathway is dependent on Elk, because the intact TCF binding site within the SRE is required for a full transcriptional response to glutamate stimulation, and coexpression of Elk-1 further enhances SRE-mediated transcription initiated by SRF.

The SRF-dependent but Elk-independent mechanism of glutamate-mediated transcriptional activation is not used by all extracellular stimuli that can induce c-*fos*. For example, in studies similar to those described here, we have discovered that brain-derived neurotrophic factor stimulates SRE-dependent transcription in cortical neurons by a mechanism that is tightly dependent on the presence of both SRF and Elk (data not shown), as was reported previously for nerve growth factor (NGF) in PC12 cells (Misra et al., 1994; Bonni et al., 1995; Miranti et al., 1995). The SRF-dependent but Elk-independent pathway that we described here may serve as a major mechanism to induce transcription from those SRE-containing promoters that lack a functional TCF binding site. This pathway may also provide a mechanism for the regulation of those genes that are selectively induced by calcium but not neurotrophic factors (Vician et al., 1995).

Although previous work showed that ERKs are activated by glutamate stimulation, the physiological function of ERKs in glutamate signaling was unclear. In this report, we demonstrated that the two ERK phosphorylation sites at the C terminus of Elk-1, Ser^{383}, and Ser^{389} are necessary for the ability of Elk to confer glutamate-induced transcription in neurons. In addition, coexpression of a phosphatase specific for the MAP kinase/ERKs, MKP-1, prevented Elk-1 from enhancing SRF-activated transcription through the SRE. Collectively, these results suggest that glutamate activation of the ERK signaling pathway is an important mechanism for Elk-1-dependent transcriptional activation. Whether other TCF family members, SAP-1 or SAP-2 for example, can mediate glutamate induction of transcription, and whether the ERK signaling pathway is important for the activation of such factors, remains to be determined. The fact that ERKs are found in neuronal cell bodies and dendrites (Fiore et al., 1993a,b), and once activated are believed to translocate to the nucleus (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993), provides supportive evidence for our proposal that ERKs transduce the glutamate signal from the cytoplasm to the nucleus where ERKs regulate gene expression by phosphorylating

nuclear transcription factors such as Elk-1. Because glutamate-stimulated gene expression may be important for synaptic plasticity and other synaptic functions, our results raise the possibility that SRF, Elk, and the MAP kinase/ERKs may play a role in these processes.

Several questions remain to be answered regarding mechanisms of glutamate-regulated transcription in neurons. For example, what are the signaling molecules that couple NMDA receptor activation to the ERKs and to SRE-mediated transcription? It will also be important to characterize the signaling pathways that mediate the SRF-dependent, Elk-independent activation of transcription by glutamate. Interestingly, glutamate treatment of neurons activates Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) (Hanson and Schulman, 1992; Bading et al., 1993; Schulman, 1993). In addition, calcium activates SRF-dependent transcription in PC12 cells by a CaM kinase signaling pathway (Miranti et al., 1995). It will be worthwhile to examine whether a CaM kinase is also involved in glutamate regulation of SRF-dependent transcription in neurons. Furthermore, the Rho family of small GTP binding proteins RhoA, Rac1, and CDC42 have been implicated in regulating transcriptional activation by SRF in fibroblasts (Hill et al., 1995); whether these proteins play a role in glutamate signaling in neurons still needs to be examined.

Another important question that requires further study is how individual transcription factors function in the context of the full c-*fos* promoter. Studies using transgenic mice demonstrated that regulation of c-*fos* expression in many tissues requires the cooperative action of multiple transcription control elements (Robertson et al., 1995). In addition, in transient transfection assays in PC12 cells, SRF was found to cooperate with CREB in inducing c-*fos* expression in response to NGF (Bonni et al., 1995). CREB phosphorylation at $serine^{133}$ is induced when cortical neurons are exposed to glutamate (Fig. 3). It will be useful to determine the role that CREB plays in glutamate-regulated gene expression in neurons.

In summary, by using a modified calcium phosphate transfection method to study gene regulation in primary neurons, we have identified two pathways that couple NMDA-receptor activation to SRE-mediated c-*fos* transcription: an SRF-dependent but Elk-independent pathway and an Elk-dependent pathway. Activation of ERKs seems to transmit the glutamate signal to the nucleus by phosphorylating Elk-1 and initiating Elk-dependent transcription from the SRE. Taken together, these results provide a functional link between activation of ERKs and regulation of glutamate-induced neuronal gene expression. Because some glutamate-regulated immediate early genes (IEGs) such as Zif268 and Krox 20 are also induced by LTP paradigms, and SREs are present within the promoters of these IEGs (Changelian et al., 1989; Chavrier et al., 1989; Christy and Nathans, 1989), the mechanisms described here for the regulation of c-*fos* SRE transcription may be used generally by neurons to regulate gene expression during LTP and other adaptive neuronal responses.

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