ERK2 Prohibits Apoptosis-induced Subcellular Translocation of Orphan Nuclear Receptor NGFI-B/TR3*

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Transcription factor NGFI-B (neuronal growth factor-induced clone B), also called Nur77 or TR3, is an immediate early gene and an orphan member of the nuclear receptor family. The NGFI-B protein also has a function distinct from that of a transcription factor; it translocates to mitochondria to initiate apoptosis. Recently, it was demonstrated that NGFI-B interacts with Bcl-2 by inducing a conformational change in Bcl-2, converting it from protector to a killer (Lin, B., Kolluri, S. K., Lin, F., Liu, W., Han, Y. H., Cao, X., Dawson, M. I., Reed, J. C., and Zhang, X. K. (2004) Cell 116, 527–540). After exposing rat cerebellar granule neurons to glutamate (100 μM, 15 min), NGFI-B translocated to the mitochondria. Growth factors such as the epidermal growth factor activate the MAP kinase ERK, the activity of which may determine whether a cell survives or undergoes apoptosis. In the present study we found that the epidermal growth factor activated ERK2 in cerebellar granule neurons and that this activation prohibited glutamate-induced subcellular translocation of NGFI-B. Likewise, overexpressed active ERK2 resulted in a predominant nuclear localization of green fluorescent protein-tagged NGFI-B. Thus, activation of ERK2 may overcome apoptosis-induced subcellular translocation of NGFI-B. This finding represents a novel and rapid growth factor survival pathway that is independent of gene regulation.

Epidermal growth factor (EGF)1 as a cell survival factor is widely known to initiate the activation of three major signaling pathways, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, the phosphatidylinositide 3-OH kinase/c-Akt kinase cascade, and the phospholipase C (PLC)γ pathway. The importance of the ERK pathway in neuronal cell survival has been well documented in PC12 pheochromocytoma cells where activation of the ERK signaling pathway inhibits apoptosis, whereas the down-regulation of ERK mediated by neuronal growth factor (NGF) withdrawal leads to apoptosis (1). EGF, when added to the culture medium, significantly reduces the glutamate-induced neuronal death in dissociated cerebellar neurons from fetal rats (2, 3) as well as in other neuronal types (4). Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system but also a potent excitotoxin (5). Overstimulation of glutamate receptors has been suggested as being involved during both acute and chronic neurodegeneration in the adult brain (6) and involves cell death by apoptosis (7).

Transcription factor NGFI-B (Nur77 or TR3) is a member of the steroid-thyroid receptor superfamily, also called nuclear receptors (8). NGFI-B was originally identified by virtue of its rapid induction by serum in fibroblasts and by NGF in PC12 (9, 10).

NGFI-B is called an orphan nuclear receptor because of strong sequence homology to nuclear receptors, whereas no specific ligand has been identified (11). Transcription of the immediate early gene NGFI-B is activated transiently, rapidly, and independently of protein synthesis in a variety of cell types by multiple stimuli, including growth factors (12–14). The activity of NGFI-B as a transcriptional activator appears to be under multiple levels of control. First, the mRNA induction varies with different inducing stimuli (15). Second, the NGFI-B protein is differentially modified by multisite phosphorylation in response to different stimuli (12, 16–19), which probably is important for modulating the cellular response to environmental change. The NGFI-B protein also has a function distinct from that of a transcription factor; it translocates to mitochondria to initiate apoptosis (20). Recently, it was demonstrated that NGFI-B interacts with Bcl-2 by inducing a conformational change in Bcl-2, converting it from protector to a killer (21). The importance of NGFI-B in the apoptotic process was demonstrated earlier in knock-out experiments in T-cell hybridomas (22, 23).

EGF stimulation has been shown to lead to phosphorylation of NGFI-B in PC12 cells (12). In addition, NGFI-B is a direct substrate for the MAP kinase ERK2 in vitro (19).

The objective of the present study was therefore to investigate whether EGF may use the ERK2 survival pathway to cross-talk to the apoptosis initiator NGFI-B. Specifically, we have asked if ERK2-mediated phosphorylation of NGFI-B may be involved in the subcellular translocation of NGFI-B in neuronal cells and if this occurs in the presence of apoptosis induced by glutamate. Furthermore, we have overexpressed the ERK2 protein and mimicked the effect of EGF stimulation on NGFI-B translocation.

EXPERIMENTAL PROCEDURES

Basal Eagle’s medium, Dulbecco’s modified Eagle’s medium with 25 mM HEPES, and fetal bovine serum were obtained from Invitrogen. Poly-l-lysine, gentamicin sulfate, cytosine arabinofuranoside, trypan blue, glutamine, and 4,6-diamidino-2-phenylindole (DAPI) were from Sigma. The Nur77 primary antibody (goat) and the heat shock protein-60 (HSP60) primary antibody (rabbit) were obtained from Santa Cruz Biotechnology.
Cy-3-conjugated mouse anti-goat and Cy-2-conjugated mouse anti-rabbit antibodies were from Jackson Immunoresearch Laboratories (West Grove, Pennsylvania). The phospho-p44/42 MAPK monoclonal antibody was obtained from Amersham Biosciences. MEK inhibitor U0126 was obtained from Promega. Pure nitrocellulose membrane and the horseradish peroxidase-linked goat anti-mouse antibody were from Bio-Rad.

Cell Culture—Cerebellar granule neurons were obtained from 7–8-day-old albino rats (24, 25). Neurons were seeded on plastic dishes coated with 20 μg/ml poly-l-lysine (cell density of 2 × 10^6 cells/cm^2) and cultured in basal Eagle’s medium supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin. To prevent the growth of non-neuronal cells, cytotoxic arabinofuranoside (10 μM final concentration) was added to the cultures 16–18 h after seeding. After 7–8 days in vitro, cultures were treated with 100 μM glutamate at room temperature for 15 min in physiological buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, 5.6 mM glucose, and 5 mM HEPES, pH 7.4). At the end of glutamate exposure, cultures were washed and incubated in serum-free basal Eagle’s medium (25 mM KCl). Control cultures were exposed to physiological buffer without glutamate for 15 min at room temperature before washing and incubation in serum-free basal Eagle’s medium (25 mM KCl). In some cultures EGF (5 ng/ml) was present for 15 min (in physiological buffer) before and during the exposure of the cells. The procedure for handling animals used for cell cultures was approved by the appropriate animal care committee of the Norwegian government.

CV1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. EGF (5 ng/ml) was added directly to the medium. In experiments with the MEK inhibitor U0126, the cells were pretreated with the inhibitor in the medium for 60 min, followed by co-treatment with EGF.

Immunofluorescence Detection of NGF1-B and HSP60—Cerebellar granule cells or CV1 cells were washed twice with ice-cold phosphate-buffered saline and harvested in 2% SDS. Equal amounts of cell lysates were mixed and boiled with 2× Laemml buffer reduced with 2-mercaptoethanol and subjected to electrophoresis on a 10% polyacrylamide gel. Proteins were transferred onto pure nitrocellulose membrane. After protein transfer, the membranes were incubated in TBS-T (100 mM Tris-HCl, 100 mM NaCl, and 0.1% Tween 20) with 5% nonfat milk and an antibody recognizing the MAP kinase in activated form, i.e. specific for ERK (p44 and p42 MAPK) phosphorylated at Thr-202 and Tyr-204 (diluted 1:2000) followed by a horseradish peroxidase-linked goat anti-mouse antibody (diluted 1:1500). Immunoreactive bands were visualized with ECL Plus.

ERK kinase activity was measured as described (26, 27). In short, the cells were rinsed twice in saline and scraped into a buffer containing 25 mM Tris, pH 7.4, 25 mM NaCl, 1.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μM leupeptin, 1 μM pepstatin A, 2 mM dithiothreitol, 1 mM NaO₃V, and 10% (v/v) ethylene glycol. The lysate was centrifuged (15,000 × g) for 10 min. The supernatant was mixed with phenyl-Sepharose (Amersham Biosciences) and washed twice with each of the buffers containing 10 and 35% ethylene glycol before elution of the ERK kinases with 60% methanol. The eluate was assayed for kinase activity in the presence of an inhibitor of protein kinase A (protein kinase inhibitor Sigma P-0300) using the myelin basic protein as the substrate.

Transient Transfection of CV1 Cells—A calcium phosphate precipitation procedure was used for transient transfections. CV1 cells were co-transfected with 1 μg of the expression plasmid NGFI-B-gfp chimera (generated by insertion of NGFI-B cDNA fused to the green fluorescent protein, amplified by 

FIG. 1. Glutamate induces cell death that may be reduced by EGF in rat cerebellar granule neurons. A, rat cerebellar granule neurons (7 days in vitro) were treated with buffer, glutamate (100 μM), or EGF (5 ng/ml). The results are given as the percentage of cell death at 24 h as measured by a trypan blue exclusion assay. Values are mean ± S.E. from four experiments. *, p < 0.05, comparing EGF plus glutamate to glutamate alone. B, rat cerebellar granule cells (7 days in vitro) were treated with EGF (5 ng/ml) or EGF in the presence of the MEK inhibitor U0126 (10 μM) and harvested after 2 h. Western blots were detected using a phospho-p44/42 MAPK monoclonal antibody (p44mapk/p42mapk).

FIG. 2. NGF1-B redistributes to the same compartment as HSP60 when rat cerebellar granule neurons are treated with glutamate. Rat cerebellar granule cells (7 days in vitro) were fixed in 4% paraformaldehyde 2 h after treatment. A, cells were DAPI-stained and immunostained with an anti-Nur77 antibody followed by a Cy-3 conjugated secondary antibody. B, rat cerebellar granule cells were double-immunostained with an anti-Nur77 antibody and an anti-HSP60 antibody followed by Cy-3 and Cy-2 conjugated secondary antibodies. Con, control cells treated with buffer alone; Glu, glutamate-treated (100 μM) cells. A, shows a cell using phase contrast (PC) followed by NGF1-B and DAPI staining. B, shows the overlay of NGF1-B and DAPI staining. Right picture shows the overlay of NGF1-B and HSP60 staining.
protein (gfp) sequence from pEGFP-N1 (Clontech) into the pQBI-Ad-CMV5 mammalian transfection vector (QBI, Fremont, CA) and 5 μg of one of the three ERK2 plasmids, namely pCMV5 ERK2 wild type, pCMV5 ERK2 K52R dominant negative, or pCMV5 ERK2-MEK1R4F dominant active (kindly provided by Dr. M. Cobb, University of Texas, South Western Medical Center, Dallas, TX). Pictures were captured using a Nikon Eclipse TE300 microscope using a SPOT RT Color camera and SPOT software.

Statistics—Statistical differences between multiple comparisons were analyzed by one-way repeated measures analysis of variance followed by a subsequent Dunnett’s post hoc test (SigmaStat software). A p value of <0.05 was considered significant.

RESULTS

EGF Protects Rat Cerebellar Granule Neurons from Cell Death Induced by Glutamate—To find out if EGF protects rat granule neurons from cell death, we treated cells with glutamate in the absence and presence of EGF. EGF significantly reduced the cell death induced by glutamate when EGF was present at the time of glutamate exposure (Fig. 1A). Cerebellar granule cells were treated with EGF or EGF in the presence of the MEK inhibitor U0126 to see if EGF turned on the MEK/ERK pathway. ERK showed some phosphorylation in control cells; however, when EGF was added the phosphorylation of ERK was increased (Fig 1B). The MEK inhibitor U0126 blocked this ERK activation.

NGFI-B Translocates When Rat Cerebellar Granule Neurons Are Treated with Glutamate and EGF—In rat cerebellar granule cells that were untreated, NGFI-B co-stained with DAPI. Cerebellar granule cultures that were treated with glutamate showed NGFI-B in a different compartment than DAPI (Fig. 2A). In this case, NGFI-B co-stained with the mitochondrial marker HSP60 (Fig. 2B).

When EGF was added to glutamate-treated cells, the majority of the cells showed NGFI-B staining similar to that of control cells. By adding the MEK inhibitor U0126, EGF no longer inhibited glutamate-induced translocation of NGFI-B (Fig. 3). Interestingly, cells treated with EGF expressed brighter staining than did control at 2 h. This brighter staining was diminished by adding U0126, indicating an ERK-dependent induction of the NGFI-B protein.
We wanted to address the question of whether ERK2 was responsible for this translocation of NGFI-B. We co-transfected gfp-tagged NGFI-B together with ERK2, dominant negative ERK2 (ERK2K52R), and dominant active ERK2 (ERK2-MEK1R4F).

The cells co-transfected with gfp-tagged NGFI-B and ERK2 showed NGFI-B distribution in the entire cell. 10 min after adding EGF this distribution changed to show NGFI-B mainly in the nucleus. After 60 min following EGF addition, NGFI-B was distributed throughout the cell again (Fig. 5B).

CV1 cells co-transfected with gfp-tagged NGFI-B and dominant negative ERK2 showed a distribution of NGFI-B in the entire cell before adding EGF. EGF did not affect this distribution either at 10 min or at 60 min. CV1 cells co-transfected with gfp-tagged NGFI-B and dominant active ERK2 showed a distribution of NGFI-B in the nucleus before adding EGF. EGF treatment did not affect this nuclear location (Fig. 5B).

**DISCUSSION**

In this communication we show that EGF activates ERK2, which prohibits apoptosis-induced subcellular translocation of the orphan nuclear receptor and apoptosis initiator NGFI-B. Glutamate produces an insult-dependent continuum of apoptosis and necrosis in cultured cortical neurons, where necrosis predominates after intense insults but mild insults result in apoptotic cell death (28). In our experiments we treated the cells with 100 μM glutamate, which is a mild insult and thus initiates apoptosis rather than necrosis (29).

EGF treatment of the cerebellar granule neuron cultures protected the cells against apoptosis induced by glutamate when present during the apoptotic signal. Earlier studies have shown that EGF promotes the survival of primary cultured neurons from various regions of the brain (3, 4). When EGF was added to the culture medium 20 h prior to the addition of glutamate, glutamate-induced cell death was significantly reduced. EGF activates the MAP kinase pathway in rat cerebellar neurons, and it has been shown that activated ERK1/2 translocates into the nucleus to regulate gene expression by either direct or indirect phosphorylation (30). In contrast to the present study, these studies did not find any reduction in cell death when EGF was added only 1 h before glutamate was added. In cultured cortical neurons, EGF protected effectively against ischemia when administered 24–6 h prior to the ischemic insult, whereas the addition of EGF at the time of ischemia provided only modest protection (31). These studies indicate that the neuroprotection seen by EGF treatment in some neurons was mainly gene-regulated. In our study EGF treatment at the time of glutamate treatment resulted in a 50% protection, demonstrating the presence of a protective mechanism that is rapid and independent of prior gene regulation.

EGF stimulation has been shown earlier to lead to phosphorylation of NGFI-B in PC12 cells (12). The NGFI-B protein is modified by multisite phosphorylation in response to different stimuli (12, 16–18). This phosphorylation takes place predominantly in the amino terminus (17). Three specific phosphorylation sites have been identified. Phosphorylation of a residue in the DNA-binding region has been described in detail as having an effect on the transcriptional function of the protein (17, 32). Recently, an NGF-induced phosphorylation in the amino terminus of NGFI-B was characterized. This phosphorylation resulted in translocation of NGFI-B out of the nucleus (18). Another phosphorylation in the amino terminus of NGFI-B has been described and is mediated by ERK2, as demonstrated by phosphorylation experiments using ERK2 (mouse pp42mapk) in vitro. Recombinant ERK1 (human pp42mapk) was not able to phosphorylate NGFI-B (19). This ERK2-specific phosphorylation has later been verified in vivo (33). NGFI-B is phosphoryl-
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When rat cerebellar granule neurons were treated with glutamate, NGFI-B translocated to the mitochondria. After adding EGF to the medium, this translocation was blocked. By introducing the MEK inhibitor U0126 to the rat cerebellar granule cells, the effect of EGF on glutamate-induced translocation was eliminated. Interestingly, our results suggest that NGFI-B is induced as well as directly regulated by EGF. An increased level of the NGFI-B protein may alert the cells to respond by proliferating. NGFI-B has been reported to be necessary for apoptosis in different cell types (20, 22, 23). Mitochondrial targeting of NGFI-B induces apoptosis in a human prostate cancer cell line (20). Recently, it was demonstrated that NGFI-B interacts with Bel-2 by inducing a conformational change in Bel-2, converting it from a protector to a killer (21). NGFI-B also has a role in alternative, non-apoptotic programmed cell death (33). NGFI-B functions in the nucleus to induce proliferation, whereas it acts on mitochondria to induce apoptosis (35). MEK kinase 1 was shown to activate c-Jun amino-terminal kinase, which phosphorylated TR3, reduced its DNA binding activity, and resulted in loss of mitogenic function. The present report adds a new pathway to the sorting of NGFI-B between subcellular compartments.

In conclusion, these results suggest that EGF may exert rapid neuroprotection in rat cerebellar granule cells through the MEK/ERK pathway by the effect of ERK2 on NGFI-B localization. Thus, activation of ERK2 may overcome apoptosis-induced subcellular translocation of NGFI-B. This finding represents a novel and rapid growth factor survival pathway that is independent of gene expression.

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