Opposing Roles of Elk-1 and Its Brain-specific Isoform, Short Elk-1, in Nerve Growth Factor-induced PC12 Differentiation

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The ternary complex factor Elk-1, a major nuclear target of extracellular signal-regulated kinases, is a strong transactivator of serum-responsive element (SRE) driven gene expression. We report here that mature brain neurons and nerve growth factor (NGF)-differentiated PC12 cells also express a second, smaller isoform of Elk-1, short Elk-1 (sElk-1). sElk-1 arises from an internal translation start site in the Elk-1 sequence, which generates a protein lacking the first 54 amino acids of the DNA-binding domain. This deletion severely compromises the ability of sElk-1 to form complexes with serum response factor on the SRE in vitro and to activate SRE reporter genes in the presence of activated Ras. Instead, sElk, but not a mutant that cannot be phosphorylated, inhibits transactivation driven by Elk-1. More pertinent to the neuronal-specific expression of sElk-1, we show it plays an opposite role to Elk-1 in potentiating NGF-driven PC12 neuronal differentiation. Overexpression of sElk-1 but not Elk-1 increases neurite extension, an effect critically linked to its phosphorylation. Interestingly, in the presence of sElk-1, Elk-1 loses its strictly nuclear localization to resemble the nuclear/cytoplasm pattern observed in the mature brain. This is blocked by mutating a normally cryptic nuclear export signal in Elk-1. These data provide new insights into molecular events underlying neuronal differentiation of PC12 cells mediated by the NGF-ERK signaling cascade.

Intracellular signaling mechanisms regulate many processes, including cell proliferation, specification of cell fate, and differentiation. One universally used signaling pathway is the ligand-induced activation of receptor tyrosine kinases and their downstream signaling cascade, leading to the activation of the extracellular signal-regulated kinases (ERKs), also known as mitogen-activated protein kinases (MAPKs). ERKs propagate signals through the phosphorylation of a wide range of proteins, including other enzymes, proteins of the cytoskeleton, and transcription factors (1). In particular, activated ERKs translocate to the nucleus, where they can activate transcription factors and thereby regulate gene expression.

Elk-1, a major nuclear target of activated ERK proteins, is a member of the ternary complex factor (TCF) family that also includes Sap1 and NET/ERAP2/Elk-3 (2–7). In cultured cell lines Elk-1 functions as a nuclear transcriptional activator via its association with serum response factor (SRF) in a ternary complex on the serum response element (SRE) present in the promoter of many immediate early genes (c-fos, c-jun, egr1, egr2, pip92, and nurr77) (8). Elk-1 has three major functional domains as follows: the N-terminal Ets-DNA binding domain that recognizes the 5'-CAGGA motif of the SRE (4), a motif of 20 amino acids (the B domain) that mediates protein-protein interaction with SRF (9–11), and the C-terminal part of the protein, which contains consensus phosphorylation sites for ERK but also the closely related MAPKs c-Jun N-terminal kinase/stress-activated protein kinase and p38 (12). These cascades are activated by different extracellular signals, principally mitogens for ERKs (13) and various stresses for c-Jun (14). Upon activation MAPKs can translocate to the nucleus (16) where they phosphorylate Elk-1 on Ser389 and Ser358, which strongly potentiates SRE-dependent gene expression (6, 17–28).

We previously reported that Elk-1 was expressed, in postmitotic neurons, in both nuclear and cytoplasmic compartments (29). In the process of investigating the functional significance of this unexpected subcellular localization, we discovered a novel isoform of Elk-1, sElk-1 for short Elk-1, the expression of which is linked to the neuronal phenotype, determined in neuronal tissue as well as in NGF-differentiated PC12 cells. This isoform arises from an internal translation start site in the Elk-1 sequence and corresponds to a truncated protein lacking the first 54 amino acids of the DNA binding domain. We have investigated the molecular and functional properties of sElk-1 relative to Elk-1, and we showed they have opposite roles in the following: (i) SRE-driven gene expression and (ii) NGF-induced PC12 differentiation. Thus sElk-1 seems to be a key mediator of NGF/ERK signaling cascade specifically in neuronal cells.
EXPERIMENTAL PROCEDURES

**Immunohistochemistry**—The immunohistochemical procedure was adapted from protocols described previously (29). Briefly, free-floating sections were incubated (after rinse and saturation steps) for 72 h at 4 °C with the primary antibodies. Rabbit polyclonal antisera raised against Elk-1 (Santa Cruz Biotechnology), Zif268 (Santa Cruz Biotechnology), and STAT3 (New England Biolabs) were used at a dilution of 1:500. After rinsing the blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated antibody (1:5000, Amersham Pharmacia Biotech) before exposure to the ECL kit (Amersham Pharmacia Biotech).

**RNA Isolation and Analysis by Northern Blots**—Total RNA was isolated from mouse and rat brains using TRIzol reagent (Life Science macia Biotech).

**Statistical Analysis**—Statistical analysis was performed using StatView (SAS Institute) using unpaired Student's t test with Bonferroni correction for multiple comparisons.

**RESULTS**

**In Vitro Kinase Assays and Gel Retardation Analysis**—The reactions contained 1.0 μg of in vitro translated protein in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM EDTA, 100 μM ATP, 0.01% Brij 35, 10 μM β-glycerophosphate, 5 mM 4-nitrophenyl phosphate, and 2.5 μg/ml each of aprotinin, leupeptin, pepstatin, and antipain. Where indicated, the reaction contained 20 ng of purified and activated recombinant Erk2 (Upstate Biotechnology Inc.). After a 30-min incubation at 30 °C, the proteins were incubated under DNA binding conditions with a 32P-labeled probe containing one copy of the c-fos SRE as described elsewhere (3). In brief, reactions (7 μl) contained 0.2 ng (4 fmol) of a calf thymus DNA probe mixture, 2.5 μg/ml of poly(dI-dC), recombinant core SRF-(90–245), and 2.5 μl of the appropriate control or kinase reaction described above. After incubation for 30 min at room temperature, complexes were resolved on a 5% polyacrylamide gel containing 0.5% TBE at 1 mA/cm for 4 h. Complexes were visualized by autoradiography at room temperature and by using intensifying screens at ~70 °C.

**Luciferase Assays**—NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc.) and plated 16 h before transfection to ~30% confluence. Medium was changed 1 h before transfection, and cells were subsequently transfected with the CaPO4 method. Typically, 300 ng of 3xSRE luciferase vector, 60 ng of cytomegalovirus Ras70 of Elk-1, or 160 ng of Elk-1(1AAG1) expression vectors were cotransfected with 5 ng of SV40 Renilla luciferase expression vector and complemented to 5 μg with nonspecific DNA. 8–10 h after transfection, cells were harvested in Dulbecco’s modified Eagle’s medium without serum for 4 h, at which point cells were harvested, and luciferase activities were determined according to the supplier’s protocol (Dual Luciferase, Promega).

**Full-length Assays**—[35S]Labeled Elk-1 protein were synthesized by coupled in vitro transcription/translation in the presence of 1 mCi of [35S]methionine (1000 Ci/mmol) using the TNT-coupled reticulocyte lysate system (Promega) according to the supplier’s recommendations. GST, GST-CBP-(1100–1286), and GST-CBP-(1460–1891) (34, 35) were expressed in bacteria, then purified by metal affinity chromatography, and then directly bound to glutathione-Sepharose beads at 4 °C for 1 h. The beads were then washed three times with a 100-fold excess of RJD buffer (10 mM HEPES, pH 7.9, 5 mM MgCl2, 50 mM NaCl, 17% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05% Nonidet P-40) containing freshly added protease inhibitors (2.5 μg/ml aprotinin, leupeptin, pepstatin, 0.5 mM benzamidine, 0.5 μM phenylmethanesulfonyl fluoride) and stored short term at 4 °C. 5 μl of in vitro translated Elk-1 was added to 10 μl of a 50% slurry of protein bearing beads in a total volume of 100 μl of RJD buffer and incubated with gentle agitation at 4 °C for 4 h. After three washes with 500 μl of RJD buffer, the beads were collected, resuspended in 20 μl of 5% Laemmli buffer (0.025x Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue), denatured for 5 min at 95 °C, and bound proteins analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were visualized by Coomassie staining, followed by autoradiography of the dried gels.

**Immunofluorescence**—PC12 cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature, and stained with a monoclonal antibody raised against hemagglutinin (HA) (1:1000, Roche Molecular Biochemicals) for 1 h at room temperature. Coverslips were then rinsed in PBS and incubated for 1 h at room temperature with an anti-mouse Cy3-conjugated antibody (1:500, Sigma).
Western blotting with extracts were fractionated into nucleus and cytoplasm and analyzed by sElk-1 corresponding to a small isoform of Elk-1. The apparent molecular weight of Elk-1 protein and the other at 45 kDa. The Elk-1 C-terminal antibody reveals two bands, one at the substantia nigra pars reticulata (SNr), cerebellum, and substantia nigra pars reticulata (SNr). The Elk-1 C-terminal antibody reveals two bands, one at the apparent molecular weight of Elk-1 protein and the other at 45 kDa, corresponding to a small isoform of Elk-1 (sElk-1). C and D, cerebral extracts were fractionated into nucleus and cytoplasm and analyzed by Western blotting with Zif268 and STAT3 antibodies (C) and an Elk-1 C-terminal antiserum (D).

Immunocytochemical analyses, the presence of Elk-1 in both nuclear and cytoplasmic compartments in mature neurons (compare Fig. 1A) illustrated in Fig. 1A). A similar subcellular distribution of Elk-1 was found using various antibodies directed against N-terminal and C-terminal parts of Elk-1 (29). This contrasts with cultured cells, where Elk-1 is localized predominantly, if not exclusively, to the nucleus (36) and its activation depends on ERK nuclear translocation (32). This led us to characterize further the expression pattern of sElk-1 versus Elk-1, we analyzed Western blots from cortical extracts as well as various peripheral tissues and cell lines. Although the full-length protein was present in all tissues tested, sElk-1 was exclusively observed in cerebral tissues (Fig. 2A). Notably, sElk-1 was not detectable in either undifferentiated PC12 cells or in the cell lines MMQ, COS7, and NIH3T3 (Fig. 2A). Taken together, these data indicate that sElk-1 is specifically expressed in cerebral tissues. Since Elk-1 proteins and mRNAs are restricted to neuronal but not glial cells (29), sElk-1 seems to be linked to the neuronal phenotype.

Upon NGF treatment, PC12 cells acquire a phenotype that resembles sympathetic neurons, with large neuritic processes (compare Fig. 2, B and C). We reasoned that if sElk-1 expression is characteristic of the neuronal phenotype, it should appear in NGF-treated PC12 cells. Indeed, Western blot analysis shows that extracts prepared from NGF-treated but not untreated PC12 cells contain a version of sElk-1 that co-migrates with the version present in brain extracts (Fig. 2D).

An Internal AUG Codon Is Used to Generate sElk-1—The neuronal specific expression of sElk-1 could arise through a number of different mechanisms. One, the activation of neuronal specific gene corresponding to sElk-1, is not supported by extensive data bank queries or genomic hybridization results (31). The second possibility was an alternative spliced version of Elk-1 mRNA capable of encoding sElk-1. However, RNase protection analysis (6) and Northern blot analysis of total and poly(A)-enriched RNA from brain tissues did not reveal an mRNA that would correspond to an alternative spliced version capable of encoding this smaller form of Elk-1.

2 R. A. Hipskind, unpublished results.
MetC, 55

5192 (Met55) and a leucine-rich sequence (residues 7–16) upstream from DNA binding domain of Elk-1 contains a methionine at position 55

Elk-1: (i) the DNA binding domain (or Ets domain), (ii) the domain that extracts.

B, schematic representation of the three functional domains of Elk-1: (i) the DNA binding domain (or Ets domain), (ii) the domain that mediates interaction with the cofactor SRF, and (iii) the C-terminal regulatory domain, which contains residues targeted by MAPKs. The DNA binding domain of Elk-1 contains a methionine at position 55 (Met55) and a leucine-rich sequence (residues 7–16) upstream from Met55. C, schematic diagrams of the five expression vectors used to generate Elk-1 proteins in vitro. Elk-1 WT contains the wild type sequence of Elk-1 cDNA with the two in-frame ATGs (bold; Elk-1(AAG1) and Elk-1(AAG2)) contain the first or the second ATG converted to AAG, respectively. Elk-1 (Kozak1) contains the first ATG in a perfect Kozak consensus context. Elk-1 (NoKozak2) contains the second ATG in a poor Kozak consensus context. The dotted lines represent nucleotides sequence of the 5’- 3’-untranslated regions of Elk-1 cDNA. D, autoradiogram obtained after in vitro coupled transcription-translation of Elk-1 constructs and immunoprecipitation (IP) with a C-terminal Elk-1 antibody.

Therefore, we investigated the possibility of an alternative translation start site, since there is some precedent for this in neuronal cells (37, 38). The Met residue located at position 55 could serve as an internal translation initiation site (see Fig. 3B). In vitro transcription translation from an Elk-1 WT expression vector generated two protein products immunoprecipitated with the C-terminal Elk-1 antibody. One corresponded to Elk-1 at 52 kDa, whereas the other migrated at the same molecular weight as sElk-1 (45 kDa) (Fig. 3D, lane 1). To confirm that these two proteins arose from initiation at one or the other AUG codon, we mutated these two ATGs to lysine-encoding AAGs termed Elk-1(AAG1) and -(AAG2), respectively (Fig. 3C). The Elk-1(AAG1) mutant template failed to generate the full-length protein in vitro (Fig. 3D, lane 2), and the Elk-1(AAG2) mutant did not yield the 45-kDa protein (Fig. 3D, lane 3). We note that these two templates give rise to higher levels of translated proteins than Elk-1 WT which encodes two protein products.

Different mechanisms can account for internal initiation of translation. First, cap-dependent mechanisms including leaky ribosome scanning, where ribosomes ignore potential start sites presumably because the flanking sequence (the Kozak sequence) deviate from the rules governing faithful initiation (39). Another cap-dependent mechanism is discontinuous scanning or ribosomal shunting (40). Last are cap-independent mechanisms directed by an internal ribosomal entry site, where the ribosomal complex fails to attach to the 5’ cap. The first ATG codon in Elk-1 cDNA is surrounded by an imperfect Kozak sequence (TAGTGATGG); thus we mutated the sequence surrounding the first ATG to a perfect Kozak consensus (Kozak1, CCACCATGG) (Fig. 3C). In vitro translation of this plasmid generates high levels of the two Elk-1 products (Fig. 3D, lane 4), suggesting that sElk-1 was not generated by the leaky ribosome scanning model. In an effort to understand further how sElk-1 was generated, we then noticed that the sequence surrounding the second ATG (CCACCATGA) was in a highly favorable Kozak context (7 nucleotides over 9) to drive initiation of sElk-1. To address this, nucleotides surrounding the second ATG were mutated to obtain a “poor” context (NoKozak2, GGCAGATGA) (Fig. 3C). In this case, we completely abrogated in vitro expression of sElk-1 (Fig. 3D, lane 5). Thus, the Kozak consensus played a key role in the mechanisms of initiation at the second AUG codon (Met55).

The Subcellular Localization of Elk-1 Is Affected by sElk-1 Expression—We generated expression vectors with an N-terminal HA tag for Elk-1 WT, Elk-1(AAG2), or Elk-1(AAG1) and a GFP tag for Elk-1(AAG2) constructs. As expected, HA-Elk-1 WT encodes both isoforms, as shown by immunoprecipitation with Elk-1 antibody (Fig. 4A), but only the full-length protein bears the HA epitope (Fig. 4B). The other expression vectors encode exclusively HA-tagged (Fig. 4, A and B) or GFP-tagged (data not shown) full-length Elk-1 or sElk-1. Thus, these constructs allowed us to follow Elk-1 and sElk-1 (encoded by Elk-1(AAG2) and -(AAG1), respectively) by immunofluorescence in transfected PC12 cells. When expressed alone, both sElk-1 and Elk-1 were predominantly nuclear with the HA tag (Fig. 4C) or GFP tag (data not shown). However, coexpression of both iso-
forms, using either HA-Elk-1 WT (Fig. 4C) or cotransfection of both HA-Elk-1(AAG1) and GFP-Elk-1(AAG2) cDNAs (Fig. 4D), led to relocalization of Elk-1 to the cytoplasm. This phenomenon is linked to a leucine-rich sequence (7LWQFLLQLLR) in Elk-1 (see Fig. 3B) that resembles a nuclear export signal (NES), since mutating this sequence caused Elk-1 to remain in the nucleus in the presence of sElk-1 (Fig. 4C). These data suggest that the expression of sElk-1 relocates Elk-1 intracellularly and help explain the different subcellular localization of Elk-1 we observed in neuronal versus other cell types.

Molecular Properties of sElk-1—Deletion of the first 54 amino acids in sElk-1 removes a major portion of the DNA binding domain (see Fig. 3D) but leaves one α-helix in place, which in both Elk-1 and the highly related TCF Sap-1a contacts the major groove of DNA (41, 42). TCFs bind to the SRE through their interaction with the SRF dimer, an interaction that might suffice to stabilize sElk-1 binding. To investigate this, we tested whether in vitro translated sElk-1 formed ternary complexes together with SRF on the c-fos SRE. In vitro translated Elk-1 WT extracts generate the typical ternary complex (Elk-1 zcore SRF2 zSRE) (Fig. 5A). Similarly, the AAG2 mutant bound DNA, although more weakly than WT (data not shown). We failed to detect this complex in extracts from in vitro translated Elk-1(AAG1) (red) with 2 μg of GFP-tagged Elk-1(AAG2) (green). The nucleus of the transfected cell is pointed by an arrowhead in the Hoechst-staining panel (blue). Note the cytoplasmic localization of GFP-Elk-1(AAG2) in the presence of an excess of HA-Elk-1(AAG1).

**Fig. 4. sElk-1 expression drives Elk-1 within the cytoplasm.** A and B, in vitro coupled transcription translation from expression vectors in which a HA tag was fused to the N-terminal part of Elk-1(AAG2), Elk-1 WT, or Elk-1(AAG1). A, in vitro translated proteins were immunoprecipitated (IP) with a C-terminal Elk-1 antibody. B, immunoprecipitation with the HA antibody; note that Elk-1 but not sElk-1 is immunoprecipitated in the case of HA-Elk-1 WT. C, PC12 cells were transiently transfected with 10 μg of HA-Elk-1(AAG1), HA-Elk-1(AAG2), HA-Elk-1 WT, or HA-Elk-1 NES and treated with NGF (100 ng/ml) for 24 h before fixation and immunofluorescent detection of the HA tag (red). Nuclei were visualized on the same field by Hoechst counterstaining (blue). Nuclei of HA-immunoreactive cells (left panels) are pointed by an arrowhead. For each construct, 50–100 transfected cells with cytoplasmic and nuclear staining (black bars) or nuclear staining (gray bars) were counted from 3 independent experiments. D, cotransfection of 10 μg of HA-tagged Elk-1(AAG1) (red) with 2 μg of GFP-tagged Elk-1(AAG2) (green). The nucleus of the transfected cell is pointed by an arrowhead in the Hoechst-staining panel (blue). Note the cytoplasmic localization of GFP-Elk-1(AAG2) in the presence of an excess of HA-Elk-1(AAG1).

**FIG. 5.**
with sElk-1 (Elk-1(AAG1) mutant) (Fig. 5A). These data demonstrate that sElk-1 interacts poorly with SRF on the c-fos SRE upon ERK induction.

We then used transient transfection assays to evaluate the effect of sElk-1 on SRE reporter gene activity. To potentiate DNA binding and possible transregulation by sElk-1, we co-transfected constitutively active RasVal-12 to generate activated ERK. The transfections contained this expression vector, the SRE reporter construct, and either Elk-1 WT or Elk-1(AAG1). SRE-driven gene expression was increased by transfecting RasVal-12 alone, likely reflecting activation of endogenous TCF (Fig. 5B, 2nd lane). Whereas overexpression of Elk-1 WT potentiated SRE activity driven by RasVal-12, sElk-1 reduced this activity (Fig. 5B, 3rd lane). Furthermore, the introduction of a large excess of the sElk-1 vector significantly diminished the activity of Elk-WT on SRE-driven gene expression, an effect reversed with the Ser383/389 to Ala mutant (Fig. 5B, compare 5th lane with 6th lane).

Given the low binding properties of sElk-1 on SRE (see Fig. 5A), we asked how sElk-1 inhibited Elk-1 properties on SRE-driven gene expression. One possibility was that sElk-1 interacts with the coactivator CBP, which is necessary for optimal SRE-driven gene expression by Elk-1 (35). Pull-down experiments performed with the bromodomain of CBP clearly showed that both Elk-1 and sElk-1 interact with this region in vitro (Fig. 5C). Thus, altogether these data suggest that sElk-1 can act as a transcriptional modulator not by competing with Elk-1 on SRE but for interaction with CBP.

Opposing Roles of sElk-1 and Elk-1 in NGF-induced PC12 Differentiation—The pheochromocytoma cell line PC12 has served as a model system to analyze intracellular mechanisms underlying neuronal differentiation. Upon nerve growth factor (NGF) treatment, PC12 cells stop proliferating and acquire a phenotype characteristic of sympathetic neurons including extension of neurites (43). NGF-driven PC12 differentiation critically depends on ERK signaling to Elk-1 (44). This lead us to examine the respective roles of Elk-1 and sElk-1 in NGF-driven PC12 differentiation. We transfected PC12 cells with expression vectors coding for green fluorescent protein (GFP) and either Elk-1 WT, Elk-1(AAG1) or Elk-1(AAG2). Neuronal differentiation was evaluated by scoring GFP-positive cells with neuritic extensions longer than the cell body 48 and 96 h after transfection as described previously (32, 33). The experiments were performed in the presence of NGF. After NGF addition, 30% of PC12 cells transfected with GFP alone were differentiated (data not shown). Overexpression of sElk-1 significantly increased the number of differentiated cells (Fig. 6H) in the presence (Fig. 6B, Elk-1 WT construct) or absence (Fig. 6D, Elk-1(AAG1) construct) of Elk-1. Elk-1 alone (Fig. 6F, Elk-1(AAG2) construct) failed to potentiate NGF-induced differentiation (Fig. 6H).

Since phosphorylation of Elk-1 and sElk-1 are critical for their molecular properties, we tested the effect of overexpressing the same proteins inactivated by mutating Ser383/389 to Ala. Inactive sElk-1 (Fig. 6, C–E) no longer potentiated neurite extension (Fig. 6F), in direct contrast to Elk-1 (Fig. 6G), where the inactivating mutations led to a significant increase in neuronal differentiation (Fig. 6F).

In conclusion, these data demonstrate that overexpression of sElk-1 alone at early stages of NGF treatment facilitates neuronal differentiation, an effect that is critically linked to its capacity to be activated by ERKs. They also indicate that Elk-1 and sElk-1 play opposite roles in neuronal differentiation.

DISCUSSION

We describe a novel, neuronal-specific isoform of Elk-1, sElk-1, that originates from translation initiation at an alternative downstream start site in Elk-1 mRNA. Whereas viruses commonly produce several variants of a protein through internal initiation on the same transcript, this mechanism is rarely documented in vertebrate cells (45). Instead, alternative splic-
s-Elk-1 Potentiates Neuronal Differentiation

**Fig. 6. Overexpression of sElk-1 facilitates PC12 neuronal differentiation.** A–G, PC12 cells were transiently transfected with 2 μg of GFP alone (A) or in the presence of 10 μg of each Elk-1 construct: Elk-1WT (B), Elk-1WT 2Ala (C), Elk-1(AAG1) (D), Elk-1(AAG1) 2Ala (E), or Elk-1(AAG2) (F), and Elk-1(AAG2) 2Ala (G) (magnification, × 400). H, after 48–96 h of treatment with NGF (100 ng/ml), GFP-positive cells (green) were counted, and the number of cells with neurites were scored. To be counted, the neurite had to be longer than the cell body. Similar results were found after 48 and 96 h of transfection. Results are expressed as percent increase of neuronal differentiation when compared with cells transfected with GFP alone (n = 4 independent experiments at least for each group of transfection). Statistical comparisons: ***p < 0.001, unpaired Student’s t test when comparing GFP alone with GFP + another construct; Δ, p < 0.05 when comparing Elk-1 WT with Elk-1WT 2Ala; ○, p < 0.05 when comparing Elk-1(AAG1) with Elk-1(AAG1) 2Ala; and #, p < 0.05 when comparing Elk-1(AAG2) with Elk-1(AAG2) 2Ala.

...ing is frequently used to generate protein isoforms from a single gene. Nevertheless, internal initiation is used in some instances to generate protein isoforms in mammalian cells, as is the case for the transcription factors c/EBP (46, 47), sCREM (37), and Egr3 (38). Notably, the truncated versions show markedly different properties as transcriptional regulators. We propose a similar mechanism for sElk-1.

Elk-1 belongs to the TCF subfamily of transcription factors that are characterized by the presence of a highly conserved Ets-DNA binding domain in their N-terminal region (8). This adopts a “winged-helix-turn-helix” structure due to the multiple interactions of three α-helices with four anti-parallel β-strands (48). The α3-helix sits in the DNA major groove and interacts with the core Ets recognition sequence GGA. The deletion in sElk-1 of the first 54 amino acids in the DNA binding region removes two α-helices and two β-strands, which strongly impairs binding of sElk-1 to the SRF-SRE binary complex. Nevertheless, according to the crystal structure of the the Elk-1 Ets domain bound to DNA, all of the amino acids in the α3 helix that contact the GGA core sequence are still present in sElk-1 (42), as are the majority of DNA backbone contacts. These residues may suffice to generate an extremely weak ternary complex with SRF on the SRE that we could only visualize upon sElk-1 phosphorylation. Thus, as with Elk-1, phosphorylation of sElk-1 induces conformational changes that modify its binding properties.

An isoform of the TCF protein Net-b (49) is encoded by an alternatively spliced mRNA and has a truncated C-terminal activation domain. This splicing variant is insensitive to the Ras-MAPK pathway and functions as a constitutive competitor for binding by other TCFs. This contrasts with the mechanism we propose for sElk-1, which still contains its phosphorylation sites, which are essential for its activity but is strongly compromised in DNA binding. Thus, sElk-1 represents a novel type of MAPK-dependent Ets protein that, while nuclear, functions independently of DNA binding. It could act as a transcriptional modulator directly, for example by competing with Elk-1 for interaction with the coactivator CBP, and/or indirectly, by causing Elk-1 relocalization to the cytoplasm (see below).

Transfection of PC12 cells with the Elk-1 WT expression vector that encodes both full-length and short Elk-1 enhanced NGF-driven differentiation, as measured by neurite extension. This effect can be attributed to sElk-1, since its overexpression, but not that of full-length Elk-1, potentiated differentiation to nearly the same extent. Moreover, mutation of the major ERK phosphorylation sites at Ser383/389 to Ala compromised potentiation in DNA binding. Thus, sElk-1 represents a novel type of MAPK-dependent Ets protein that, while nuclear, functions independently of DNA binding. It could act as a transcriptional modulator directly, for example by competing with Elk-1 for interaction with the coactivator CBP, and/or indirectly, by causing Elk-1 relocalization to the cytoplasm (see below).

Although sustained activation of ERK by NGF gives rise to neuronal differentiation in PC12 cells (45), in other cell systems signals that cause sustained activation of ERK are implicated in cell proliferation (51). One difference between NGF-treated PC12 cells and other cells could be the presence of sElk-1. By inhibiting Elk-1 transactivating properties at the SRE site, sElk-1 could block the activation of genes implicated in cell proliferation. In support of this, c-Fos has been argued to play a key role in cell cycle progression (52, 53). Although NGF treatment of PC12 cells leads to a transient activation of c-fos (54), microinjection of antibodies to c-Fos in PC12 cells significantly increases neuronal differentiation after exposure to NGF (52). Indeed, early induction of c-fos could be related to the mitogenic effect of NGF observed on the first days of treatment (55, 56). Then, expression of sElk-1 could accelerate ne-
Elk-1 is classically described as a nuclear target of activated ERK. However, we show here that the scenario is different in cells of neuronal phenotype, since we found it in both nuclear and cytoplasmic compartments, including neuritic extensions. This redistribution was related to Elk-1 expression. Extensive deletion studies have shown that at least two regions of Elk-1 are required for nuclear translocation (36). One is located in the N-terminal part of Elk-1 (amino acids 34–83) and contains a consensus sequence for nuclear translocation (47GLRKNKTN54) that is deleted in sElk-1. The reason why Elk-1 relocalizes upon the amino acids 137–157, would appear to be sufficient for nuclear retention of sElk-1. However, the other domain, spanning amino acids 158–190, is required for nuclear translocation (36). One is located in the C-terminal part of Elk-1 (amino acids 191–250) and contains a consensus sequence for nuclear translocation (47GLRKNKTN54) that is deleted in sElk-1. The presence of Elk-1 in cytoplasmic compartments could be related to a specific function of ERK signaling in neuronal cells. Indeed, we have previously shown that phosphorylation of both ERK and Elk-1 occurred in dendritic, cytoplasmic, and nuclear compartments upon in vivo electric stimulation (29). The dendritic and nuclear phosphorylation of Elk-1 was blocked by PD98059, an inhibitor of ERK activity. In NGF-differentiated PC12 cells, strong activation of ERK by phorbol ester leads to nuclear translocation of Elk-1 (data not shown). This raises the interesting possibility that dendritic activation of Elk-1 leads to its nuclear translocation, where it could serve as a signal transducer and transcriptional activator mediating a later phase of gene induction. Given the strong link between Elk-1 and ERK in neuronal cells in vitro (28) as well as in vivo (29), Elk-1 could conceivably translocate together with activated ERK in the nucleus. Further experiments should clarify this intriguing hypothesis.

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