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ETS-domain transcription factor Elk-1 mediates neuronal survival: SMN as a potential target

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ABSTRACT

Elk-1 belongs to the ternary complex factors (TCFs) subfamily of the ETS domain proteins, and plays a critical role in the expression of immediate-early genes (IEGs) upon mitogen stimulation and activation of the mitogen-activated protein kinase (MAPK) cascade. The association of TCFs with serum response elements (SREs) on IEG promoters has been widely studied and a role for Elk-1 in promoting cell cycle entry has been determined. However, the presence of the ETS domain transcription factor Elk-1 in axons and dendrites of post-mitotic adult brain neurons has implications for an alternative function for Elk-1 in neurons other than controlling proliferation. In this study, possible alternative roles for Elk-1 in neurons were investigated, and it was demonstrated that blocking TCF-mediated transactivation in neuronal cells leads to apoptosis through a caspase-dependent mechanism. Indeed RNAi-mediated depletion of endogenous Elk-1 results in increased caspase activity. Conversely, overexpression of either Elk-1 or Elk-VP16 fusion proteins was shown to rescue PC12 cells from chemically-induced apoptosis, and that higher levels of endogenous Elk-1 correlated with longer survival of DRGs in culture. It was shown that Elk-1 regulated the *Mcl-1* gene expression required for survival, and that RNAi-mediated degradation of endogenous Elk-1 resulted in elimination of the *mcl*-1 message. We have further identified the survival-of-motor neuron-1 (*SMN1*) gene as a novel target of Elk-1, and show that the *ets* motifs in the *SMN1* promoter are involved in this regulation.

1. Introduction

The ternary complex factor (TCF) subfamily of ETS domain transcription factors, including Elk-1, SAP-1 and SAP-2/ERP/Net, have been extensively studied with respect to the serum response at the immediate-early gene (IEG) promoters, most notable that of *c-fos* (reviewed in [1]). Within minutes of serum stimulation, phosphorylation of the ETS domain factor Elk-1 takes place, which can then form a ternary complex with the serum response factor (SRF) on the serum response element (SRE) of the *c-fos* promoter, thereby activating its transcription [2,3]. In addition to their role in the immediate growth response, ETS domain transcription factors have been either implicated or shown to function in many developmental processes, from hematopoiesis to forming functional neural circuits (reviewed in [1,4]).

Interestingly, Elk-1 has been shown to reside in axons and dendrites of adult rat brain cells, and to be phosphorylated upon stimulation in these largely post-mitotic neurons [5]. In these neurons Elk-1 was found to regulate transcription of immediate early genes such as *c-fos* and *zif268*, possibly regulating synaptic plasticity [5]. Furthermore, full-length Elk-1 and an alternatively-translated isoform, short Elk-1, have been shown to have opposing roles in PC12 cells. The short Elk-1 isoform (sElk-1) is primarily involved in neurite extension in NGF-induced differentiation of PC12s [6]. More recently, Elk-1 was shown to interact with neuronal microtubules [7], and phosphorylated Elk-1 was shown to translocate to the nucleus upon stimulation [7,33]. Phosphorylation of Elk-1 on Serine 383/389 was further shown to regulate dendritic elongation and cytoskeletal dynamics, as well as SRF and actin levels [33]. SUMOylation mutant of Elk-1, on the other hand, was shown to both cause faster nuclear translocation of Elk-1, and consequent neuronal differentiation [22]. Taken together, these findings suggest that Elk-1 can play additional roles in neurons beyond a cell cycle response [7,8,22,33]. Indeed, Elk-1 and TCFs were indicated to have an anti-apoptotic role in HEK293 cells [8], and as such a similar role for Elk-1 could be envisaged in neurons.

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In this study we have investigated the potential function of Elk-1 in neuronal survival in a range of neuronal model cells including both rat pheochromacytoma (PC12) and human neuroblastoma (SH-SY5Y) model systems, as well as primary dorsal root ganglia (DRG) cultures. We present evidence that Elk-1 can enhance survival of these cells, either by depleting *elk-1* mRNA in SH-SY5Y cells through RNA interference experiments, or by overexpressing a dominant negative form, ElkEN (engrailed fusion) in PC12 cells. We further show that exogenous expression of Elk-1 in PC12 cells can protect the cells from chemically-induced apoptosis. This suggests a potential role of Elk-1 in protecting the cells from apoptosis, or maintaining survival. To that end, we have investigated two candidate targets for Elk-1, the anti-apoptotic gene *MCL-1*, which was previously reported to be regulated by Elk-1 in other cell types [8,9], and a potential novel target human *SMN1*. Our results show that *MCL-1* and *SMN1* are Elk-1 target genes in SH-SY5Y neuroblastoma cells and hence may contribute to the anti-apoptotic activities of Elk-1 in neuronal cells.

2. Materials and methods

2.1. Materials

Epidermal growth factor (EGF, Sigma E4127) was typically used at 100 nM concentration. Nerve growth factor (2.5 S, Promega) was used at 50 ng/ml, unless otherwise noted. Cobalt chloride (Sigma) was used at a final concentration of 750 μM. 1-methyl-4-phenylpyrinidium iodide (MPP^+ iodide, Sigma) was used at a final concentration of 1 μM. Caspase inhibitor Z-VAD-fmk was supplied from Promega and used at a final concentration of 20 μM.

pSRE-Luc (pAS821) contains two copies of the *c-fos* SRE (nucleotides −357 to −275, containing both an SRF binding site and an adjacent *ets* motif) upstream of a minimal *tk* promoter and the luciferase gene [8]. pMcl-1-Luc (pAS2156) contains the human *Mcl-1* promoter (−3893 to +25) upstream from the firefly luciferase gene (kindly provided by Steve Edwards). Renilla luciferase was used as an internal control plasmid. pRSV–Elk-1–VP16 (pAS348) is a Rous sarcoma virus (RSV) promoter-driven vector encoding full-length wildtype human Elk-1 fused to residues 410 to 490 of a VP16 C-terminal sequence [10]. His-FLAG-tagged Elk-1 (pAS278) was reported previously [8]. For *in vivo* expression in mammalian cells, pAS1402 (encoding RSV-driven Elk-(L158P)VP16), pElk-EN, and pElk-(L158P)EN have been described elsewhere [8,11]. pCDNA3 rIAP-1 construct was kindly obtained from Dr. M. Holcik. The expression plasmid encoding human wild-type SMN gene was constructed as follows: Total RNA was isolated from SH-SY5Y cells with High Pure RNA Isolation Kit (Roche) and 1 μg RNA was converted into cDNA by High Fidelity cDNA Synthesis Kit (Roche) using oligo-dT primers. Then the SMN coding region was amplified by using 5′-AGATC **GGA TCC** TTT GCT ATG GCG ATG AGC AG-3′ and 5′-AGATC **AAG CTT** ATT TAA GGA ATG TGA GCA CCT TCC-3′ primers and the resulting 884 bp product was inserted into BamHI/HindIII site of the pCMV-3-tag-6 vector (Stratagene).

The *SMN* promoter region spanning −553 to +100 with respect to transcriptional initiator was amplified from the human genomic DNA as follows: genomic DNA from SH-SY5Y neuroblastoma cells was isolated using Wizard SV Genomic DNA Purification System (Promega). A 663 bp PCR fragment of *SMN* promoter was amplified by 5′-ACGAGACGGTACC-CATTCTGACGACAGAGCGAG-3′ and 5′-ACGAGAC AAGCTTTTCTGG-GAGCGGAACAGTAC primers using *Pfu* DNA polymerase (Fermentas) using the following PCR protocol: 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, and a final elongation at 72 °C for 5 min. The resulting promoter DNA was cloned into KpnI and HindIII restriction sites of pGL2 plasmid (Promega). Two predicted *ets* binding domains were deleted using the Gene-Tailor Mutagenesis System (Invitrogen). The *SMN* promoter, cloned into pGL2 was methylated with DNA methylase and this methylated DNA was used as the template for the subsequent PCR reactions performed with Long PCR Enzyme Mix (Fermentas). 5′-AGGATCTGCCTTCCCCTGCCCCATGTT-3′ and 5′-AAGGCA-GATCCTTAAACACTAGAAG AT-3′ primers were used for the deletion at +47 to +50 (deletion 1) and 5′-CAAACAAAAAAAAAAAGGGGAAATA-TAACACAGTG-3′ and 5′-TTTTTTTTT TTGTTTGTTTGTTTTGAGAC-3′ primers were used for the deletion at +457 to +459 TT (deletion 2). The resulting clones carrying the relevant mutations were confirmed by sequence analysis.

2.2. Tissue culture, cell transfection and reporter gene assays

Rat pheochromacytoma PC12 cells were routinely maintained in high-glucose DMEM supplemented with 10% Horse Serum, 5% Fetal Calf Serum, 1× Penicillin/Streptomycin antibiotics and 1× L-glutamine. SH-SY5Y human neuroblastoma cells were maintained in low-glucose DMEM supplemented with 10% Fetal Calf Serum, 1× Penicillin/Streptomycin antibiotics and 1× L-glutamine. SH-SY5Y cells were maintained in DMEM with 10% Fetal Bovine Serum, supplemented with L-glutamine and antibiotics Penicillin/Streptomycin, at 5% CO_2.

Transient transfections with PC12 cells were performed using 500 ng total DNA with Effectene reagent (Qiagen) or more routinely using 1 μg total DNA with TransFast reagent (Promega) following the manufacturers' instructions. SH-SY5Y cells were plated into 24 well plates at 5×10^4 cells 1 day before transfection and the next day they were transfected using the TransFast transfection reagent (Promega) according to the manufacturer's instructions. Usually 400 ng reporter construct, 200 ng pRL-TK *Renilla* luciferase plasmid as internal control, and varying amounts of expression plasmids were transfected into cells plated as above. After transfection, the cells were maintained in growth medium for a further 48 h. Then SH-SY5Y cells were scraped from the plates, spun down, and washed once with ice-cold PBS. For the luciferase assays, cells were lysed in 100 μl passive lysis buffer (Promega) and 50 μl of the lysate was placed in 96-well plates, and luciferase reporter assays were carried out using the dual luciferase assay system (Promega) according to the manufacturer's instructions. The assay was monitored and quantified in Thermo Luminoskan Ascent and analyzed in MS Excel. Assays were commonly performed in triplicates, and repeated at least twice.

2.3. Cell survival scoring

The cells were commonly seeded on coverslips in 24-well or 12-well plates at 5×10^4 cells/ml. For immunofluorescence-based cell survival experiments, the coverslips were fixed in methanol and mounted on glass slides using mowiol mounting medium containing Hoechst stain (Sigma). Visualization and scoring of nuclear staining was carried out in pseudo-confocal fluorescence microscope (Leica). Pictures were generated using Picture Publisher software (IPLab). For differentiation, neurite extension beyond almost 2 cell body length was assessed qualitatively and the number of differentiated cells was counted versus total cells, in at least 3 different fields per experiment; % differentiation was then reported. For apoptosis scoring, apoptotic and total nuclei were counted on duplicate coverslips, for at least three different fields per coverslip, and % apoptosis was calculated. For mitotic index estimation, cells in various stages of mitosis were counted on duplicate coverslips, at least three different fields per coverslip. Mitotic index (MI) was calculated as the average % of mitotic cells in the population, and average and standard deviation calculated using MS Excel software. For surviving cell counts, Trypan Blue exclusion assay was used for counting live cells over a period of days and reported in MS Excel software.

2.4. RNA interference

For RNA interference of *elk-1* message, psiSTRIKE hMGFP vector was used as per manufacturer's instructions (Promega), and the constructs siElk-1 (targeting human Elk-1) and scrRNA (scrambled RNA) have been described previously [12]. These resulting plasmids were