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Nerve growth factor (NGF) induces mRNA expression of the new transcription factor protein p48ZnF

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Abbreviations: AD, Alzheimer's disease; GFP, green fluorescent protein; IAP, inhibitors of apoptosis proteins; NGF, nerve growth factor; p48ZnF, zinc finger protein of 48 kilo Dalton; p60TRP, transcription regulator protein of 60 kilo Dalton; RT-PCR, reverse transcription-polymerase chain reaction; Smac, second mitochondria-derived activator of caspases.

Abstract

Apoptosis, the cell's intrinsic death program, plays a crucial role in the regulation of tissue homeostasis, and abnormal inhibition of apoptosis is an indicator of cancer and autoimmune diseases, whereas excessive cell death is implicated in neurodegenerative disorders such as Alzheimer's disease (AD). Using cDNA subtraction analysis, we compared p60TRP (p60 transcription regulator protein) expressing cells with control cells during the process of apoptosis and we identified the new zinc-finger protein p48ZnF that is predominantly located in the cytoplasm of the cell. Additionally, we demonstrate here that p48ZnF is up-regulated in rat neuronal PC12 cells upon stimulation with the neurotrophic factor NGF (50 ng/ml). These findings point to a possible pivotal role of p48ZnF in the control of neuronal survival.

Keywords: Alzheimer's disease, apoptosis, neurodegeneration, neurotrophins, transcription

Introduction

Neuronal cell death is known to be executed by an endogenous cell suicide program called apoptosis. The importance of apoptosis resides in the fact that several steps involved in the modulation of apoptosis are susceptible to therapeutic intervention. Apoptosis is of crucial importance not only during embryonic and neuronal development but also in maintenance of the nervous system and in pathological situations such as neuronal degeneration in Alzheimer's disease (AD) (Selkoe, 2001; Heidenreich, 2003).

Recently, we have identified several new proteins, such as CGI-94 (comparative gene identification-94), rTid-1 (rat homolog of the Drosophila tumor suppressor l(2)tid gene) and p60TRP (p60 transcription regulator protein) that show an altered expression in AD brain and are probably involved in AD-related cell death (Heese *et al*., 2002a; b; 2004; Fujita *et al*., 2004).

The present study aimed at gaining more insight into the possible potential anti-apoptotic signaling of p60TRP. Applying the cDNA-subtraction technology we found and described in detail the new zinc-finger protein p48ZnF. After characterizing its protein sequence we have subcloned the open reading frame of p48ZnF in-frame with the green fluorescent proteins (GFP) to study the subcellular localization of p48ZnF by fluorescent light microscopy as a visual classification approach (Heese *et al*., 2002a, b).

To further explore the physiological function of p48ZnF, this protein was expressed and analyzed in PC12 cells that are known to differentiate into neuronal cells in response to NGF (nerve growth factor). Previously, PC12 cells have been used as a model for the analysis of the effect of NGF on survival, apoptosis and differentiation and to elucidate the specific intracellular signaling cascade involved in these responses (Götz, 2000; Heidenreich, 2003). We demonstrate that *p48ZnF's* mRNA expression is significantly up-regulated in PC12 cells after stimulation with NGF.

Materials and Methods

Reagents

Unless indicated, all reagents used for biochemical methods were purchased from Sigma-Aldrich (Tokyo, Japan).

cDNA-subtraction analysis

The application of PCR-Select-cDNA subtraction (Clontech, Tokyo, Japan) enabled us to compare two po-

pulations of mRNA and to obtain clones of genes that were expressed in one population (p60TRP-transfected cells) but not in the others (control sample). For these studies rat B104 neuroblastoma cells were incubated without FCS±p60TRP for 60 h and thereafter, cDNA-subtraction was performed as reported previously (Heese *et al*., 2000). Differentially expressed genes were identified by 5'-/3'-RACE-PCR (using a rat brain cDNA library (Marathon-Ready (Clontech) and pAP3neo (TaKaRa, Otsu, Shiga, Japan), Southern-blots and sequencing. Thereafter, differentially expressed genes were confirmed by RT-PCR as described previously (Heese *et al*., 2000).

Reverse transcription-polymerase chain reaction (RT-PCR)

The quantitative RT-PCR method was used for mRNA expression analyses and *p48ZnF* isolation, as described previously (Heese *et al*., 2000; 2002a), using cDNA-library- and rat *p48ZnF*-specific primers (for isolation: sense: 5'-gcg taa tac gac tca cta tag gga att cga cgt-3', anti: 5'-cgc gac gta cga ttt aaa tta acc ctc act aaa-3'; r-sense: 5'-atg ccc ccc aag aaa cag gct cag gcc ggg ggc agc aag aag gcg-3', r-anti: 5'-tca ttc ttc taa atc aag tgt gtt taa ttc ttc ttc tag ttc atc-3'. The amplification steps involved denaturation at 94°C for 0.5 min, annealing for 50 s at 65°C (AnnT) and extension for 2 min at 68°C (AnnT: 65°C/24 cycles; for tissue analysis: 28 cycles). PCR amplification of the constitutively expressed ribosomal protein S12 or the GAPDH (AnnT: 60°C/21 cycles) cDNA were used as a measure of input RNA. The PCR reactions were analyzed with specific fluorescein-labelled DNA probes on a Southern-blot. Detection and appropriate analysis of the membranes were done with the Fluor Imager 595/Image Quant ver. 5.0 (Molecular Dynamics, Tokyo, Japan). In addition to non-parametric statistical testing (Kruskal-Wallis test) and a t-test analysis, statistical evaluation of results was performed by one-way analysis of variance (ANOVA) and the statistical error was indicated as the SEM (standard error of the mean) (Heese *et al*., 2000; 2002a).

P48ZnF cDNA and protein analyses

P48ZnF cDNA and protein sequences were used as search tools in the National Center for Biotechnology information (NCBI) Blastp 2.0 program against non redundant GenBank CDS translations+PDB+SwissProt +PIR+PRF databases, in addition to the UniGene database (NCBI) (Altschul *et al*., 1997). Protein sequence motif searching was performed with the Prosite-, Profile-, Blocks-, ProDom-, Prints-, Pfam- and PsortII-programs (Horton and Nakai, 1997; Bateman *et al*., 1999). Additionally, protein sequence analysis was performed using 'Toolbox' at the European Bioinformatics Institute (EBI, www.ebi.ac.uk) and the Amino Acid Composition Search program at the ExPASy-www-server (http://www.expasy.ch).

Tissue-specific p48ZnF expression analysis

For the tissue-specific gene expression analysis of p48ZnF, Rapid-Scan™-Gene-Expression panels (Origene Technologies, Rockville, MD) were used as ready to use tissue cDNAs to perform a semi-quantitative RT-PCR analysis (pre-standardized cDNA panels). PCR products were analyzed by using a standard 1.5% DNA electrophoretic agarose E-gel™ (Invitrogen) (Heese *et al*., 2002a).

Cell culture

PC12 cells were propagated in Dulbecco's Modified Eagle Medium (D-MEM)/F12 (1:1) containing N2-supplement and 10% fetal calf serum (FCS; Gibco BRL, Grand Island, NY) at 37°C in humidified 5% CO_2/95% air. CHO cells were cultured in D-MEM plus 10% FCS. For cell-transfections, a p48ZnF expression construct was generated by inserting rat *p48ZnF* cDNA in-frame with the green fluorescent protein (GFP) (pcDNA3.1CT-GFP-TOPO®, Invitrogen) at the C-terminus of p48ZnF (p48ZnF-GFP). CHO or PC12 cells were transiently transfected with p48ZnF-GFP, GFP (Clontech) expression vectors or empty plasmid (controls) using SuperFector transfection reagent (according to the manufacturer's protocol; B-Bridge, San Jose, CA) and maintained in D-MEM/F12(1:1)/N2 medium containing 10% FCS (Gibco) (Heese *et al*., 2002a, 2004). 24 h after transfection PC12 cells were stimulated with NGF (murine NGF 2.5 S, 50 ng/ml; Invitrogen): for a time period of 96 h to study *p48ZnF*'s mRNA expression upon NGF stimulation and for 120 h to characterize neurite outgrowth in p48ZnF-positive PC12 cells. Thereafter, cell survival & neurite-outgrowth was examined by fluorescence microscopy (Olympus IX70, Olympus, Tokyo, Japan) (Heese *et al*., 2000; 2004).

Results

Isolation of p48ZnF and characteristic features of p48ZnF

After performing the cDNA subtraction analysis comparing p60TRP transfected cells with control cells during the process of apoptosis, *p48ZnF* was isolated by 5'-RACE-RT-PCR from rat neuronal B104 cells and a rat brain cDNA library as described in material and methods (Heese *et al*., 2002a; 2004). In addition, among the differentially expressed genes, we could identify neurotrophic factors such as the new neuro-

Rat p48ZnF [AY377983], a 426 aa [E, K-rich (12-14%)] protein: MW: 48.3 kDa; pI: 5.21; aliphatic index: 67.07; half-life: 30 hs; cellular distribution: nuclear/cytoplasm

MPPKKQAQAGGSKKAEQKKKEKIIEDKTFGLKNKKGAKQQKFIKAVTHQVKFGQ
QNPRQVAQSEAEKKLKKDDKKKELQELNELFKPVVAAQKISKGADPKSVVCAFFK
QGQCTKGDKCKFSHDLTLERKCEKRSVYIDARDEELEKDTMDNWDEKKLEEVVN
KKHGEAEKKKPKTQIVCRHFLEAIENNKYGWFWVCPGGGDNCMYRHALPPGFVL
KKDKKKEEKEDEISLEDLIERERSALGPNVTKITLESFLAWKKRKRQEKIDKLEQD
MERRKADFKAGKALVISGREVFEFRPELVNDDDEEADDTRYIQGTGGDEVDDSVGV
NDIDLSLYVPRDVEETGITVASLERFSTYASDKDENKLSEASGGLAENGERSDLDEDS
GGGGQENGSIDAVPVDENLFTGEDLDELEEELNTLDLEE

- aa100 – aa125 / aa175 – aa211: Zinc finger C-x8$_{(7)}$-C-x5-C-x3-H type;
- aa147 – aa188: Myb-like DNA-binding domain;
- aa4 – aa175: Lysine-rich region profile;
- aa285 – aa289: cyclin-recognition site, aa290 – aa293: CK2-phosphorylation site, aa368 – aa371: CK2-phosphorylation site;
- aa26 – aa29, aa229 – aa232, aa239 – aa242, aa324 – aa327, aa333 - aa336: Polo-like kinase (Plk)1-phosphorylation sites;

- aa4 - aa21 / aa19 - aa36 / aa262 - aa262 - aa279: Bipartite nuclear localization signal (NLS), low probability;

No Nuclear-Receptor (NR-) box: Lxx(x)LL motifs; No specific R/G-rich RNA binding motif; no R/S-domain for nuclear speckles or splicing factors; No specific nuclear export signal (NES); no N-terminal signal sequence; no transmembrane sequence; no SH2/SH3 domain.

Figure 1. Characteristic features of p48ZnF.

trophic protein NNT-1 and other (known) zinc-finger proteins (not shown).

Protein sequence analysis revealed that p48ZnF is a glutamate-/lysine-rich and zinc-finger-domain-containing protein (Figure 1).

P48ZnF is ubiquitously expressed

By using the RT-PCR method we analyzed the expression pattern of p48ZnF mRNA and detected it in all tissues examined (Figure 2). Higher expression levels could be observed in brain; by contrast, only low p48ZnF mRNA levels could be detected in lung or liver.

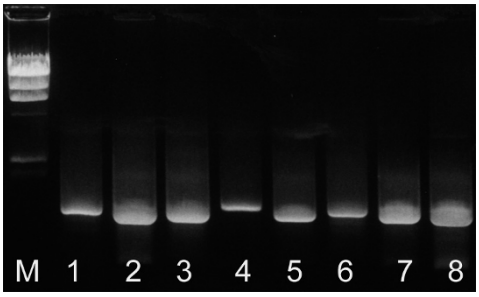

Figure 2. Expression of *p48ZnF* mRNA in various tissues: semi-quantitative mRNA expression analysis by RT-PCR. 1=heart, 2=brain, 3=kidney, 4=liver, 5=placenta, 6=lung, 7=skeletal muscle, 8=pancreas.

Expression of p48ZnF in CHO and PC12 cells

To investigate the possible physiological function of p48ZnF we transfected CHO and PC12 cells with a p48ZnF-GFP-fusion protein. The characterization by fluorescence microscopy demonstrates that p48ZnF is primarily located in the cytoplasm (Figure 3a and b). However, as shown in figures 3c-3l, it could also be detected in the nucleus of the cell. No difference could be observed if we changed GFP from the C-terminus to the N-terminus of p48ZnF (not shown). The observation that p48ZnF-GFP induced a neurite-like outgrowths in CHO cells (Figure 3c-3g) led us to transfect it into PC12 cells. In contrast to neuroblastoma cells, PC12 cells are more suitable to start a protein-characterization (Gotz *et al.*, 2000), because PC12 cells show a quick response to neurotrophins such as NGF (and to other mitogenic factors such as EGF (epidermal growth factor) or bFGF (basic fibroblast growth factor)). After transfection, PC12 cells do not show a spontaneous outgrowth of neurites (Figure 3h-3j). However, after treatment with NGF (50 ng/ml) p48ZnF-positive cells showed typical neurite outgrowths of PC12 cells.

P48ZnF mRNA is up-regulated in neuronal PC12 cells upon NGF stimulation

To further explore a possible link between p48ZnF and neuronal survival or differentiation, we stimulated PC12 cells with NGF. As shown in Figure 4, *p48ZnF* mRNA was apparently up-regulated in PC12 cells upon NGF stimulation. Maximum induction (about 3-fold of basal-expression levels) could be observed

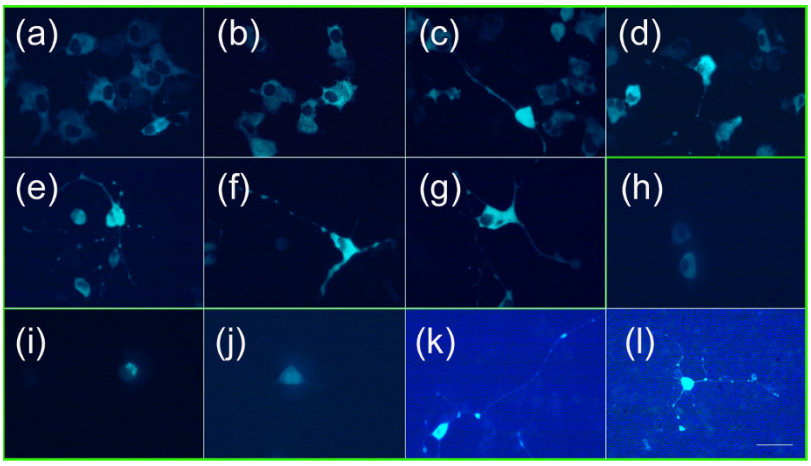

Figure 3. Fluorescence localization of p48ZnF-GFP expression in CHO and PC12 cells. CHO and PC12 cells transiently transfected with p48ZnF-GFP were visualized directly by p48ZnF-GFP-green fluorescence showing a cytoplasmic or nuclear p48ZnF expression pattern. (a-g) CHO cells. (h-j) PC12 cells. (k, l) Expression of p48ZnF in NGF-activated PC12 cells. p48ZnF does not inhibit NGF-induced neurite outgrowth in neuronal PC12 cells. 24 h post-transfection, cells were treated for 120 h with NGF (50 ng/ml); Scale bar represents 50 µm.

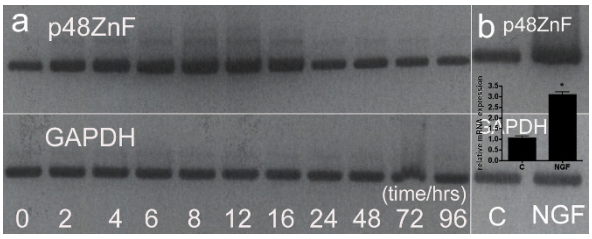

Figure 4. Southern blot analysis of *p48ZnF* mRNA expression in rat neuronal PC12 cells upon NGF (50 ng/ml) stimulation - time course. (a) Top: *p48ZnF* PCR-products, bottom: PCR-products of reference-gene GAPDH. (b) Quantitation of *p48ZnF* transcripts 8 hrs post NGF-stimulation (C, control cells; NGF, NGF-activated cells). Values are the ratio of densitometric scores for *p48ZnF*- and *GAPDH*-PCR-products ± SEM of six independent experiments (*$P < 0.05$, compared to controls).

after 8-12 h NGF-stimulation and it declines to basal level after an incubation period of about 72 h. After stimulation of PC12 cells with EGF or bFGF, only a slight but not significant effect (increase) on *p48ZnF* mRNA expression levels could be detected (data not shown)

Discussion

Programmed cell death is an essential and widespread physiological process that results in the elimination of cells. Genes required to carry out this process have been identified, and many of these remain the subjects of intense investigation. In general, the molecular mechanism of apoptosis involves the activation of caspases (cysteine proteases) and the cascades of several specific caspases ultimately converge on a common pathway in which the final morphological and biochemical alterations characteristic of apoptosis take place (Heidenreich, 2003).

In neurons such as PC12 cells, the rescue of these cells from apoptosis by NGF seems to be critically dependent on signaling mechanisms that involve the kinases PI-3K and PKB (Akt). Akt suppresses apoptotic death via phosphorylation of the pro-apoptotic regulator Bad and the caspase-9 (Gotz, 2000; Heidenreich, 2003).

In the mitochondrial pathway, an apoptotic insult causes the mitochondria to release cytochrome-c along with Smac/DIABLO. While Smac/DIABLO promotes caspase-9 activation by binding to IAP (inhibitors of apoptosis proteins), thus removing its inhibition, released cytosolic cytochrome-c forms an activation complex with apaf-1 (apoptotic protein activating factor-1) and caspase-9. This complex facilitates the activation of effector caspases to execute downstream apoptotic changes (Heidenreich, 2003).

Recently, we have described the new transcription regulator p60TRP and have determined its possible involvement in apoptotic signaling and neurodegenerative diseases such as AD. In particular, we have shown a possible link between p60TRP and the mitochondrial-derived Tid-1 protein that is a key regulator of mitochondria-dependent apoptosis (Fujita *et al.*, 2004; Heese *et al.*, 2004).

In the present study we describe p48ZnF as a new ubiquitously expressed zinc-finger protein which is localized in the cytoplasm and nucleus, respectively. Taking into account that p48ZnF may be involved in p60TRP's multifunctional signaling pathways it is tempting to speculate that p48ZnF may also fulfill a more versatile role as transcription regulator during apoptosis as it has been described, for instance, for the zinc-finger protein NRIF (neurotrophin receptor interacting factor), a key protein involved in NGF-mediated apoptotic signaling (Rabizadeh and Bredesen, 2003).

In conclusion, since p48ZnF was up-regulated by NGF and could interfere with p60TRP-mediated sig-

nalling, p48ZnF appears to be involved in the regulation of neuronal survival. Thus, the connection between p48ZnF's and p60TRP's signal-transductions will be an interesting topic for future investigations and further experiments are necessary to clarify p48ZnF's physiological role and its function during apoptotic processes in neurodegenerative diseases.

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