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Characterizing the New Transcription Regulator Protein p60TRP

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Abstract Active cell death ('apoptosis' or 'programmed cell death') is essential in the development and homeostasis of multicellular organisms and abnormal inhibition of apoptosis is an indicator of cancer and autoimmune diseases, whereas excessive cell death might be implicated in neurodegenerative disorders such as Alzheimer's disease (AD). Using bioinformatics-, Western-blotting-, yeast-two-hybrid-system-, polymerase chain reaction (PCR)-, and fluorescence microscopy-analyses, we demonstrate here that the neuroprotective protein p60TRP (p60-transcription-regulator-protein) is a basic helix–loop–helix (bHLH) domain-containing member of a new protein family that interacts with the Ran-binding-protein-5 (RanBP5) and the protein-phosphatase-2A (PP2A). The additional findings of its influence on NNT1 and p48ZnF (new-neurotrophin-1, p48-zinc-finger-protein)-signaling and its down-regulation in the brain of AD subjects point to a possible pivotal role of p60TRP in the control of cellular aging and survival. J. Cell. Biochem. 91: 1030–1042, 2004.

Key words: apoptosis; nerve-growth-factor; neurodegeneration; phosphatase; importin

Apoptosis is an active form of cell death with an important role in development and homeostasis in multicellular organisms. Cell death by apoptosis comprises a sequence of events leading to the activation of caspases, which execute the fragmentation of the cellular protein and DNA leading to disintegration of the cell. Physiological neuronal apoptosis allows the nervous system to eliminate excess neurons. In addition, apoptotic cell death has been observed in a variety of neuronal degenerations such as Alzheimer's disease (AD) [Leist and Jäättelä, 2001; Selkoe, 2001; Shi, 2002].

Recently, we have identified several new proteins, such as CGI-94 (comparative gene identification-94), p18AβrP (p18-amyloid-beta-responsive-protein), and rTid-1 (rat homolog of the *Drosophila* tumor suppressor *l(2)tid* gene) that show an altered expression in AD brain and/or are probably involved in AD-related cell death [Hata et al., 2001; Heese et al., 2002a,c,d; Fujita et al., 2003].

In the present study, we describe for the first time in detail the new basic helix–loop–helix (bHLH) motif-containing protein p60TRP (p60 *t*ranscription *r*egulator *p*rotein) as a member of a new protein family. We found that its mRNA expression is significantly reduced in the brain of AD patients. After characterizing the protein sequence we have subcloned the open reading frame of p60TRP in-frame with the green and red fluorescent proteins (GFP & DsRed1) to study the subcellular localization of p60TRP by fluorescent light microscopy as a visual classification approach [Heese et al., 2002c].

Moreover, we investigated the signaling pathways of p60TRP by the two-hybrid-system analysis, which is an in vivo yeast-based system that identifies the interaction between two

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proteins (X and Y) by reconstituting an active transcription factor. We identified protein-phosphatase-2A (PP2A) and Ran-binding protein-5 (RanBP5) as new p60TRP-interacting proteins.

To further explore the (patho-) physiological function of p60TRP this protein was expressed in PC12 cells. PC12 cells are known to differentiate into neuronal cells in response to nerve growth factor (NGF) and have been used as a model for the analysis of the effect of NGF on survival, apoptosis, and differentiation and to elucidate the signaling cascade involved in these responses [Götz, 2000].

RNA intereference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing, initiated by double-stranded RNA (dsRNA) [Elbashir et al., 2001]. Here we show that small interfering RNA (siRNA) duplexes specifically suppress the expression of endogenous *p60TRP* in rat neuronal PC12 cells which slightly reduces neuronal survival. Moreover, we could identify NNT1 and p48ZnF (new-neurotrophin-1, p48-zinc-finger-protein) as two new proteins, which are probably involved in p60TRP signaling during neurodegenerative processes.

MATERIALS AND METHODS

Brain Materials

Patients with sporadic AD (early stage; low incidence; Table I) received a pathological diagnosis of AD according to the criteria of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [Mirra et al., 2000] and the Braak stage [Braak and Braak, 1991]. Controls were elderly patients without significant neurological disorders (Table I). The brains were obtained from the brain bank of the Choju Medical Institute of Fukushimura Hospital (Toyohashi, Aichi, Japan) and protocols used were approved independently by the local ethics committees of the BF Research Institute and Fukushimura Hospital. The scientific use of this human material was conducted in accordance with the Declaration of Helsinki and informed consents were obtained from the guardians of the patients. Brains were weighed at autopsy and snap frozen with liquid nitrogen and stored at -80°C.

Reagents

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

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Rat and human *p60TRP* cDNA were obtained by 5′-RACE-RT-PCR (RACE, rapid amplification of cDNA ends) with primers derived from rat/human brain cDNA library (pAP3neo, TaKaRa, Otsu, Shiga, Japan) [Heese et al., 2002a,c]. The RT-PCR method was used for mRNA expression analyses and *p60TRP* isolation as described previously [Heese et al., 2002a,c]. Briefly, total cellular RNA was isolated according to the TRIzol® reagent-protocol (Gibco-BRL, Grand Island, NY). After extraction with chloroform, RNA was precipitated by adding 1 volume isopropyl alcohol to the aqueous phase, washed with 75% ethanol, resolved in RNase-free water, and quantified spectrophotometrically by absorbance at 260 nm. Total RNA (0.2 μg/μl) of each sample was first reverse-transcribed into cDNA (oligo (dT)-primed-SMART™-cDNA-synthesis (Clontech, Tokyo,

TABLE I. Characteristics of Brain Tissue Samples

Subjects	Gender	Age	PMI (h)	NFT stage (I–VI)	Stage of amyloid deposits (none, A, B, C)	Neuropa-thological diagnosis
Control						
Patient 1	Female	81	1	I	None	Massive cerebral infarct, physiological aging
Patient 2	Female	91	6	I	A	Cerebral arteriosclerosis
Patient 3	Female	88	2	I	None	Physiological aging
Patient 4	Female	94	2	I–II	None	Physiological aging
AD						
Patient 5	Female	86	2	IV	C	SDAT infarction at ACA region
Patient 6	Male	86	2	IV	C	SDAT widespread amyloid angiopathy
Patient 7	Female	97	9	IV	C	SDAT (rather AD)
Patient 8	Female	90	9	IV	C	SDAT (rather AD)

AD, Alzheimer's disease.
Hippocampus (H)/parietal cortex (Cx).

Japan); Superscript II™-reverse-transcriptase (RT) (Invitrogen, Tokyo, Japan)) according to the manufacturer's protocol, which (0.5 μl) in turn was subjected to PCR amplification (25 μl reaction-volume) using cDNA-library- and rat/human *p60TRP*-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 act ggc tca aag aat aag gct cgg gct cag gct aaa ctg-3′, r-anti: 5′-tta cat tct ttc aat aat ccc ttt aac ttc acg gaa tat ggc agt-3′; h-sense: 5′-atg gct ggg act aag aat aag aca aga gcc cag gcc aaa ac-3′, h-anti: 5′-cat tgt ttc aat aat ctc ttt aac ttc cct gaa aat ggc cat gag-3′). The numbers of cycles used to amplify each cDNA were chosen to allow the PCR to proceed in a linear range according to the Elongase™ enzyme mix-protocol (Gibco-BRL). The amplification steps involved denaturation at 94°C for 0.5 min, annealing for 50 s at 65°C (AnnT) with specific primers and extension for 2 min at 68°C (AnnT: 65°C/24 cycles). PCR amplification of the constitutively expressed ribosomal protein *S12* (AnnT: 60°C/16 cycles) cDNA was used as a measure of input RNA. Controls using RNA samples without RT or controls without RNA were used to demonstrate the absence of contaminating DNA. The PCR reactions were analyzed by electrophoresis in 1.5% agarose gels followed by alkaline blotting of the fragments onto nylon membranes and subsequent hybridization with specific fluorescein-labeled DNA probes. Detection and appropriate analysis of the membranes were done with the Fluor Imager 595/Image Quant version 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 standard error of the mean (SEM) [Heese et al., 2000, 2002a].

P60TRP cDNA and Protein Analyses

P60TRP 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]. Homology searching was performed using the Blast and FASTA (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI) algorithms and hits were aligned using BestFit (Wisconsin Package Version 10.0, GCG). Protein sequence motif searching was performed with the Prosite-, Profile-, Blocks-, ProDom-, Prints-, Pfam-, and PsortII-programs [Horton and Nakai, 1996, 1997; Bateman et al., 1999]. Phosphorylation sites were searched using NetPhos 2.0 protein phosphorylation prediction server [Blom et al., 1999]. Additionally, protein sequence analysis was performed using 'Toolbox' at the European Bioinformatics Institute (EBI, www.ebi.ac.uk/) and the following programs at the ExPASy-www-server (http://www.expasy.ch): softberry: http://www.softberry.com/index.html; and Amino Acid Composition Search (AACompIdent): http://kr.expasy.org/tools/aacomp/.

Tissue-Specific *p60TRP* Expression Analysis

For the tissue-specific gene expression analysis of *p60TRP* Rapid-Scan™-Gene-Expression panels (Origene Technologies, Rockville, MD) were used as ready to use tissue cDNAs to perform a RT-PCR analysis. 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) at 37°C in humidified 5% CO_2/95% air. CHO cells were cultured in DMEM plus 10% FCS.

Transfection

A *p60TRP* expression construct was generated by inserting rat *p60TRP* cDNA in-frame with (a) the green fluorescent protein (GFP) (pcDNA3.1CT-GFP-TOPO®, Invitrogen) or (b) the DsRed1-protein (using NheI and Hind III restriction-enzyme-cloning sites, Clontech) at the C-terminus of p60TRP (p60TRP-CT-GFP or p60TRP-CT-DsRed1). In addition, *p60TRP* was subcloned into pIRES2-EGFP (Clontech) to co-express (co-translate) p60TRP with GFP from the same mRNA (p60TRP-IRES-GFP).

CHO or PC12 cells were transiently transfected with p60TRP-DsRed1, p60TRP-GFP, p60TRP-IRES-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-BRL) at 37°C in humidified 5% CO_2/95% air [Heese et al., 2002a,b]. Additionally, transfection efficiency (about 50–60%) was always confirmed by co-transfection of GFP reporter transcripts with p53-/PKC-DsRed1 (Clontech) transcripts as reported recently [Heese et al., 2002b,c]. Cell survival of p60TRP-DsRed1/p60TRP-GFP or p60TRP-IRES-GFP positive cells was assessed 48 h post-transfection by fluorescence microscopy (Olympus IX70, Olympus, Tokyo, Japan). 24 h after transfection cells were stimulated with NGF (murine NGF 2.5S, 50 ng/ml; Invitrogen) for 120 h. Thereafter, cell survival was measured using the Cell-Titer 96® AQ_{ueous} Assay (Promega, Madison, WI) and examined by fluorescence microscopy [Heese et al., 2000, 2002a].

ProQuest™ Two-Hybrid-System With Gateway™ Technology

The two-hybrid-system is an in vivo yeast-based system that identifies the interaction between two proteins (for instance X = p60TRP p60TRP and Y = cDNA library or PP2A) by reconstituting an active transcription factor. The active transcription factors are formed as a dimer between two fusion proteins, one of which contains a DNA-binding domain (db) fused to the first protein of interest (db-X; also known as the "bait") and the other, an activation domain (ad) fused to the second protein of interest (ad-Y; also known as the "prey" or "target protein"). db-X:ad-Y interaction reconstitutes a functional transcription factor that activates chromosomally integrated reporter genes driven by promoters containing the relevant db binding sites. When a selectable marker such as *HIS3* is used as a reporter gene, two-hybrid-dependent transcription activation can be monitored by growth of cells on plates lacking histidine, thereby providing a means to detect protein:-protein interactions genetically. This method can be used to test whether two known proteins interact with each other or detect an unknown protein encoded by a cDNA library that interacts with a protein (p60TRP) of interest [Fields and Song, 1989].

The two-hybrid-system analysis was performed according to the manufacturer's protocol (Invitrogen) [Heese et al., 2002a]. Briefly, *p60TRP* was sub-cloned from pENTR/D-TOPO® into the pDEST™32-vector (Invitrogen) containing the GAL4 DNA binding domain. pEXP-AD502 was used as an activation domain expression vector containing the ProQuest two-hybrid brain cDNA library (Invitrogen). The used yeast strain for ProQuest system was MaV203.

For selection three reporter genes were used: single copies of each of three reporter genes (*HIS3*, *URA3*, and *lacZ*) are stably integrated at different loci in the yeast genome. The promoter regions of *URA3*, *HIS3*, and *lacZ* are unrelated (except for the presence of GAL4 binding sites). In the ProQuest two-hybrid-system, in comparison to standard two-hybrid-systems, false positives are reduced because three independent transcription events (from distinct promoters) must occur at independent chromosomal loci. Induction of the *HIS3* and *URA3* reporter genes allow two-hybrid-dependent transcription activation to be monitored by cell growth on plates lacking histidine or uracil, respectively. Induction of the *lacZ* gene results in a blue color when assayed with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Moreover, two-hybrid-dependent induction of *URA3* results in conversion of the compound 5-fluoroorotic acid (5FOA) to 5-fluorouracil, which is toxic. Hence, cells containing interacting proteins grow when plated on medium lacking uracil, but growth is inhibited when plated on medium containing 5FOA.

This system, therefore, reduces false positives by providing four phenotypes [His^+ ($3AT^R$), β-gal, Ura^+, and $5FOA^S$] for assessing true interactors and by using low-copy-number (ARS/CEN) vectors that reduce expression levels and toxicity.

Positive clones were confirmed by retransformation assay: yeast cells containing potentially interacting proteins harbor both db-*p60TRP* and ad-Y (Y = e.g.: *PP2A*). Plasmid DNA isolated from yeast cells containing db-*p60TRP* and ad-Y (Y = e.g.: *PP2A*) was introduced into *E. coli* by electroporation and transformants containing ad-Y (Y = e.g.: *PP2A*) were selected with ampicillin (or db-*p60TRP* with gentamicin). The plasmid DNA ad-Y (Y = e.g.: *PP2A*) from these E. coli cells was transformed into MaV203 together with pDBLeu or db-*p60TRP* and tested for induction of the reporter genes. True positives induced the reporter genes with pdb-*p60TRP* but not with the pdbLeu control vector alone.

Anti-p60TRP Antibody

Affinity purified rabbit anti-p60TRP antibody was raised against the N-terminal domain of p60TRP (peptide: aa35–aa45 (RGAGKNR-DKGK-cys) was used for immunization (Sigma Genosys, Tokyo, Japan)).

Protein Precipitation

Protein precipitation was performed as follows [Heese et al., 2002c]: 24 h after transient transfection of about 5.0×10^6 PC12 cells with p60TRP-IRES-GFP (6-well plate) cells were incubated for additional 48 h. Cells were washed (2×) with Tris-saline buffer (TBS) pH 7.2 and 0.5 ml ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP40, 2% glycerol, 0.001% SDS, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate) was used to lyse the cells at 4°C. Cell debris were removed in a microcentifuge at high speed for 10 min at 4°C. After 2 h incubation at 4°C with the capture antibody (anti-p60TRP) 500 μl of a 50% solution of protein A Sepharose® CL-4B was added for additional 2 h. Immunoprecipitates were washed three times before adding 50 μl 1× sample Laemmli-protein buffer (Bio-Rad, Tokyo, Japan) and samples were applied to Western blot analysis.

Western-Blot Analysis

Total protein cell lysate/protein precipitation was subjected to Western blot analysis as described previously [Heese et al., 2002c]. In brief, proteins were separated by electrophoreses in a 10% polyacrylamide gel. The proteins were then transferred to a polyvinylidene fluoride membrane (Bio-Rad), immunoreacted with an anti-PP2A (regulatory subunits) antibody (SantaCruz Biotechnology, Santa Cruz, CA) and exposed to a second fluorescein-linked anti-goat antibody and a third alkaline phosphatase-conjugated anti-fluorescein antibody before incubating with alkaline phosphatase substrate according to manufacturer's instructions (ECF™ Western-blotting Kit, Amersham/Pharmacia, Tokyo, Japan). A suitable anti-RanBP5-specific antibody for the Western-blot analysis is not yet available.

Survival Assay

The siRNA method of assessing gene function involves the introduction of siRNA duplexes into cells. These short duplexes are 21–23 nucleotides in length and are designed to specifically target and silence a particular messenger RNA (mRNA) of interest.

PC12 cells were regularly passaged to maintain exponential growth. Twenty four hours before transfection at 50–80% confluency, cells were trypsinized and diluted 1:5 with fresh medium without antibiotics ($1–3 \times 10^5$ cells/ml) and transferred into 24-well plates (500 μl/well). For the 'standard' serum-withdrawal-assay, cells were non-transfected (=control-1), or transfected with GFP-(=control-2)-p60TRP-expression vectors as described previously [Heese et al., 2002b]. Survival was measured 24 h upon serum withdrawal [Heese et al., 2000]. For the siRNA-assay, transfection of 0.5 μg siRNA-duplex/well was carried out with oligofectamine as described by the manufacturer for adherent cells (Invitrogen) [Heese et al., 2002d]. Transfection efficiencies were determined by fluorescence microscopy after co-transfection of 1 μg GFP-expression vector and 0.2 μg siRNA-duplex/well as described recently [Elbashir et al., 2001; Heese et al., 2002b,d]. Serum-withdrawal- and specific silencing of target gene-(siRNA) assays were confirmed by eight independent experiments, each done in duplicate (two factor ANOVA and unpaired *t*-test). For the siRNA-assay, cell survival was measured after 48 h by Cell-Titer 96 AQ_{ueous} One solution assay (Promega) [Heese et al., 2000]. For controls, the single-stranded sense, complementary RNA oligos (=control-1, single-stranded siRNAs) and a duplex with the inverted *p60TRP* sequence (=control-2) were used. The siRNA sequence targeting *p60TRP* was from position nt 310–330 relative to the start codon. *P60TRP* specific 21-nucleotide siRNA-duplex ready for transfection was obtained from Dharmacon Research (Lafayette, CO; B-Bridge International, Tokyo, Japan).

cDNA-Subtraction Analysis

The application of PCR-Select-cDNA subtraction (Clontech), a technique based on selective amplification of differentially expressed sequences, enabled us to compare two populations of mRNA and to obtain clones of genes that were expressed in one population (p60TRP-transfected cells) but not in the other (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]. Both mRNA populations were converted into cDNA by 'SMART-PCR-cDNA'-synthesis (Clontech). A modified oligo-(dT)-primer (CDS-primer) primed the first strand synthesis reaction. The SMART-oligo-nucleotide-anchor sequence and the polyA$^+$ sequence served as universal priming sites for end-to-end cDNA amplification (LD-PCR). P60TRP-activated- and control-cDNAs were hybridized, and the hybrid sequences were then removed. Consequently, the remaining unhybridized cDNAs represented genes that were expressed in the p60TRP-activated population, but were absent from the control mRNA. The subtracted cDNA was cloned into a T/A-cloning-vector (Clontech) and differentially expressed genes were identified by 5′-/3′-RACE-PCR (using a rat brain cDNA library (Marathon-Ready (Clontech) and pAP3neo (TaKaRa; Otsu))), Southern-blots and sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer); sequencer: ABI PRISM Model 310).

RESULTS

Isolation of *p60TRP* and Characteristic Features of p60TRP

p60TRP was isolated by 5′-RACE-RT-PCR from rat PC12 cells co-transfected with *p18Aβ rP* and a brain cDNA library as described in Material and Methods. Protein sequence analysis revealed that p60TRP is a member of a new protein family. Interestingly, p60TRP contains a myc-type bHLH domain at its C-terminus (Fig. 1 and Table II).

P60TRP mRNA Is Down-Regulated in AD Brain

RT-PCR analysis of brain samples from eight subjects (four AD and four control brains, Table I) was applied to explore that *p60TRP* mRNA was generally down-regulated in the hippocampus and parietal cortex of AD brains. As shown in Figure 2, almost the same levels of *p60TRP* mRNA in the hippocampus or parietal cortex were seen in control brains whereas *p60TRP* mRNA was apparently down-regulated in the hippocampus and parietal cortex of all AD brains examined.

P60TRP Interacts With PP2A and RanBP5

Using the yeast two-hybrid-system and Western-blot analysis, we found two new p60TRP-interacting proteins such as (Table III and Fig. 3).

1) Ran-binding-protein-5 (RanBP5) and
2) protein-phosphatase-2A.

P60TRP Is not Ubiquitously Expressed

By using the RT-PCR method, we analyzed the expression pattern of *p60TRP* and detected that its mRNA is not expressed in all tissues investigated (Fig. 4). High expression levels could be observed in brain; by contrast, *p60TRP* could not be detected in lung or liver.

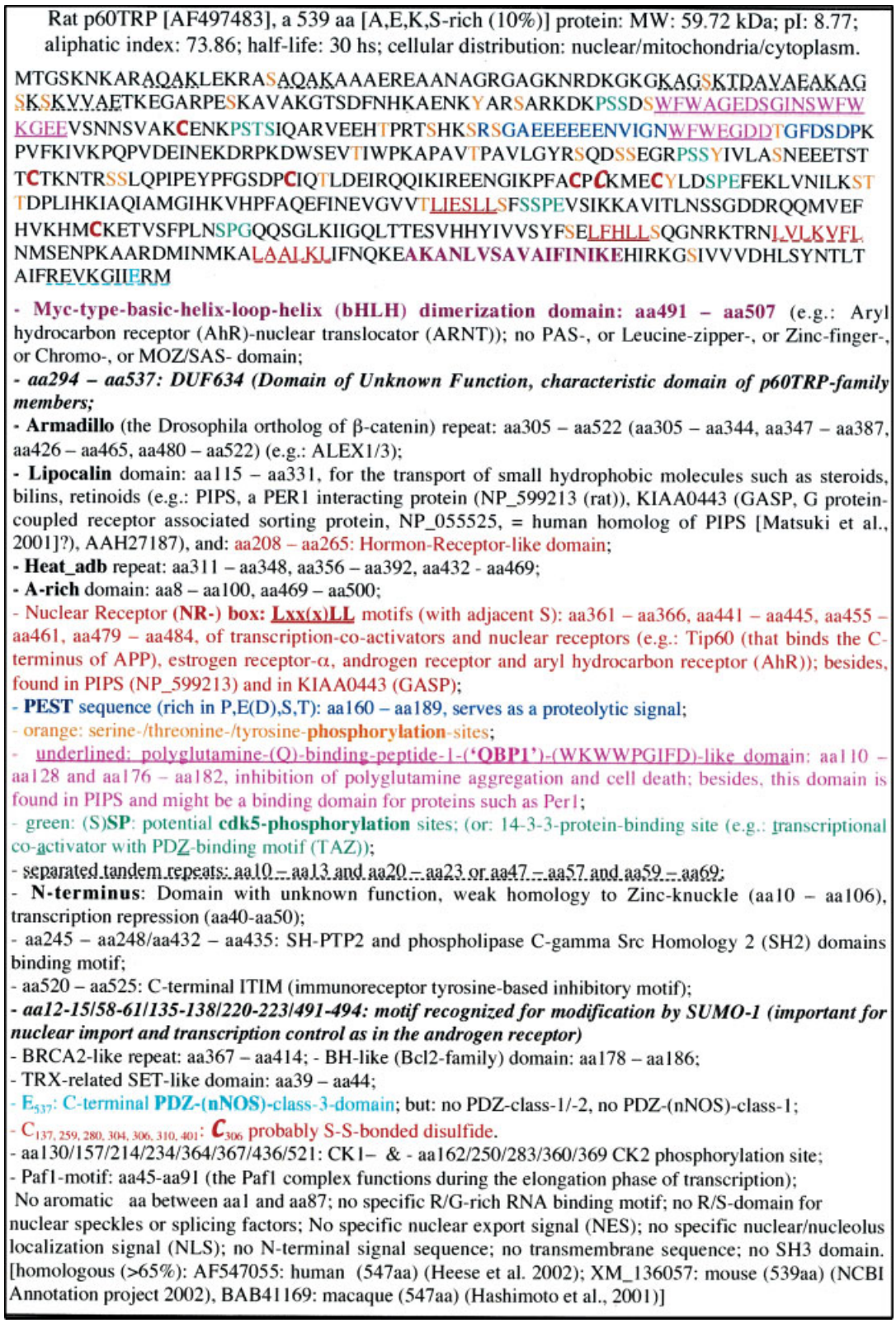
Expression of p60TRP in CHO Cells

To investigate the possible physiological function of p60TRP, we transfected CHO cells with a p60TRP-GFP- or p60TRP-DsRed1-fusion protein. The characterization by fluorescence microscopy demonstrates that p60TRP is particularly located in the cytoplasm (Fig. 5A,B). However, as shown in Figure 5C–F, it could also be detected in the nucleus of the cell.

Survival Assay Upon Serum Withdrawal and by Using Small Interfering RNA (siRNA) and cDNA Subtraction Analysis

To investigate the possible (patho-) physiological function of p60TRP, we applied a 'classical' apoptosis assay (serum-withdrawal), the siRNA- and the cDNA-subtraction technologies. On the one hand, p60TRP could not significantly protect neuronal PC12 cells from cell death induced by serum-withdrawal (Fig. 6A); on the other hand, we demonstrate in Figure 6B that 21-nucleotide siRNA duplexes specifically suppressed endogenous *p60TRP* gene expression in rat neuronal PC12 cells. This specific *p60TRP* mRNA suppression resulted in neuronal cell death. Additionally, we studied the functional role of p60TRP by analyzing the (patho-) physiological outcome of neuronal p60TRP-expression and its effect on NGF-induced neurite outgrowth. Expression of p60TRP in neuronal PC12 cells neither induced (not shown) nor inhibited neuronal neurite outgrowth (Fig. 7).

To further explore the possible mechanism of p60TRP-action during apoptosis, we performed the cDNA-subtraction analysis and found two proteins (Table IV and Fig. 8) potentially engaged in the p60TRP signaling cascade: (a) the new neurotrophin NNT1 [Senaldi et al.,



Rat p60TRP [AF497483], a 539 aa [A,E,K,S-rich (10%)] protein: MW: 59.72 kDa; pI: 8.77; aliphatic index: 73.86; half-life: 30 hs; cellular distribution: nuclear/mitochondria/cytoplasm.

MTGSKNKARAQAKLEKRASAQAKAAAEREAANAGRGAGKNRDKGKGKAGSKTDAVAEAKAG
SKSKVVAETKEGARPESKAVAKGTSDFNHKAENKYARSARKDKPSSDSWFWAGEDSGINSWFW
KGEEVSNNSVAKCENKPSTSIQARVEEHTPRTSHKSRSGAEEEEEENVIGNWFWEGDDTGFDSDPK
PVFKIVKPQPVDEINEKDRPKDWSEVTIWPKAPAVTPAVLGYRSQDSSEGRPSSYIVLASNEEETST
TCTKNTRSSLQPIPEYPFGSDPCIQTLDEIRQQIKIREENGIKPFACPCKMECYLDSPEFEKLVNILKST
TDPLIHKIAQIAMGIHKVHPFAQEFINEVGVVTLIESLLSFSSPEVSIKKAVITLNSSGDDRQQMVEF
HVKHMCKETVSFPLNSPGQQSGLKIIGQLTTESVHHYIVVSYFSELFHLLSQGNRKTRNLVLKVFL
NMSENPKAARDMINMKALAALKLIFNQKEAKANLVSAVAIFINIKEHIRKGSIVVVDHLSYNTLT
AIFREVKGIIERM

- **Myc-type-basic-helix-loop-helix (bHLH) dimerization domain: aa491 – aa507** (e.g.: Aryl hydrocarbon receptor (AhR)-nuclear translocator (ARNT)); no PAS-, or Leucine-zipper-, or Zinc-finger-, or Chromo-, or MOZ/SAS- domain;
- ***aa294 – aa537: DUF634 (Domain of Unknown Function, characteristic domain of p60TRP-family members;***
- **Armadillo** (the Drosophila ortholog of β-catenin) repeat: aa305 – aa522 (aa305 – aa344, aa347 – aa387, aa426 – aa465, aa480 – aa522) (e.g.: ALEX1/3);
- **Lipocalin** domain: aa115 – aa331, for the transport of small hydrophobic molecules such as steroids, bilins, retinoids (e.g.: PIPS, a PER1 interacting protein (NP_599213 (rat)), KIAA0443 (GASP, G protein-coupled receptor associated sorting protein, NP_055525, = human homolog of PIPS [Matsuki et al., 2001]?), AAH27187), and: aa208 – aa265: Hormon-Receptor-like domain;
- **Heat_adb** repeat: aa311 – aa348, aa356 – aa392, aa432 - aa469;
- **A-rich** domain: aa8 – aa100, aa469 – aa500;
- Nuclear Receptor (**NR-**) **box: Lxx(x)LL** motifs (with adjacent S): aa361 – aa366, aa441 – aa445, aa455 – aa461, aa479 – aa484, of transcription-co-activators and nuclear receptors (e.g.: Tip60 (that binds the C-terminus of APP), estrogen receptor-α, androgen receptor and aryl hydrocarbon receptor (AhR)); besides, found in PIPS (NP_599213) and in KIAA0443 (GASP);
- **PEST** sequence (rich in P,E(D),S,T): aa160 – aa189, serves as a proteolytic signal;
- orange: serine-/threonine-/tyrosine-**phosphorylation**-sites;
- underlined: polyglutamine-(Q)-binding-peptide-1-(**'QBP1'**)-(WKWWPGIFD)-like domain: aa110 – aa128 and aa176 – aa182, inhibition of polyglutamine aggregation and cell death; besides, this domain is found in PIPS and might be a binding domain for proteins such as Per1;
- green: (S)**SP**: potential **cdk5-phosphorylation** sites; (or: 14-3-3-protein-binding site (e.g.: transcriptional co-activator with PDZ-binding motif (TAZ));
- separated tandem repeats: aa10 – aa13 and aa20 – aa23 or aa47 – aa57 and aa59 – aa69;
- **N-terminus**: Domain with unknown function, weak homology to Zinc-knuckle (aa10 – aa106), transcription repression (aa40-aa50);
- aa245 – aa248/aa432 – aa435: SH-PTP2 and phospholipase C-gamma Src Homology 2 (SH2) domains binding motif;
- aa520 – aa525: C-terminal ITIM (immunoreceptor tyrosine-based inhibitory motif);
- ***aa12-15/58-61/135-138/220-223/491-494: motif recognized for modification by SUMO-1 (important for nuclear import and transcription control as in the androgen receptor)***
- BRCA2-like repeat: aa367 – aa414; - BH-like (Bcl2-family) domain: aa178 – aa186;
- TRX-related SET-like domain: aa39 – aa44;
- E_{537}: C-terminal **PDZ-(nNOS)-class-3-domain**; but: no PDZ-class-1/-2, no PDZ-(nNOS)-class-1;
- $C_{137, 259, 280, 304, 306, 310, 401}$: C_{306} probably S-S-bonded disulfide.
- aa130/157/214/234/364/367/436/521: CK1– & - aa162/250/283/360/369 CK2 phosphorylation site;
- Paf1-motif: aa45-aa91 (the Paf1 complex functions during the elongation phase of transcription);

No aromatic aa between aa1 and aa87; 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 specific nuclear/nucleolus localization signal (NLS); no N-terminal signal sequence; no transmembrane sequence; no SH3 domain. [homologous (>65%): AF547055: human (547aa) (Heese et al. 2002); XM_136057: mouse (539aa) (NCBI Annotation project 2002), BAB41169: macaque (547aa) (Hashimoto et al., 2001)]

Fig. 1. Characteristic features of p60TRP.

TABLE II. p60TRP-Protein-Family Members

O43168	Q96D09	Q9BVZ3	Q8IZC3
Q9CVV3	Q9H969	Q920R4	Q91VP8
Q9BE11	Q9C0G2	Q9CXQ7	Q91VZ8
Q9UJC4	Q96LA1	O60267	Q83103
Q9NPE4/Q9UH62	Q9NTS2	Q9BTM6	Q8R0B3
Q9H2Q0	Q9P291	Q9NWJ3	Q8R095
Q9CXI9	Q9DC32	Q9CZ87	Q8NAB4
Q9CUN3	Q9D0L7	Q9CS81	Q8N8W9
Q9CX83	Q8BHS6	Q8BJ81	Q8N2F6
Q8BJ82	Q8BTE8	Q8BTE9	Q8K3A6
Q8BYK9	Q8CHF4	Q8IZC1	Q8K2R3
Q8IZC2			

Protein-family members of p60TRP (numbers referred to: European Bioinformatics Institute (EBI)). Protein family was generated at 'EBI' according to Enright et al. [2002]; all proteins are unknown and have at least one unknown functional domain (DUF634) in common with each other.

1999] and (b) the new zinc-finger transcription factor p48ZnF [Gregory et al., 2000].

DISCUSSION

A number of eukaryotic transcription regulators share a conserved HLH domain that mediates protein-dimerization and they are classified into discrete categories according to dimerization, DNA binding and developmental-/tissue-expression characteristics. The HLH proteins lacking the basic domain (for instance: Emc (extramacrochaetae protein), Id (inhibitor of DNA-binding)) function as negative regulators since they form heterodimers, but fail to bind DNA. The hairy-related proteins (hairy, E(spl), deadpan) also repress transcription although they can bind DNA [Atchley and Fitch, 1997].

Cell death by apoptosis comprises a sequence of events leading to the activation of cysteine proteases, called caspases, that are synthesized as inactive precursors and that are proteolytically processed to generate active subunits until caspase-3, the final executioner, is activated. The mitochondrial pro-apoptotic protein Smac/Diablo (second mitochondria-derived activator of caspases) has recently been shown to potentiate apoptosis by counteracting the anti-apoptotic function of the inhibitor of apoptosis proteins (IAPs) (Fig. 8) [Leist and Jäättelä, 2001; Shi, 2002].

In the present study, we describe p60TRP as a new bHLH containing transcription-regulator that belongs to a new class of protein family. Our data indicate that p60TRP might be involved in regulating neuronal survival through its interaction with RanBP5 and PP2A, two important enzymes in intracellular signaling:

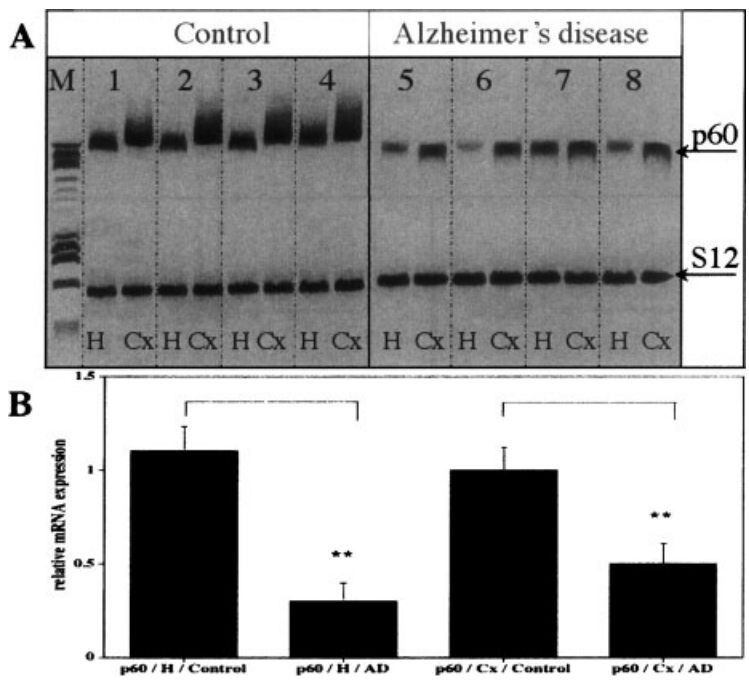


Fig. 2. Southern blot analysis of *p60TRP*mRNA expression in the brain of Alzheimer's disease (AD) patients and control subjects. **A**: analysis of polymerase chain reaction (PCR)-products; **left**: control group, **right**: AD group; **top**: *p60TRP* PCR-products, **bottom**: PCR-products of reference-gene *S12*. H, hippocampus; pCx, parietal cortex; M, DNA molecular weight marker; 1–8, patient number 1–8 of Table I. **B**: quantitation of *p60TRP* transcripts. Values are the ratio of densitometric scores for *p60TRP*- and *S12*- PCR-products ± standard error of the mean (SEM, $^{**}P < 0.01$, compared to controls).

TABLE III. P60TRP-Interacting Proteins

P60TRP-interacting proteins	Locus ID/GenBank accession number
PP2A (regulatory subunit-B56 (epsilon))	#5529/NM_006246
RanBP5	#3843/NM_002271

The yeast-two-hybrid-system analysis reveals two new P60TRP-interacting proteins.

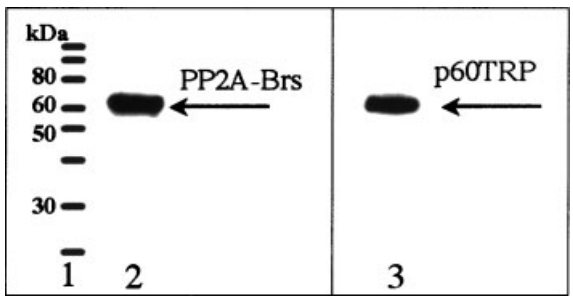


Fig. 3. Immunoprecipitation and Western blot analysis of PP2A. Western blotting was performed as described in Materials and Methods. **Lane 1**: Molecular weight marker. **Lane 2**: Protein precipitation from PC12 cells transfected with p60TRP-IRES-GFP and detected with polyclonal goat anti-PP2A (regulatory subunits) antibody. In **lane 3**, a single band at approximately 60 kDa could be detected with a polyclonal rabbit anti-p60TRP antibody.

1) RanBP5 is a recently identified protein that belongs to the importin-β-like transport receptors—(namely importin-β itself, transportin, RanBP5 and RanBP7)—and

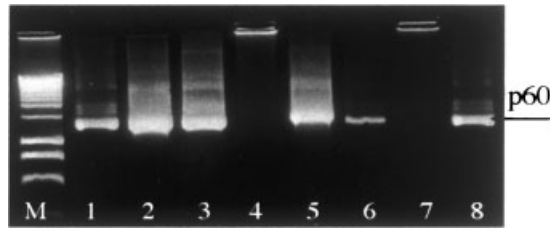


Fig. 4. Expression of *p60TRP* mRNA in various tissues: mRNA expression analysis by RT-PCR. **Lane 1**: Heart; **lane 2**, brain; **lane 3**, kidney; **lane 4**, liver; **lane 5**, spleen; **lane 6**, pancreas; **lane 7**, lung; **lane 8**, skeletal muscle.

it is an important protein involved in transporting HLH-transcription factors (for instance the sterol regulatory element-binding protein-2 (SREBP-2)) from the cytoplasm into the nucleus [Deane et al., 1997; Nagoshi and Yoneda, 2001]. Like importin-β, RanBP5 strongly binds the GTP-bound form of Ran. Importin-β, the key mediator of nuclear transport, is one of the best characterized import receptors and has been found to be unique in the receptor family because it recognizes nuclear proteins not only via adapter molecules, such as importin-α, but also directly. SREBP-2 is transported through the direct interaction with importin-β in a Ran-dependent manner. The importin-β-binding domain of

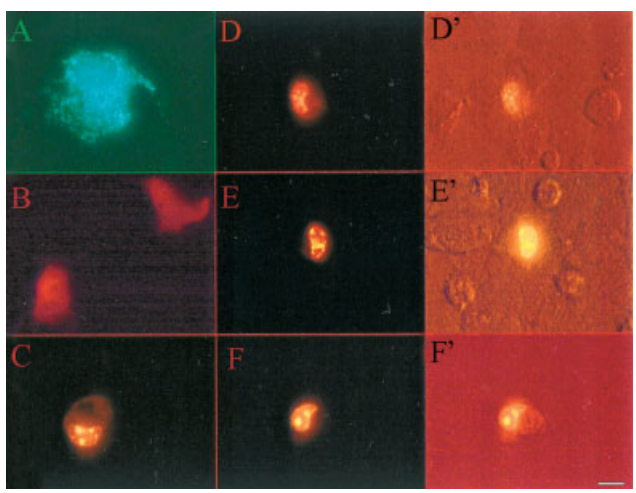


Fig. 5. Fluorescence localization of p60TRP-GFP and p60TRP-DsRed1 expression in CHO cells. CHO cells transiently transfected with p60TRP-GFP or p60TRP-DsRed1 were visualized directly by p60TRP-GFP-green or p60TRP-red fluorescence showing a cytoplasmic (**A**) p60TRP-GFP, (**B**) p60TRP-DsRed1 or nuclear p60TRP expression pattern (**C**–**F**) p60TRP-DsRed1; (**D′**–**F′**) same as in D–F but using more light to show the cellular context and to indicate that the fluorescent signal is located in the nucleus). The bar indicates 50 μm.

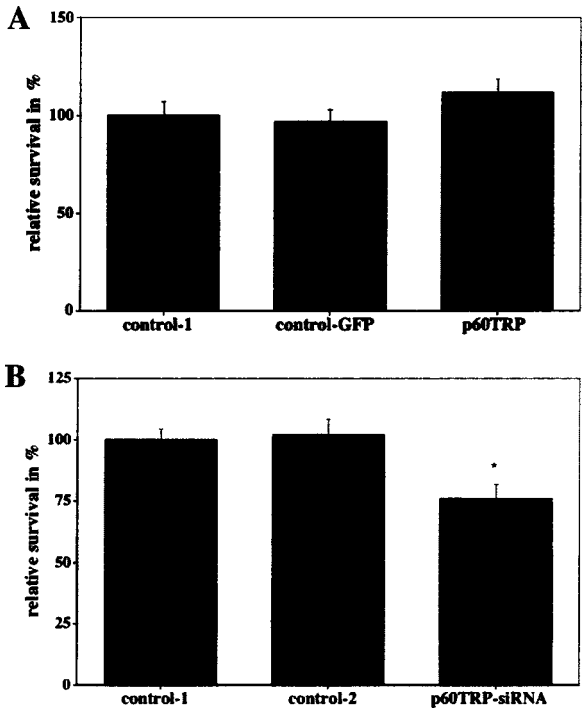


Fig. 6. Effect of p60TRP expression on survival/proliferation of PC12 cells. ELISA-Cell-Titer 96® AQ_{ueous} Assay (Promega, Madison, WI). Cells were incubated as described in Material and Methods. **A**: Serum-deprivation assay. **B**: siRNA assay. Data are shown as mean ± SEM of eight independent experiments, each done in duplicate (*$P < 0.05$, compared to controls).

SREBP-2 was mapped on the HLH-domain, which serves as a nuclear localization signal (NLS).

2) PP2A is a key-enzyme in intra-cellular signaling and, particularly, a key-phosphatase in regulating tau-phosphorylation. The importance of phosphorylation and dephosphorylation in intracellular signaling pathways has long been recognized, although attention has focused mainly on kinases and, therefore, phosphatases have been neglected for a long time. Recent studies have highlighted the importance of serine/threonine protein-phosphatases in many processes including apoptosis [Janssens and Goris, 2001]. PP2A is a heterotrimer that contains a catalytic C-subunit, a structural A-subunit, and a regulatory B-subunit. Recent results indicate that expression of the PP2A regulatory subunit-B promotes neuronal differentiation by activating the MAP kinase cascade [Strack, 2002] and that the regulatory B56-subunit (highly expressed in brain) is a candidate regulatory subunit of the physiologic Bcl2 phosphatase [Li et al., 2002]. The phosphorylation state of anti-apoptotic ((B-cell-lymphoma-2) Bcl-2, Bcl-X(L)) and pro-apoptotic (BAD, Bid, Bik) Bcl-2 proteins regulates their cellular activity and, therefore, cell survival and cell death. Interestingly, it has been reported that PP2A function is disturbed in AD leading to hyperphosphorylation of tau. To address the role of PP2A in AD brain, Kins et al. produced transgenic mice that expressed a dominant negative mutant form of PP2A [Kins et al., 2001]. In these mice the PP2A activity was reduced twofold and endogenous tau was

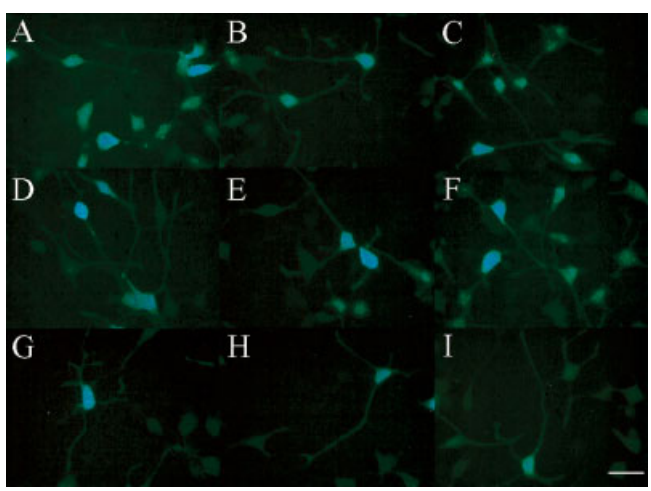


Fig. 7. P60TRP does not inhibit NGF-induced neurite outgrowth in neuronal PC12 cells. Co-expression of p60TRP and GFP (p60TRP-IRES-GFP) in PC12 cells. Twenty-four hours post-transfection, cells were treated for 120 h with NGF (50 ng/ml). **A–I**: Eight cell culture dishes were treated by NGF separately; thus, avoiding observations based on artifacts. Scale bar represents 50 μm.

TABLE IV. New Proteins Probably Involved in p60TRP-Signaling

Proteins	Locus ID/GenBank accession number
Rat NNT-1	#56708 (mouse), 23529 (human)/AY365006 (rat)[a]
Rat p48ZnF	#69082 (mouse)/AY377983 (rat)[a]

[a]Submitted by Heese et al. [2003].

hyperphosphorylated and co-localized with ubiquitin—thus targeted for degradation. Moreover, it has been demonstrated that hyperphosphorylation of tau is probably based on a general disturbed PP2A activity in AD [Vogelsberg-Ragaglia et al., 2001]. These results support the hypothesis that PP2A is involved in phosphorylation of tau in vivo and it suggests that a defect in PP2A activity contributes to the pathogenesis in AD.

Interestingly, the B56-associated PP2A is required for survival and protects from apoptosis in *Drosophila melanogaster*. Global knockdown of PP2A activity or specific loss of redundant B56 regulatory subunits caused cell death with the morphological and biochemical changes characteristic of apoptosis in cultured *Drosophila* S2 cells. B56:PP2A-regulated apoptosis required caspases and the upstream regulators dark, reaper, head involution defective, and dp53. In *Drosophila* embryos, knockdown of B56-regulated PP2A activity resulted in apoptosis and failure of gastrulation, an effect that was blocked by concurrent RNAi of the caspase DRICE: therefore, B56-regulated PP2A activity appears to be required upstream of dp53 to maintain a critical proapoptotic substrate in a dephosphorylated, inactive state, thereby preventing apoptosis in *Drosophila* S2 cells [Li et al., 2002]. In contrast to PP2A, ablation of protein phosphatase 4/5 caused only a very slight reduction in cell growth and had no effect on MAP kinase signaling, cell growth, or apoptosis. These observations [Li et al., 2002] indicated that PP2A has a powerful anti-apoptotic activity that is specifically mediated by the B56 regulatory subunit.

Taking into account that importins may contain a HLH-domain (RanBP6), interact with PP2A [Lubert and Sarge, 2003] and function as cytoplasmic chaperones for exposed basic domains [Jäkel et al., 2002], it is tempting to speculate that p60TRP may also fulfill a versatile function as transcription regulator, nuclear import receptor and, just as heat shock proteins function as chaperones for exposed hydrophobic patches, p60TRP may act as an anti-aggregation chaperon.

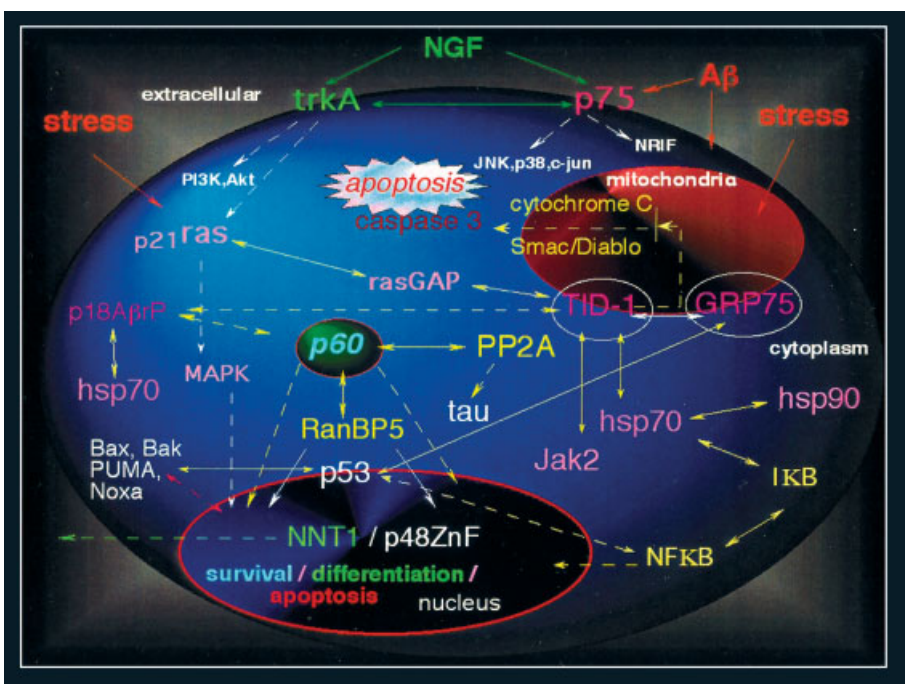


Fig. 8. Schematic illustration of p60TRP-activity during apoptotic processes induced, for instance, by stress or growth factor deprivation.

In conclusion, since p60TRP (a) could interfere with NNT1-/p48ZnF-/p18AβrP-mediated signaling (Fig. 8) [Heese et al., 2002a], (b) is down-regulated in the hippocampus/parietal cortex of early stage AD brain, and (c) interacts with PP2A/RanBP5, p60TRP appears to be involved in the regulation of neuronal aging and survival. Thus, the connection between p60TRP's PP2A- and RanBP5-binding activity and its other signaling functions will be an interesting topic for future investigations and further experiments are necessary to clarify p60TRP's physiological role and its possible function in neurodegenerative diseases.

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