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Both the N-terminal fragment and the protein–protein interaction domain (PDZ domain) are required for the pro-apoptotic activity of presenilin-associated protein PSAP

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Abstract

Presenilin-associated protein (PSAP) was originally identified as a PS1-associated, PDZ domain protein. In a subsequent study, PSAP was found to be a mitochondrial apoptotic molecule. In this study, we cloned the *PSAP* gene and found that it is composed of 12 exons and localizes on chromosome 6. To better understand the structure and function of PSAP, we have generated a series of antibodies that recognize different regions of PSAP. Using these antibodies, we found that PSAP is expressed in four isoforms as a result of differential splicing of exon 8 in addition to the use of either the first or the second ATG codon as the start codon. We also found that all these isoforms are localized in the mitochondria and are pro-apoptotic. Furthermore, our data revealed that the PDZ domain and N-terminal fragment are required for the pro-apoptotic activity of PSAP.

Keywords: Apoptosis; Mitochondria; Caspase; Cytochrome *c*; Cell death; Alzheimer's disease

1. Introduction

Apoptosis, one of the mechanisms of cell death, has been implicated in the neurodegeneration observed in Alzheimer's disease (AD) and other neurodegenerative disorders [1–7]. This apoptotic cell death has been reported to be affected by presenilins (presenilin-1 [PS1] and PS2. The involvement of presenilin in apoptosis was first suggested by a study showing that ALG-3, a mouse homolog of the C-terminal fragment of PS2, rescues a T-cell hybridoma from Fas-induced apoptosis [8]. Interestingly, as in the case of PS2, the C-terminal fragment of PS1 was also found to inhibit Fas-induced apoptosis in Jurkat cells [9]. In an effort to understand the apoptotic regulatory effect of the C-terminal of PS1, we have identified a novel presenilin-associated protein (PSAP), which interacts with the C-terminal of PS1 [10]. Interestingly, in a subsequent study we found that PSAP is a pro-apoptotic protein that causes apoptosis when it is overexpressed [11]. This finding provides a direct molecular link between presenilin and the apoptotic pathway and also places PSAP in an important position in apoptosis.

Two major pathways lead to apoptotic cell death: namely, the cell surface death receptor-mediated pathway and the mitochondria-mediated pathway [12]. In the death receptor-mediated pathway, extracellular stimuli activate apoptotic cascades via binding and activation of cell surface death receptors. On the other hand, in the mitochondria-mediated pathway, apoptotic cascades are activated by small molecules, such as cytochrome *c* and apoptosis-inducing factor (AIF), released from the mitochondrial intermembrane space into the cytoplasm. In this regard, it is notable that PSAP is localized in mitochondria and that overexpression of PSAP causes release of cytochrome *c* from mitochondria [11], suggesting that PSAP causes apoptosis through a mitochondria-mediated pathway.

Since the report of the identification of PSAP, several groups have reported the cDNAs for PSAP from different species by directly depositing the DNA sequence information into the

Abbreviations: AD, Alzheimer's disease; PS, presenilin; PSAP, presenilin-associated protein; siRNA, small interfering RNA; FISH, fluorescence in situ hybridization; SMART, Simple Modular Architecture Research Tool

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GenBank. Now the *PSAP* gene is also known as mitochondrial carrier homolog 1 (Mtch1). These DNA sequences revealed an important fact that some of the reported cDNAs are missing 51 nucleotides in the 3′-half of the coding region, suggesting the presence of different splicing isoforms of mRNA for PSAP. This information prompted us to determine the structures of the gene and protein of PSAP and their relationship with the biological function of PSAP. In the current work, we report that PSAP is expressed in two isoforms as a result of alternative splicing and that both isoforms are apoptotic active. We sought to discover whether these potential isoforms are indeed expressed under physiological conditions and whether these isoforms may have different biological functions. In addition, we sought to determine the relationship between the structure and function of PSAP.

2. Materials and methods

2.1. Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen). PSAP–myc fusion protein expression plasmid was constructed as described previously [11]. All deletion and truncation mutants were constructed using the site-directed mutagenesis kit (Stratagene).

2.2. Mitochondria isolation and proteinase treatment

Mitochondria were isolated as described previously [11]. Briefly, HEK 293 cells were harvested and washed with phosphate buffered saline (PBS) once and swelled with 10 volumes of Swell buffer A (20 mM HEPES, pH7.9, 1.5 mM NaCl, 10 mM KCl, 0.5 mM β-mercaptoethanol) for 5 min on ice. After 500 ×*g* centrifugation at 4 °C for 5 min, the cells were resuspended in 10 volumes of Swell buffer A and ruptured with 5 or 6 strokes with a Dounce Homogenizer using a tight-fitting pestle (pestle B). Sucrose (166.35 μl, 60%) was added into each 1 ml of ruptured cells to reach a final concentration of 250 mM (isoosmolar solution). After centrifugation at 800 ×*g* for 10 min at 4 °C to remove the unbroken cells and nuclei, the supernate was layered on top of the sucrose gradients consisting of 30%, 35%, 40%, 43%, 46%, 50%, 55% and 60% sucrose, each in 4 ml of 10 mM Tris–HCl, pH 7.4, 1 mM EDTA. The mixture was centrifuged at 60,000 ×*g* in a Beckman SW 28.1 Rotor for 45 min at 4 °C. Ten fractions or layers were collected using a 20-gauge needle, and the mitochondria-containing fractions were determined by Western blotting for the presence of cytochrome *c*. The intact mitochondria were collected from layers 3 and 4 (damaged or ruptured mitochondria go to the lower layer). After dilution with 3 volumes of distilled water, mitochondria were pelleted by centrifugation at 9600 ×*g* at 4 °C for 15 min. The pellet was then resuspended in Buffer B (250 mM sucrose, 10 mM Tris–HCl, pH 7.4). The integrity of mitochondria was determined by measuring the activity of citrate synthase in the presence and absence of 1% Triton X-100 (w/v), following the method of Robinson et al [13]. The presence of 1% Triton X-100 (w/v) has been shown to completely damage mitochondria [13,14]. After confirming their integrity, the intact mitochondria were treated with trypsin (20 μg/ml, at 37 °C), chymotrypsin (25 μg/ml, at 37 °C), and proteinase K (5 μg/ml, at 4 °C) in a time course. The ruptured mitochondria were prepared by passing the isolated mitochondria in solution B through a 22-gauge hypodermic needle 100 times; the complete rupture of mitochondria was confirmed by measuring the activity of citrate synthase.

2.3. Immunoprecipitation and Western blotting

For routine Western blot analysis, cells or purified mitochondria were lysed by sonication for 20 s on ice in Western blot lysis buffer (50 mM Tris–HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitor mixture from Roche Applied Science). The brain tissues from humans and other animals were first homogenized in Western blot lysis buffer using a Lab homogenizer (Polyton Kinematica, Switzerland) before sonication. After adding 4× SDS sample buffer and incubating at 65 °C for 15 min, samples were subjected to SDS-PAGE (8% for PARP and full-length PSAP, 10–14% for truncated PSAP mutants, 10–16% for cytochrome *c*) and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membranes were probed with appropriate antibodies (monoclonal antibodies against epitope myc (Santa Cruz), PARP (7D3–6, BD PharMingen), and cytochrome *c* (PharMingen) and visualized by ECL-Plus (GE Healthcare Biosciences) as described previously [11].

For co-immunoprecipitation, cells were harvested and homogenized in buffer A (30 mM HEPES, pH 7.5, 10 mM KCl, and protease inhibitor mixture) by passing through a 20-gauge needle 30 times. The homogenized samples were centrifuged at 800 ×*g* for 10 min to remove the unbroken cells and nuclei. The post nuclear supernates were centrifuged at 20,000 ×*g* for 1 h, and the resulting membrane pellets were resuspended in 1 ml IP buffer (1% CHAPSO, 30 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA containing cocktail protease inhibitors) and sonicated for 10 s on ice at 80% power employing a Fisher Sonic Dismembrator Model 300 with a 3.5 mm diameter tip. The lysates were cleared by centrifuging at 14,000 ×*g* for 5 min at 4 °C, and the supernates were subjected to co-immunoprecipitation using appropriate antibodies, followed by Western blot analysis as described previously [11].

2.4. Immunofluorescence microscopy

HEK293 cells were plated on poly-L-lysine-coated coverslips and grown in DMEM containing 10% fetal bovine serum. The wild type PSAP construct and its mutants were transfected into HEK293 cells on the second day. 12 h after transfection, the medium was replaced with fresh medium containing 100 nM MitoTracker Red (Invitrogen, Carlsbad, CA) and incubated for 30 min to label the mitochondria. Cells were fixed in pre-warmed 3.7% paraformaldehyde in PBS at 37 °C for 20 min, followed by permeablization with 0.2% Triton X-100 for 4 min. The fixed and permeablized cells were washed four times with PBS and blocked with 1% bovine serum albumin and 5% normal goat serum in PBS for 45 min. The coverslips were incubated overnight with a monoclonal anti-myc antibody (1:130, Santa Cruz Biotechnology, Santa Cruz, CA.), and then rinsed four times with PBS. After incubation with green-fluorescent Alexa Fluor 488 dye conjugated goat anti-mouse IgG (1:150, Invitrogen) for 1 h, the coverslips were washed four times with PBS and mounted with Supermount mounting medium (BioGenex, San Ramon, CA). Coverslips were examined under a fluorescence microscope (Nikon Eclipse E600) equipped with a digital camera.

2.5. Genomic DNA isolation and fluorescence in situ hybridization (FISH)

To isolate the genomic DNA for PSAP, a human genomic DNA bacteriophage P1 library (Genome Systems, Inc., St. Louis, MO) was screened by PCR using a pair of primers designed based on the cDNA sequence of PSAP (custom service provided by Genome Systems, Inc., St. Louis, MO). One of the two clones, which contain the full-length genomic DNA for PSAP, was used as a probe in a FISH analysis to determine the chromosomal localization of the *PSAP* gene (custom service provided by Genome Systems, Inc., St. Louis, MO). DNA was labeled with digoxigenin-dUTP by nick translation. The labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from human phytohemagglutinin-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated anti-digoxigenin antibodies followed by counterstaining with 4,6-diamidino-2-phenylindole.

2.6. RNA interference

A small interfering RNA (siRNA) duplex was generated by Qiagen, Inc. (Valencia, CA) against the mouse *PSAP* (CTGGGATTCTTCGTTGGCTTA),