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Short communication

Ectopic Expression of Mitochondria Endonuclease Pnu1p from *Schizosaccharomyces pombe* Induces Cell Death of the Yeast

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Endonuclease G (EndoG) is a mitochondrial non-specific nuclease that is highly conserved among the eukaryotes. Although the precise role of EndoG in mitochondria is not yet known, the enzyme is released from the mitochondria and digests nuclear DNA during apoptosis in mammalian cells. *Schizosaccharomyces pombe* has an EndoG homolog Pnu1p (previously named *Sp*Nuc1) that is produced as a precursor protein with a mitochondrial targeting sequence. During the sorting into mitochondria the signal sequence is cleaved to yield the functionally active endonuclease. From the analogy to EndoG, active extramitochondrial Pnu1p may trigger cell killing by degrading nuclear DNA. Here, we tested this possibility by expressing a truncated Pnu1p lacking the signal sequence in the extramitochondrial region of *pnu1*-deleted cells. The truncated Pnu1p was localized in the cytosol and nuclei of yeast cells. And ectopic expression of active Pnu1p led to cell death with fragmentation of nuclear DNA. This suggests that the Pnu1p is possibly involved in a certain type of yeast cell death *via* DNA fragmentation. Although expression of human Bak in *S. pombe* was lethal, Pnu1p nuclease is not necessary for hBak-induced cell death.

Keywords: Cell death, Ectopic expression, Endonuclease, Mitochondria

Introduction

Endonuclease G (EndoG) is a non-specific DNA/RNA nuclease in mitochondria that is highly conserved among the eukaryotes. EndoG is encoded by a nuclear genome and was originally thought to be involved in the replication and maintenance of the mitochondrial genome (Low, 2003, Irvine *et al*., 2005; Huang *et al*., 2006). Recently, it was shown that EndoG is released from the mitochondria and translocates to the nucleus during apoptosis in mammalian and a nematode *C. elegans* cells (Li *et al*., 2001; Parrish *et al*., 2001; van Loo *et al*., 2001). EndoG digests nuclear DNA as an apoptotic nuclease in the absence of caspase activity or the caspase-activated DNase. In healthy cells degradation of nucleic acids by the mature nuclease is prevented through the compartmentalization of EndoG in the mitochondrial intermembrane space (Ohsato *et al*., 2002).

A fission yeast *Schizosaccharomyces pombe* has an EndoG homolog Pnu1p (otherwise known as *Sp*Nuc1), which is encoded by the nuclear genome and produced as a 36.4 kDa precursor protein with a mitochondrial targeting sequence at its N-terminus (Ikeda and Kawasaki, 2001; Nakashima *et al*., 2002; Sakem and Kohli, 2007). During sorting into mitochondria the signal sequence is cleaved by a certain proteinase to yield the functionally active endonuclease of 32 kDa. The full-length recombinant Pnu1p exhibited very little endonuclease activity by a zymogram assay, but N40d-Pnu1p with truncation of N-terminal 40 amino acids degraded DNA and RNA in a non-specific manner depending on Mg^{2+} (Ikeda and Kawasaki, 2001). The precise role of Pnu1p in the yeast mitochondria is not yet known. In analogy with the case of EndoG, the endonuclease activity may be involved in degradation of nuclear DNA during cell death of the yeast. In this study, we tested this possibility by expressing the mature and endonuclease active Pnu1p in the extramitochondrial region of *pnu1*-disrupted cells (*pnu1*Δ). The truncated Pnu1p was localized in the cytosol and nuclei, and ectopic expression of active Pnu1p triggered cell killing. This indicates that Pnu1p is possibly involved in a certain type of yeast cell death *via* fragmentation of nuclear DNA.

Materials and Methods

Strains and media. Yeast strains used in this study are derivatives of the wild type strains ED0665 (ATCC no. 96993; h^-, *ade6*-M210,

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leu1-32, *ura4*-D18). The strains were routinely grown on YE medium (0.5% yeast extract and 3% glucose) or SD medium (0.67% yeast nitrogen without amino acids and 2% glucose) containing appropriate supplements. To stain dead cells on an agar plate phloxine B was added to the medium to a final concentration of 5 mg/ml.

Disruption of *pnu1* genes. The gene cassette for disruption of the *pnu1* gene was produced by cloning the *ura4*$^+$ fragment into a pGEM-T plasmid (Promega, Madison, USA) with the *pnu1* genomic sequence (pGEN-pnu1). Briefly, the pUR18 plasmid (ATCC no. 77297) was digested with *Cla*I, and the recessive ends were filled with KOD polymerase (TOYOBO, Osaka, Japan). A 1.7-kb fragment containing the *ura4*$^+$ gene was excised by *Hin*dIII digestion and subcloned into pGEM-pnu1, which had been successively processed by *Bsm*FI digestion, blunting of the ends, and *Hin*dIII digestion, in progress. The *pnu1*::*ura4* DNA fragment was excised from the plasmid by *Mun*I and *Bst*EII digestion, separated from the vector by electrophoresis, and used to transform haploid strain ED0665. Clones that were *ura*$^+$ were selected by PCR and Southern analysis.

Localization of Pnu1p. The entire coding region of *pnu1* cDNA was amplified by PCR with a primer set (forward, 5'-cgc ggc cat ATG TCT AGT AAT CTT ATC CAA-3'; reverse, 5'-c ggc tcg agc TTT ACC ATG ATT CTT TTG T-3'), and cloned into pREP41GFPC (Craven *et al*., 1998) using the *Nde*I and *Xho*I sites. The resulting plasmid, pPnu1-GFP, expresses Pnu1p fused with C-terminal GFP using the thiamine-repressible *nmt1* promoter. To make a truncated Pun1p (N40d) with GFP fusion, PCR was carried out using another forward primer 5'-g tgc agc cat ATG AGG TAT ACC AAA TTT GAC-3'. Recombinant plasmids were introduced into *S. pombe* cells using the EZ-yeast transformation kit (QbioGene, Carlsbad, USA). For visualization of Pnu1p-GFP, cultures of *S. pombe* ED0665 carrying the GFP plasmid were grown in EMMG medium containing appropriate supplements in the absence of thiamine at 28°C. Cells were examined using a fluorescence microscope (Axioskop, Carl Zeiss MicroImaging GmbH, Germany) equipped with a cooled CCD camera (Quantix 1400, PhotoMetrics Inc., Huntington Beach, USA).

Plasmid construction for expression of Pnu1p and hBak in *S. pombe* cells. Full length of Pnu1p was expressed using the pREP41GFPC vector by replacing the GFP-coding region with *pnu1* cDNA. The pREP41GFPC was successively processed by digestion with *Nco*I, blunting the ends with KOD polymerase, and *Nde*I digestion. The entire coding region of *pnu1* cDNA was amplified by PCR with a primer set (forward, 5'-g gct cgc cat ATG TCT AGT AAT CTT ATC-3'; reverse, 5'-tc ccc cgg gta TCA TTT ACC TTG ATT C-3'), digested with *Nde*I and *Sma*I, and ligated with the vector. To express the truncated Pun1p (N40d), the DNA fragment encoding this region was amplified by PCR using a primer set (forward, 5'-at ccg ctc gag ATG AGG TAT ACC AAA TTT GAC-3'; reverse, 5'-cg cgg atc cta TCA TTT ACC TTG ATT C-3') and cloned into pREP41X (ATCC no. 87605) using the *Xho*I and *Bam*HI sites. The cDNA of hBak was purchased from Open Biosystems (Clone ID 2819507; Huntsville, USA). To express the hBak protein, the entire coding region of the cDNA was amplified by PCR using a primer set (forward, 5'-at aag ctc gag ATG GCT TCG GGG CAA GGC-3'; reverse, 5'-cgc gga tcc TCA TGA TTT GAA GAA TCT TC-3') and cloned into pREP41X using the *Xho*I and *Bam*HI sites.

Immunological detection of Pnu1p and hBak expressed in yeast cells. *S. pombe* cells carrying the expression plasmid were grown at 28°C in SD medium with appropriate supplements. After washing the cells with phosphate buffered saline (PBS) the recombinant proteins were expressed in the absence of thiamine by culturing the cells in EMMG medium at 28°C overnight. A whole cell extract of *S. pombe* was prepared by disrupting the cells using glass beads in a sampling buffer for SDS-polyacrylamide gel electrophoresis. Antibody against Pnu1p was prepared by immunization of rabbits with a recombinant protein of Pnu1p expressed in *E. coli* using the pET16b vector (Novagen). Monoclonal antibody against hBak (TC-100) was purchased from BIO-MOL International, L.P. (Plymouth Meeting, USA). Proteins were fractionated by SDS-PAGE (12.5% polyacrylamide), and transferred onto Hybond-P blotting membrane (GE Healthcare Bio-Sciences Corp., Piscataway, USA). The Pnu1p band was visualized using anti-Pnu1p antibody (1 : 1,000), horseradish peroxidase-labeled anti-rabbit Ig antibody (1 : 2,000) and ECL Western Blotting Detection Reagents Kit (GE Healthcare Bio-Sciences). The hBak band was visualized using anti-hBAK antibody (1 : 1,000) and horseradish peroxidase-labeled anti-mouse Ig antibody (1 : 2,000). Imaging of the chemiluminescence was performed with a LAS-1000 Plus luminescent image analyzer system (FUJIFILM, Tokyo, Japan).

Viability test. Cells were grown at 28°C overnight in SD medium. They were washed with PBS, resuspended at a density of 1.0×10^8 cells/ml, and diluted 10-fold with PBS. Dilutents (10 µl) containing 10 to 10^6 cells were spotted on the EMMG agar plate with or without thiamine (10 µM). Photographs of colonies were taken after 3 to 4 days of growth at 28°C.

TUNEL assay. The *S. pombe* cells (about 10^7) were washed twice with PBS, resuspended with 0.1 ml of 3.7% formaldehyde, and fixed at 4°C for 1 h. After washing three times with PBS, the cells were resuspended with 0.5 ml sorbitol buffer (1.2 M sorbitol, 50 mM Tris-HCl, pH 7.5, 100 mM EDTA) containing 28 µg/ml zymolyase 20T (Seikagaku Corp., Tokyo, Japan), and incubated at 37°C for 2 h, The cells were washed with PBS, incubated in 1 ml of a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, and washed twice with PBS. The TUNEL reaction with TdT and immunological detection of incorporated dUMP by FITC-labeled antibody were carried out using a kit purchased from CHEMICON International Inc. (ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit; Temecula, USA) according to the supplier's instruction manual. The cells were then applied to microscopic slides and examined using a fluorescence microscope.

Results and Discussion

Intracellular localization of full-length and truncated Pnu1p was observed by fusion with GFP-tag using fluorescence microscopy. Expression of full-length Pnu1p-GFP in the yeast

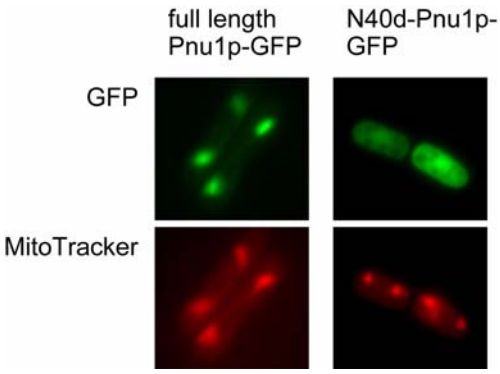

Fig. 1. Localization of Pnu1p in *S. pombe* cells. Full length and truncated (N40d) Pnu1p with a GFP tag at their C-terminals were expressed in *S. pombe* cells, and the localization of each protein was then observed. The cells were also stained with MitoTracker Red CMXRos (Invitrogen Corp., Carlsbad, CA) to visualize the mitochondria as described previously (Ikeda and Kawasaki, 2001). (left panels) full length Pnu1p-GFP, (right panels) N40d Pnu1-GFP.

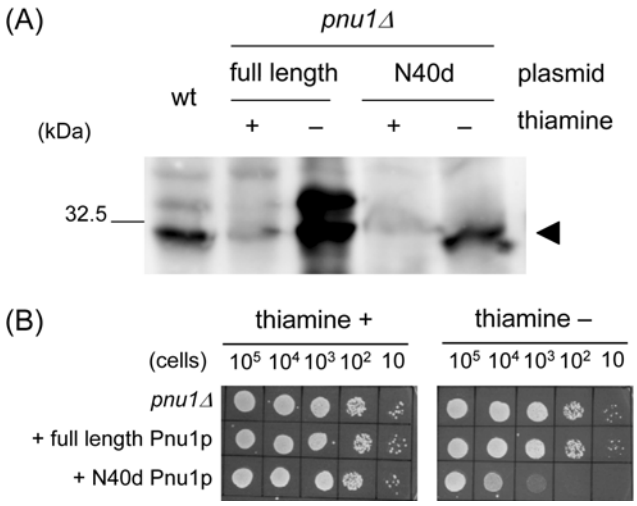

Fig. 2. Expression of Pnu1p and N40d Pnu1p in *pnu1*Δ cells and the effect of the expression of these proteins on cell survival. (A) Plasmids for expression of Pnu1p and N40d-Pnu1p were introduced into *S. pombe* cells lacking the *pnu1* gene, and the cells were cultured in EMMG medium containing 60 μg/ml adenine and 20 μg/ml uracil in the presence or absence of thiamine (10 μM) at 28°C overnight. Whole cell extracts were separated on SDS-PAGE and Pnu1p was immunologically detected using anti-Pnu1p antibody. Left lane was the positive control, i.e. cell extract from wild type cells. (B) The *pnu1*Δ cells carrying the plasmid for expression of Pnu1p and N40d-Pnu1p were serially diluted and spotted on an EMMG agar plate with or without thiamine (10 μM). The cells were cultured at 28°C for 3 days.

cells, as previously described (Ikeda and Kawasaki, 2001), leads to the mitochondrial accumulation of the fusion protein (Fig. 1). In contrast truncated Pnu1p lacking N-terminal 40 amino acids (N40d-Pnu1p-GFP) was found in the cytosol and nucleus of the yeast cells. This indicated that a mitochondrial targeting sequence of Pnu1p is present within the N-terminal 40 amino acids. Therefore, N-terminal truncation of Pnu1p leads to disclosure of its endonuclease activity while remaining in an extramitochondrial region.

Full length and truncated Pnu1p were expressed under the regulation of the *nmt1* promoter in *pnu1*Δ cells. Immunoblots using anti-Pnu1p antibody showed that Pnu1p in wild type cells is present as a matured mitochondrial form of 32 kDa (Fig. 2A). In the presence of thiamine no Pnu1p band was observed in *pnu1*Δ cells. By removing thiamine from the medium the cells carrying the plasmid of full length *pnu1* cDNA produced two forms of Pnu1p: unprocessed form (36 kDa) and the matured form. The former form probably remains in the cytosol and exhibits no endonuclease activity, because removal of the N-terminal signal sequence is required for activation of the endonuclease activity (Ikeda and Kawasaki, 2001). Cells carrying truncated *pnu1* cDNA expressed N40d-Pnu1p with nearly the same size as that of the matured form.

The viability of cells expressing full length or truncated Pnu1p was measured on agar plates with or without thiamine (Fig. 2B). The *pnu*1Δ cells expressing full length Pnu1p exhibited the same viability as did the cells without plasmid. In contrast the expression of N40d-Pnu1p, which is localized to the extramitochondrial region, extremely reduced cell viability. And phloxin B stained only the cells expressing the truncated Pnu1p to pink (data not shown). Cell staining *in situ* with the TUNEL assay was employed to examine whether the endonuclease activity of Pnu1p is essential or not for cell death induced by ectopic expression of truncated Pnu1p. Cleavage of DNA by EndoG and its homologs produces free 3'-OH termini, which can be effectively labeled by fluorescently tagged nucleotides in a process catalyzed by terminal deoxynucleotidyl transferase. The majority (over 50%) of the N40d-Pnu1p-expressing cells have an intense nuclear stain, corresponding to a strong fragmentation (Fig. 3, upper). Control cells in the presence of thiamine show no detectable fluorescence. These results indicated that the truncated Pnu1p in the cytosol and nuclei leads to cell killing probably *via* endonuclease activity. A similar result was obtained for mammalian EndoG (Schafer *et al*., 2004). The expression of bovine EndoG in the extramitochondrial region of HeLa and CV1 cells induced death of these cells.

Heterologous expression of the pro-apoptotic proteins Bak and Bax is lethal for fission yeast, and cell death was suppressed by coexpression of anti-apoptotic protein Bcl-XL (Ink *et al*., 1997; Jurgensmeier *et al*., 1997; Torgler *et al*., 1997). To examine whether Pnu1p is involved in hBak-induced death we expressed hBak cDNA in wild type and *pnu1*Δ cells. In the absence of thiamine the hBak band was observed at 22 kDa in both wild type and *pnu1*Δ cells by Western blotting (Fig. 4A). Expression of hBak extremely reduced the cell viability of both wild type and *pnu1*Δ cells to the same extent (Fig. 4B). And phloxin B staining also

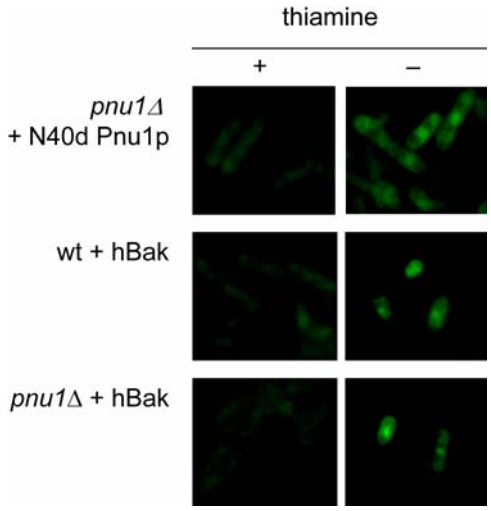

Fig. 3. TUNEL assay of the cells expressing N40d-Pnu1p and hBak. The *pnu1*Δ cells carrying the plasmid for expression of N40d-Pnu1p were cultured in EMMG medium with or without thiamine (10 μM) at 28°C overnight (upper panels). The cells were stained *in situ* by the TUNEL assay as described in the Materials and Methods section. The wild type cells (middle panels) or *pnu1*Δ cells (lower panels) carrying the hBak expression plasmid were cultured and attained by TUNEL assay as above.

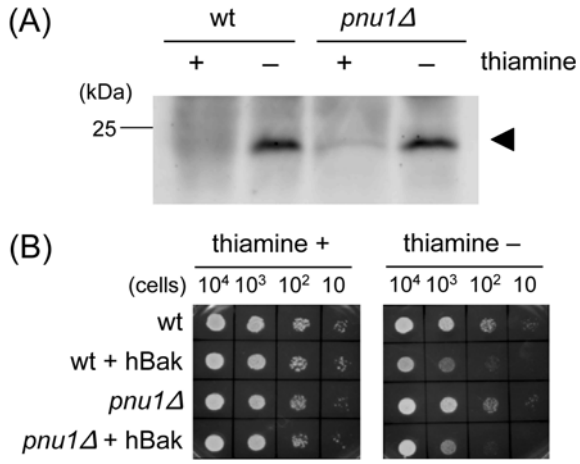

Fig. 4. Expression of hBak in *S. pombe* cells and the effect of the hBak expression on cell survival. (A) Plasmid for expression of hBak were introduced into *S. pombe* wild type and *pnu1*Δ cells, and the cells were cultured in EMMG medium with or without thiamine (10 μM) at 28°C overnight. Whole cell extracts were separated on SDS-PAGE and hBak protein was immunologically detected using anti-hBak antibody. (B) The cells (wild type or *pnu1*Δ) carrying the hBak expression plasmid were serially diluted and spotted on an EMMG agar plate with or without thiamine (10 mM). The cells were cultured at 28°C for 3 days.

showed the cell death of both cell types (data not shown). These results suggest that Pnu1p is not necessary for hBak-induced death. In the TUNEL assay the majority of wild type and *pnu1*Δ cells expressing hBak have an intense nuclear stain, as is the case of ectopic expression of Pnu1p (Fig. 4, middle and lower). Control cells in the presence of thiamine show no detectable fluorescence. Previously, Ink *et al*. (1997) observed the cleavage of nuclear DNA into high-molecular weight fragments in Bak-expressing cells by pulse-field gel electrophoresis. Our result does not conflict with theirs. However, the yeast nuclease involved in DNA fragmentation during hBak-induced cell death suicide is not yet known.

Unicellular organisms such as yeasts experience cell death with morphological changes similar to those experiencing apoptosis in mammalian cells (Madeo *et al*., 2004; Low *et al*., 2005). In *S. pombe* cells expression of pro-apoptotic proteins (human Bax, Bak, and nematode CED-4) from metazoans conferred a lethal phenotype (for review, see Low *et al*., 2005). Moreover, *S. pombe* cells with perturbation in triacylglycerol synthesis can undergo cell death with prominent apoptotic markers including the fragmentation of nuclear DNA and exposure of phosphatidylserine (Zhang *et al*., 2003). In this study we demonstrated that ectopic expression of the *S. pombe* Pnu1p produced a cytotoxic effect in yeast cells with nuclear DNA fragmentation. This suggests that Pnu1p possibly contributes to a certain type of cell death if the matured nuclease is released from the mitochondrial intermembrane space, as is the case of mammalian EndoG. Previous observation of Bak-induced death cells by electron microscopy showed that the features with extensive cytosolic vacuolization and multifocal chromatin condensation are readily distinguishable from those of mammalian apoptotic cell death (Jurgensmeier *et al*., 1997). Further studies, especially the observation of ultrastructure of the cell, will characterize the type of death induced by ectopic expression of Pnu1p.

In mammalian cells EndoG is released from mitochondria *via* Bax/Bak-mediated permeabilization (Arnoult *et al*., 2003). And some reports have suggested that mitochondrial nuclease is released in the process of autophagy of unicellular organisms. During conjugation of a protozoan *Tetrahymena* an EndoG homolog contributes to an apoptosis-like process, which is called programmed nuclear degradation (Kobayashi and Endoh, 2005; Endoh and Kobayashi, 2006). In the first step of the death process, the parental macronucleus is engulfed by a large autophagosome together with many mitochondria. EndoG is then released from the simply broken mitochondria and is responsible for the generation of the DNA ladder. Pnu1p of *S. pombe* has been demonstrated to contribute to drastically decrease the amount of cellular RNA during nitrogen starvation, probably *via* RNA-degrading activity (Nakashima *et al*., 2002). The increase in RNA-degrading activity was strongly dependent on *isp6*, a gene for a vacuolar protease, which is involved in both autophagy and sexual development (Nakashima *et al*., 2006). Our result showed that Pnu1p nuclease activity in the extramitochondrial region caused cytotoxic effects in *S. pombe* cells. The manner in which the nuclease is released from mitochondria, e.g. through channels or during autophagy, seems to affect the physiological role of

the nuclease. Expression of GFP-tagged Pnu1p, shown in this study, will be useful to search what kinds of stimuli trigger the release of nuclease from mitochondria. Our expression system using the truncated Pnu1p will also provide a model to identify and characterize the effector molecules involved downstream of mitochondrial nuclease-induced cell death.

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