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Human Polynucleotide Phosphorylase, hPNPase, is Localized in Mitochondria

Jan Piwowarski, Pawel Grzechnik, Andrzej Dziembowski Aleksandra Dmochowska, Michal Minczuk and Piotr P. Stepien*

Department of Genetics Warsaw University and Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawinskiego 5A 02-106 Warsaw, Poland

**Corresponding author*

The human gene encoding a polynucleotide phosphorylase (hPNPase) has been recently identified as strongly up-regulated in two processes leading to irreversible arrest of cell division: progeroid senescence and terminal differentiation. Here, we demonstrate that the hPNPase is localized in mitochondria. Our finding suggests the involvement of mitochondrial RNA metabolism in cellular senescence.

Keywords: mitochondria; polynucleotide phosphorylase; PNPase; RNA degradation

The relationship between cell proliferation, differentiation and aging is still poorly understood. The intense scientific effort currently put into this area is fueled by cancer research as well as a growing interest in understanding and interfering with the aging processes. Premature aging in progeria seems to be associated with the changed ability of cells to proliferate. Normal human fibroblasts can achieve about 60 population doublings in culture, while cells from progeria patients can only double approximately 20 times before they lose their proliferative potential and enter the state called cellular senescence.[1] Another type of growth arrest exhibited by normal cells is called terminal cell differentiation.[2] This state can be also artificially induced in metastatic human melanoma *in vitro* by treating cells with a combination of interferon-beta and the protein kinase C activator mezerein (MEZ), which in effect causes a loss of their tumorigenic properties.[3]

In a recent study by Leszczyniecka *et al*.[4] a genetic screen was used in order to identify genes which are simultaneously up-regulated in both pathways: cellular senescence and terminal differentiation. The results revealed that the expression of the nuclear gene coding for polynucleotide phosphorylase (hPNPase) is very strongly induced in both of these processes.

PNPases are evolutionary conserved and known to exist in a variety of organisms. The best characterized are PNPases from *Escherichia coli*[5] and plant chloroplasts.[6] In *E. coli* PNPase was found together with RNA helicase and enolase in the multi-protein degradosome complex, responsible for RNA turnover in the cell.[7] Experimental data indicate that bacterial and plant PNPases function as phosphate dependent 3′–5′ exoribonucleases, and polymerases adding poly(A) tails to RNA. In both systems the addition of poly(A) tails constitutes the signal for RNA degradation.[8–11]

RNA degradation enzymes in the mitochondria of yeast and human cells have been a focus of our research for some time.[12–17] We have characterized the yeast mitochondrial degradosome and the human homologue of one of its components (hSuv3p). In order to study the RNA metabolism in human mitochondria we have cloned a full-length cDNA coding for human PNPase and expressed the protein in a heterologous system to study its sub-cellular localization and activity. While our work was in progress, two groups reported their data on human PNPase: Raijmakers *et al*.[18] on the basis of *in silico* analysis of the PNPase protein sequence have suggested its mitochondrial localization, although no experimental data were presented. On the other hand, Leszczyniecka *et al*.[4] have shown a strong induction of hPNPase mRNA in terminal differentiation and progeroid senescence, but have suggested that the protein is localized in the cytosol. In this work we show that the nuclear-encoded hPNPase is, in fact, localized in mitochondria and we discuss the biological significance of this finding.

We were looking for a homologue of known

Abbreviations used: PNPase, polynucleotide phosphorylase; hPNPase, human polynucleotide phosphorylase; FITC, fluorescein-5-isothiocyanate.

E-mail address of the corresponding author: stepien@ibb.waw.pl

PNPases as a candidate for the RNA degrading enzyme in human mitochondria by analysis of genomic databases. We have found a putative protein (TrEMBL Q96T05) with two characteristic PH domains, but lacking the C-terminal domains of PNPases.[5] Using the human EST sequences we assembled the full-length cDNA sequence coding for a protein with all domains characteristic for PNPases. The full-length hPNPase cDNA was PCR amplified from a HeLa D98-H2 cDNA library[19] using a 5′ primer: CATGGATCCGATGG CGGCCTGCAGGTACTG and a 3′ primer: TATAG TCGACCTGAGAAATTAGATGATGACTGTG. The PCR product was cloned into the *Bam*HI and *Sal*I restriction sites of pUC18. Sequence analysis of the cloned cDNA revealed five missense mutations, when compared to assembled full-length cDNA. The mutations were replaced by standard site-directed mutagenesis, and verified by DNA sequencing.

The N-terminal mitochondrial targeting pre-sequence of a protein can be predicted *in silico*, although such predictions are not always consistent with experimental data. We analyzed the N-terminal pre-sequence for human, mouse, fruit fly and *Caenorhabditis elegans* PNPases using several computer programs (PSORT;[20] iPSORT;[21] TargetP v1.0;[22] Predotar†; MitoProt II 1.0a4;[23] SignalP v1.1[24]). Most of them identified a putative N-terminal mitochondrial targeting sequence, indicating the mitochondrial localization of PNPases. Two most probable cleavage sites of the mitochondrial leader peptide were found: the first located at the 24th amino acid residue from the translation start site, and the second one in proximity of the 45th amino acid residue from the start codon.

In order to get direct experimental evidence for mitochondrial localization of human PNPase, specific antibodies were raised. The recombinant hPNPase was expressed in *E. coli*, and the purified protein was used to raise rabbit polyclonal antibodies. Sub-cellular fractions of HeLa cells were analyzed by Western blots for the presence of hPNPase. Results presented in Figure 1 show that hPNPase is present in the mitochondrial, but not in the cytoplasmic fraction. The anti-hPNPase antibodies were used for *in situ* localization of the protein. Co-localization of hPNPase and the mitochondrial marker MitoTracker CMXRos was observed (Figure 2).

Additional evidence for mitochondrial localization was provided by over-expression of the recombinant hPNPase fused to a c-myc tag at the C terminus (Figure 3). HeLa cells were transiently transfected with the gene fusion and probed with anti-c-myc antibodies. Immunofluroscence microscopy showed that the hPNPase co-localized with the mitochondrial marker MitoTracker CMXRos (Figure 3). In order to confirm that the

† http://www.inra.fr/predotar/

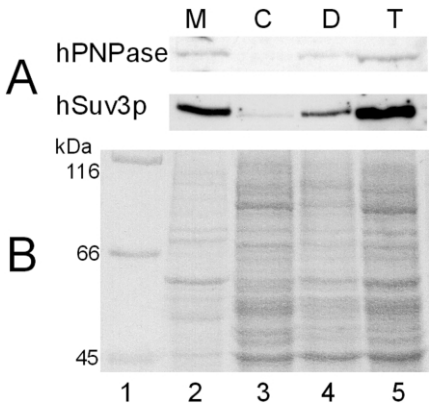

Figure 1. Human PNPase is localized in the mitochondrial fraction. Sub-cellular fractionation of HeLa cells was performed as described[12] and analyzed by Western blotting with the anti-hPNPase serum. To obtain the rabbit serum anti-hPNPase recombinant protein was expressed in *E. coli* BL21 using the pET15b (Novagen) derivative. The protein was purified from inclusion bodies under denaturing conditions using Ni-NTA agarose (Qiagene) followed by electroelution after SDS-PAGE. Rabbits were immunized as described.[12] The obtained serum at a dilution of 1:1000 recognized a single band in total protein extract from HeLa cells migrating at about 90 kDa, which was not observed when a pre-immune serum was used (results not shown). A, Western-blot analysis of cellular fractions. Blots were probed with anti-hPNPase serum and serum against well-characterized mitochondrial protein hSuv3p.[12] M, mitochondria; C, cytoplasm; D, cellular debris; T, total cells. B, Ponceau-S staining as a loading control. Lane 1, protein marker; lanes 2, 3, 4, 5, correspond to M, C, D, T in (A), respectively.

N-terminal sequence is necessary for the transport of PNPase into mitochondria, the same experiment was performed for truncated protein, without the first 52 amino acid residues. The truncated hPNPase was found not to co-localize with mitochondria (Figure 3). Similar results were obtained for the COS-1 cell line (results not shown). This experiment confirms the role of the N-terminal sequence in directing hPNPase into mitochondria.

Our data differ from the results obtained by Leszczyniecka *et al.*,[4] who reported cytosolic localization of hPNPase. The reason for the discrepancy could be that the protein fusion used in their study had the GFP tag at the N terminus of the protein, which could interfere with the import to mitochondria.

The recombinant hPNPase expressed in *E. coli* was assayed for its enzymatic activity *in vitro* according to the protocol described for rat PNPase.[25] The protein purified from *E. coli* was found to degrade RNA in the direction from 3′ to 5′ in the phosphorolytic reaction yielding nucleoside diphosphates (data not shown) similarly to hPNPase expressed in baculovirus.[4] Coupled with the localization studies, our data strongly suggest that hPNPase is involved in the degradation of mitochondrial RNA.

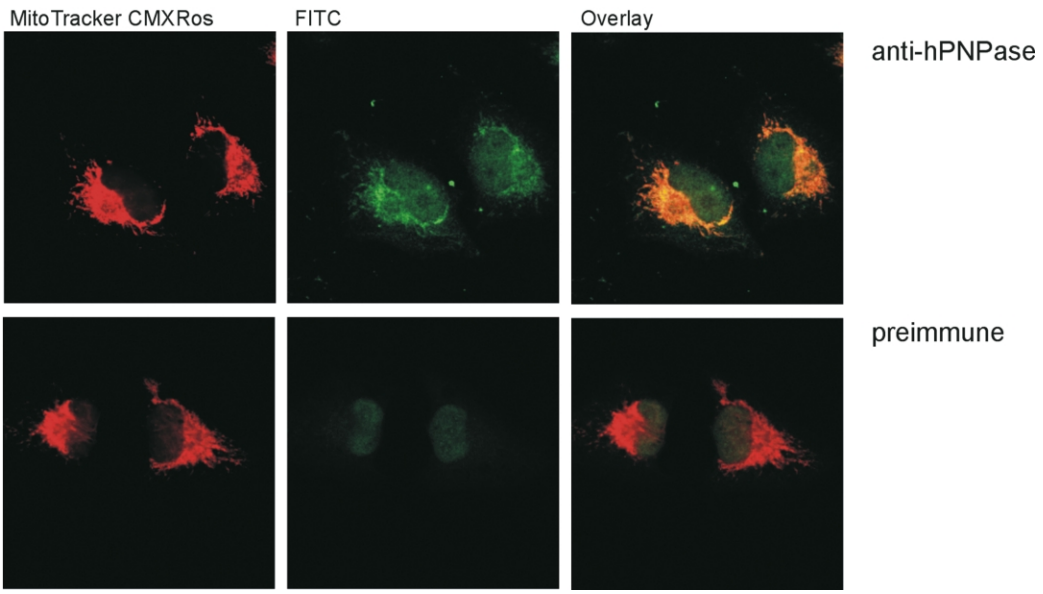

Figure 2. *In situ* localization of endogenous hPNPase. HeLa cells were grown on coverslips, incubated with the mitochondria-specific label MitoTracker CMXRos (Molecular Probes; 300 nM), fixed with 4% (v/v) formaldehyde and permeabilized with 1% (v/v) Triton X-100. Immunostaining was performed with anti-hPNPase serum (1:100) and FITC-conjugated goat anti-rabbit IgG (Molecular Probes; 1:100). The green fluorescence of hPNPase and red of mitochondrial marker were visualized using a confocal microscope.

Our finding that human PNPase is localized in mitochondria, together with the data of Leszczyniecka *et al*.,[4] suggest an important link between mitochondria and cellular senescence. Mitochondria have a crucial role in apoptotic cell death, and therefore our data could indicate that an apoptotic mechanism is involved in the phenomena described by Leszczyniecka *et al*.[4] In particular, they found strong up-regulation of PNPase after the treatment of melanoma cells with interferon-beta. Moreover, they observed that PNPase over-expression using a viral vector resulted in cell growth inhibition. The authors suggested that this could be due to apoptosis, although no experiments were reported. In the light of our finding, this suggestion seems to be

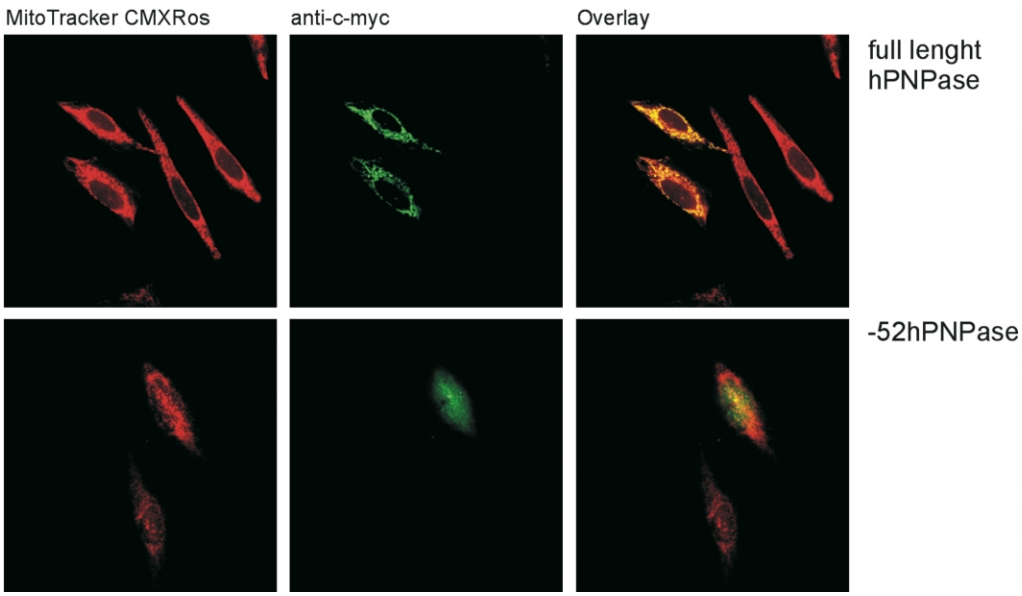

Figure 3. Localization of over-expressed hPNPase-c-myc fusion with or without the N-terminal sequence. HeLa cells were grown on coverslips and transiently transfected using FuGene6 reagent (Roche) with constructs based on pcDNA3.1(−) vector and encoding either full-length hPNPase or a truncated version without the 52 N-terminal amino acid residues (−52hPNPase), both with a c-myc tag at the C terminus. Cells were probed with the MitoTracker CMXRos and immunostained as for Figure 2, except that anti-c-myc 9E10 monoclonal antibody (Santa Cruz Biotechnology; 1:200) and FITC-conjugated goat anti-mouse IgG (Molecular Probes; 1:200) were used.

very plausible, especially as interferon-beta has been shown to induce apoptosis.[26] In addition, interferon-beta was shown to reduce mitochondrial mRNA levels[27] and it is possible that this is a result of the increased RNA degrading activity due to hPNPase up-regulation. We are currently investigating if changes in hPNPase levels can indeed induce lower steady-state levels of mitochondrial transcripts and the mitochondrial apoptotic pathway.

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References

1. Faragher, R. G., Kill, I. R., Hunter, J. A., Pope, F. M., Tannock, C. & Shall, S. (1993). The gene responsible for Werner syndrome may be a cell division "counting" gene. *Proc. Natl Acad. Sci. USA*, **90**, 12030–12034.
2. Gagna, C. E., Kuo, H. R., Florea, E., Shami, W., Taormina, R., Vaswani, N. *et al.* (2001). Comparison of apoptosis and terminal differentiation: the mammalian aging process. *J. Histochem. Cytochem.* **49**, 929–930.
3. Madireddi, M. T., Dent, P. & Fisher, P. B. (2000). Regulation of mda-7 gene expression during human melanoma differentiation. *Oncogene*, **19**, 1362–1368.
4. Leszczyniecka, M., Kang, D. C., Sarkar, D., Su, Z. Z., Holmes, M., Valerie, K. & Fisher, P. B. (2002). Identification and cloning of human polynucleotide phosphorylase, hPNPase$^{old\text{-}35}$, in the context of terminal differentiation and cellular senescence. *Proc. Natl Acad. Sci. USA*, **99**, 16636–16641.
5. Symmons, M. F., Williams, M. G., Luisi, B. F., Jones, G. H. & Carpousis, A. J. (2002). Running rings around RNA: a superfamily of phosphate-dependent RNases. *Trends Biochem. Sci.* **27**, 11–18.
6. Hoffmann, M., Kuhn, J., Daschner, K. & Binder, S. (2001). The RNA world of plant mitochondria. *Prog. Nucl. Acid Res. Mol. Biol.* **70**, 119–154.
7. Mohanty, B. K. & Kushner, S. R. (2000). Polynucleotide phosphorylase functions both as a 3′ → 5′ exonuclease and a poly(A) polymerase in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **97**, 11966–11971.
8. Yehudai-Resheff, S., Hirsh, M. & Schuster, G. (2001). Polynucleotide phosphorylase functions as both an exonuclease and a poly(A) polymerase in spinach chloroplasts. *Mol. Cell. Biol.* **21**, 5408–5416.
9. Carpousis, A. J. (2002). The *Escherichia coli* RNA degradosome: structure, function and relationship in other ribonucleolytic multienzyme complexes. *Biochem. Soc. Trans.* **30**, 150–155.
10. Mohanty, B. K. & Kushner, S. R. (2002). Polyadenylation of *Escherichia coli* transcripts plays an integral role in regulating intracellular levels of polynucleotide phosphorylase and RNase E. *Mol. Microbiol.* **45**, 1315–1324.
11. Lisitsky, I., Kotler, A. & Schuster, G. (1997). The mechanism of preferential degradation of polyadenylated RNA in the chloroplast. The exoribonuclease 100RNP/polynucleotide phosphorylase displays high binding affinity for poly(A) sequence. *J. Biol. Chem.* **272**, 17648–17653.
12. Minczuk, M., Piwowarski, J., Papworth, M. A., Awiszus, K., Schalinski, S., Dziembowski, A. *et al.* (2002). Localisation of the human hSuv3p helicase in the mitochondrial matrix and its preferential unwinding of dsDNA. *Nucl. Acids Res.* **30**, 5074–5086.
13. Dziembowski, A., Piwowarski, J., Hoser, R., Minczuk, M., Dmochowska, A., Siep, M. *et al.* (2003). The yeast mitochondrial degradosome. Its composition, interplay between RNA helicase and RNase activities and the role in mitochondrial RNA metabolism. *J. Biol. Chem.* **278**, 1603–1611.
14. Dziembowski, A. & Stepien, P. P. (2001). Genetic and biochemical approaches for analysis of mitochondrial degradosome from *Saccharomyces cerevisiae*. *Methods Enzymol.* **342**, 367–378.
15. Dmochowska, A., Golik, P. & Stepien, P. P. (1995). The novel nuclear gene DSS-1 of *Saccharomyces cerevisiae* is necessary for mitochondrial biogenesis. *Curr. Genet.* **28**, 108–112.
16. Stepien, P. P., Kokot, L., Leski, T. & Bartnik, E. (1995). The suv3 nuclear gene product is required for the *in vivo* processing of the yeast mitochondrial 21 S rRNA transcripts containing the r1 intron. *Curr. Genet.* **27**, 234–238.
17. Dziembowski, A., Malewicz, M., Minczuk, M., Golik, P., Dmochowska, A. & Stepien, P. P. (1998). The yeast nuclear gene DSS1, which codes for a putative RNase II, is necessary for the function of the mitochondrial degradosome in processing and turnover of RNA. *Mol. Gen. Genet.* **260**, 108–114.
18. Raijmakers, R., Egberts, W. V., van Venrooij, W. J. & Pruijn, G. J. (2002). Protein–protein interactions between human exosome components support the assembly of RNase PH-type subunits into a six-membered PNPase-like ring. *J. Mol. Biol.* **323**, 653–663.
19. Minet, M. & Lacroute, F. (1990). Cloning and sequencing of a human cDNA coding for a multifunctional polypeptide of the purine pathway by complementation of the ade2-101 mutant in *Saccharomyces cerevisiae*. *Curr. Genet.* **18**, 287–291.
20. Nakai, K. & Horton, P. (1999). PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* **24**, 34–35.
21. Bannai, H., Tamada, Y., Maruyama, O., Nakai, K. & Miyano, S. (2002). Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics*, **18**, 298–305.
22. Emanuelsson, O., Nielsen, H., Brunak, S. & von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* **300**, 1005–1016.
23. Claros, M. G. & Vincens, P. (1996). Computational

method to predict mitochondrially imported proteins and their targeting sequences. *Eur. J. Biochem.* **241**, 770–786.
24. Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**, 1–6.
25. See, Y. P. & Fitt, P. S. (1972). Partial purification and properties of rat liver mitochondrial polynucleotide phosphorylase. *Biochem. J.* **130**, 343–353.
26. Chawla-Sarkar, M., Leaman, D. W. & Borden, E. C. (2001). Preferential induction of apoptosis by interferon (IFN)-beta compared with IFN-alpha2: correlation with TRAIL/Apo2L induction in melanoma cell lines. *Clin. Cancer Res.* **7**, 1821–1831.
27. Shan, B., Vazquez, E. & Lewis, J. A. (1990). Interferon selectively inhibits the expression of mitochondrial genes: a novel pathway for interferon-mediated responses. *EMBO J.* **9**, 4307–4314.

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