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Deletion of the mitochondrial NADH kinase increases mitochondrial DNA stability and life span in the filamentous fungus *Podospora anserina*

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ABSTRACT

In the filamentous fungus *Podospora anserina*, aging is systematically associated with mitochondrial DNA (mtDNA) instability. A causal link between deficiency of the cytochrome respiratory pathway and life-span extension has been demonstrated. Knock out of the cytochrome respiratory pathway induces the expression of an alternative oxidase and is associated with a reduction in free radical production. The question of the links between mtDNA stability, ROS generation and lifespan is therefore clearly raised in this organism. NADPH lies at the heart of many anti-oxidant defenses of the cell. In *Saccharomyces cerevisiae*, the mitochondrial NADPH is largely provided by the Pos5 NADH kinase. We show here that disruption of *PaNdk1* encoding the potential mitochondrial NADH kinase of *P. anserina* leads to severe somatic and sexual defects and to hypersensitivity to hydrogen peroxide and paraquat. Surprisingly, it also leads to a spectacular increase of mtDNA stability and lifespan. We propose that an adaptative metabolic change including the induction of the alternative oxidase can account for these results.

1. Introduction

Mitochondria play a major role in the intermediary metabolism of eukaryotic cells and are the principle site for energy transduction, through electron transport and oxidative phosphorylation. However, mitochondria are also the main site for production of reactive oxygen species (ROS) in the cell (de Souza-Pinto and Bohr, 2002). It is known that under normal physiological conditions, about 1–2% of the mitochondrial oxygen consumption leads to superoxide ($O_2^{\cdot -}$) formation (Klein and Ackerman, 2003). While ROS, such as superoxide or hydrogen peroxide (H_2O_2) are mediators of oxygen toxicity, they are also involved in intracellular signaling through the activation of response pathways (D'Autreaux and Toledano, 2007; Veal et al., 2007 for reviews). It is the presence of highly efficient scavenging mechanisms that most likely enabled cells to overcome toxicity and led to the use of these molecules as signal transducers. Thus physiological ROS levels are under tight homeostatic control achieved by a variety of anti-oxidant defense systems such as glutathione peroxidases (GPX), thioredoxins (TRX), superoxide dismutases (SOD) and catalases (CAT) (Scandalios, 2005). It is generally admitted that situations in which ROS levels exceed the capacity of anti-oxidant defenses have a causative role in aging and associated diseases (Sohal and Weindruch, 1996; Finkel and Holbrook, 2000).

After their formation, superoxide anions ($O_2^{\cdot -}$) are reduced to H_2O_2 by superoxide dismutases. The mitochondrial matrix contains a specific form of SOD, MnSOD, with manganese in the active site (Fridovich, 1995). Because catalase is absent in the mitochondria of most animal cells, the GPX and TRX play a key role in reducing H_2O_2 in this compartment (Jo et al., 2001). These two anti-oxidant enzymes are dependant on the reduced form of gluthatione and thioredoxin to be regenerated and participate to another cycle of detoxification. This essential step requires the presence of NADPH and the action of NAPDH-requiring glutathione and thioredoxin reductases, respectively. Thus the production of NADPH is a vital step in the detoxification of the mitochondrial ROS. Furthermore NADPH supports several reductive biosynthetic reactions (Grose et al., 2006). In yeast and mammals, cytosolic NADPH is provided primarily by enzymes in the pentose phosphate pathway and by the cytosolic NADP pool through the cytosolic NAD kinases (Grabowska and Chelstowska, 2003; Pollak et al., 2007). However, the sources of mitochondrial NADPH are divergent. In mammalian cells, mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (NADP-IDHm) has been reported to be an important source of mitochondrial NADPH (Jo et al., 2001) while in *Saccharomyces cerevisiae*, the NADH kinase encoded by the *POS5* gene, is the major source of mitochondrial NADPH (Outten and Culotta, 2003; Strand et al., 2003).

The NAD(H) kinases are the only known enzymes which convert NAD(H) to NADP(H) (McGuinness and Butler, 1985; Kawai et al.,

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2001; Lerner et al., 2001). Based on their preference for the oxidized or reduced form of NAD(H), NAD(H) kinases have been classified as NAD kinases and NADH kinases, respectively (Chai et al., 2006). Genes encoding NAD(H) kinases have been reported from bacteria (Kawai et al., 2001) to archae (Sakuraba et al., 2005) and humans (Lerner et al., 2001). Based on sequence homology, three NAD(H) kinases were identified in the filamentous fungus *Podospora anserina*.

In *P. anserina*, the mitochondrial function and specifically the respiratory function plays a fundamental role in controlling life span. (Lorin et al., 2006; Scheckhuber and Osiewacz, 2008 for reviews). In this organism, aging is systematically associated with mitochondrial DNA (mtDNA) instability and accumulation of specific mtDNA rearrangements. Besides this aging process that occurs systematically in all the wild-type cultures of *P. anserina*, several genetically controlled syndromes responsible for premature death have also been identified in this organism. That is the case for strains carrying certain mutations in the gene encoding a cytosolic ribosomal protein and for strains carrying certain "pathogenic" mutations in the ADP/ATP translocator. Remarkably, in these mutants, premature death is also associated with mtDNA instability though the mtDNA rearrangements are different from those observed during aging (Belcour et al., 1991; Contamine et al., 1996, 2004; El-Khoury and Sainsard-Chanet, 2009). These data indicate that in *P. anserina*, there is a strong link between mtDNA instability and death and that life span is an indicator of the mtDNA stability. Mutants, which are characterized by high mtDNA stability, are also characterized by an increased lifespan whereas mutants that are characterized by low mtDNA stability are also characterized by a reduced lifespan.

The causes of mtDNA instability and accumulation of mtDNA rearrangements during aging in *P. anserina* are poorly understood. Several non exclusive possibilities have been suggested, including mitochondrial intron mobility (Sellem et al., 1993), mtDNA repair deficiency (Soerensen et al., 2009), accumulation of ROS-mediated lesions (Soerensen et al., 2009; Gredilla et al., 2006).

Here, we report the properties of a strain deleted for the mitochondrial *PaNdk1* homologous to the *S. cerevisiae* mitochondrial NADH kinase Pos5p. We show that the deletion of the *PaNdk1* gene is responsible for impairment of vegetative growth and sexual development associated with an increased sensitivity to oxidative stress generated by H_2O_2 and paraquat and surprisingly, for a spectacular lifespan extension and mtDNA stability.

2. Materials and methods

2.1. P. anserina strains, growth conditions, life span measurements and transformation experiments

General properties of the organism used in this study, the ascomycete *P. anserina*, have been reviewed by Esser (1974). Strain *s* is a well-known laboratory strain of this species (Rizet, 1952). Cultures were grown on standard minimal synthetic (M2) medium at 27°. When necessary, hygromycin 100 μg/ml (Boehringer-Mannheim), phleomycin 10 μg/ml (Boehringer-Mannheim) or nourseothricin 50 μg/ml (Werner BioAgents) were added to the medium. Medium for germination contains ground corn meal (50 g/l), agar (12.5 g/l) and ammonium acetate (6 g/l).

Lifespans were measured on M2 medium in race tubes at 27° in the dark, for three to five subcultures derived from 5 to 10 independent spores of a given strain. The lifespan of a strain is defined as the mean time (given with standard errors) of growth of parallel cultures between the inoculation and the death of the culture.

Protoplast preparation and transformation experiments were conducted as described previously (Berges and Barreau, 1989; El-Khoury et al., 2008).

2.2. Deletion of the PaNdk1 gene

To delete the *PaNdk1* gene, targeting fragments of 0.75 kb (5′) and 1.1 kb (3′) flanking the *PaNdk1* gene were prepared by PCR on genomic DNA using the primer pairs F-5′UTR (5′-GACTAGTGCC GATCAGTTGTGCAAG-3′), R-5′UTR (5′-CATACCATGGCTCGGATAGC AC CCTGA-3′) and F-3′UTR (5′-AAGAATGCGGCCGCGCTTTGCATGGC ATTTGA-3′), R-3′UTR (5′-GACTAGTCGCGGGTGTTGAAGAACT-3′), respectively. The two fragments bear SpeI–NcoI and NotI–SpeI restriction sites and were introduced sequentially into the corresponding sites of the polylinker of pAPI508 plasmid that contains a nourseothricin resistance cassette (El-Khoury et al., 2008). The resulting vector pPAPI–Δ*PaNdk1* was digested by SpeI and about 10 μg of linear plasmid were used to transform the *PaNdk1*$^+$ strain. The deletion of the endogenous *PaNdk1*$^+$ gene was verified by PCR and Southern blot.

2.3. Quantitative RT-PCR

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) from cultures grown for 48 h on cellophane disks overlying M2 medium. The mycelium was broken with glass beads in a Fastprep apparatus (40 s, intensity 6.5). For (q)RT-PCR, cDNA was synthesized with SuperScript™ II reverse transcriptase (Invitrogen) from 2 μg of RNA, using a T_{15} primer. Subsequent quantitative real-time PCRs were performed in a Lightcycler® (Roche), using the LightCycler® FastStart DNAMaster SYBR Green I Kit (Roche). At least 3 independent experiments were performed on one to three different RNA preparations for each strain. Primers QAOX4-F (5′-TGATCTCGCCACGAATTACA-3′) and QAOX4-R (5′-TATA-GGTGTGGACCGCTTCC-3′) were used for the *aox* gene.

2.4. Southern blot analyses

DNA restriction, gel electrophoresis and Southern blotting were performed on total DNA isolated from cultures grown on cellophane disks overlying solid M2 or MR medium, using a standard phenol/chloroform extraction according to Lecellier and Silar (1994).

2.5. Hydrogen peroxide and paraquat sensitivity tests

Hydrogen peroxide and paraquat sensitivity were tested on plates containing M2 medium to which 0.01% and 0.02% of H_2O_2 and 20 μM of paraquat were added.

2.6. ROS measurements

ROS production was estimated by histochemical detection as described by Munkres (1990). After incubation to obtain appropriate grown thalli, the plates were flooded with a 5 ml of a solution containing 2.5 mM Nitro Blue Tetrazolium (NBT) in 5 mM (*N*-morpholino) propane sulfonate–NaOH at pH7.6. Super oxide radicals that react with NBT form a blue precipitate.

3. Results

3.1. The genome of P. anserina encodes several putative NAD(H) kinases

Using the publicly available sequenced genome of the *P. anserina* wild-type strain S (Espagne et al., 2008), we identified three genes showing strong similarities with the mitochondrial NADH kinase Pos5p of *S. cerevisiae: PaNdk1* (Pa_2_4330), *PaNdk2* (Pa_1_2220) and *PaNdk3* (Pa_2_3590). The N-terminal amino acid