Mitochondrial nucleoids from the yeast *Candida parapsilosis*: expansion of the repertoire of proteins associated with mitochondrial DNA

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Molecules of mitochondrial DNA (mtDNA) are packed into nucleic acid–protein complexes termed mitochondrial nucleoids (mt-nucleoids). In this study, we analysed mt-nucleoids of the yeast *Candida parapsilosis*, which harbours a linear form of the mitochondrial genome. To identify conserved as well as specific features of mt-nucleoids in this species, we employed two strategies for analysis of their components. First, we investigated the protein composition of mt-nucleoids isolated from *C. parapsilosis* mitochondria, determined N-terminal amino acid sequences of 14 proteins associated with the mt-nucleoids and identified corresponding genes. Next, we complemented the list of mt-nucleoid components with additional candidates identified in the complete genome sequence of *C. parapsilosis* as homologues of *Saccharomyces cerevisiae* mt-nucleoid proteins. Our approach revealed several known mt-nucleoid proteins as well as additional components that expand the repertoire of proteins associated with these cytological structures. In particular, we identified and purified the protein Gcf1, which is abundant in the mt-nucleoids and exhibits structural features in common with the mtDNA packaging protein Abf2 from *S. cerevisiae*. We demonstrate that Gcf1p co-localizes with mtDNA, has DNA-binding activity *in vitro*, and is able to stabilize mtDNA in the *S. cerevisiae* $\Delta abf2$ mutant, all of which points to a role in the maintenance of the *C. parapsilosis* mitochondrial genome. Importantly, in contrast to Abf2p, *in silico* analysis of Gcf1p predicted the presence of a coiled-coil domain and a single high-mobility group (HMG) box, suggesting that it represents a novel type of mitochondrial HMG protein.

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INTRODUCTION

Mitochondrial nucleoids (mt-nucleoids) are cytological structures that represent fundamental segregating units of the mitochondrial genome (Kuroiwa, 1982; Sakai *et al.*, 2004). These nucleic acid–protein complexes undergo dynamic remodelling in response to changes in cellular metabolism (Kucej *et al.*, 2008). In baker's yeast, a number of proteins have been identified as specific components of isolated mt-nucleoids (Miyakawa *et al.*, 1995; Miyakawa &

Sato, 2001; Sato *et al.*, 2002; Sato & Miyakawa, 2004) and/or cross-linked mitochondrial DNA (mtDNA)–protein complexes (Kaufman *et al.*, 2000; Chen *et al.*, 2005). The mt-nucleoids contain proteins known to interact with mtDNA such as the non-histone protein Abf2, which is considered to be a major mtDNA packaging factor, and a set of enzymes involved in mtDNA replication, transcription and repair. In addition, a number of metabolic enzymes have been shown to be bona fide components of the mt-nucleoids, thus linking mitochondrial inheritance with cell metabolism and physiology (reviewed by Chen & Butow, 2005; Kucej & Butow, 2007). Investigations of human mt-nucleoids indicate that these complexes are linked to the mitochondrial inner membrane and have a layered structure in which mtDNA replication and transcription occur in the central core, whereas RNA processing, translation and assembly of the respiratory chain complexes may occur in the periphery (Wang & Bogenhagen, 2006; Bogenhagen *et al.*, 2008).

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; HMG, high-mobility group; mtHMG, mitochondrial HMG protein; mtDNA, mitochondrial DNA; mt-nucleoid, mitochondrial nucleoid; mtTBP, mitochondrial telomere-binding protein; NP-40, Nonidet P40; yEGFP3, yeast enhanced version 3 of GFP.

Two supplementary tables and a supplementary dataset are available with the online version of this paper.

The mtDNA packaging protein Abf2 from *Saccharomyces cerevisiae* is the best-characterized mt-nucleoid component. It is a 20 kDa DNA-binding protein that contains two high-mobility group (HMG) box domains and, in the presence of topoisomerase I, it introduces negative supercoils into a relaxed circular DNA plasmid (Caron *et al.*, 1979; Diffley & Stillman, 1991). In Abf2p-deficient yeast cells, mtDNA is rapidly lost during growth on glucose media, suggesting that Abf2p plays a key role in the maintenance of the mitochondrial genome (Diffley & Stillman, 1991, 1992). This protein is specifically associated with the isolated mt-nucleoids and has the highest affinity for dsDNA among the mt-nucleoid proteins (Miyakawa *et al.*, 1995) with a preference for GC-rich gene sequences in mtDNA (Kucej *et al.*, 2008). It is also involved in the regulation of the copy number and the recombination of mtDNA (Zelenaya-Troitskaya *et al.*, 1998).

In previous studies (Miyakawa *et al.*, 2000; Umezaki & Miyakawa, 2002) we have employed an SDS-DNA PAGE approach to detect the Abf2-like mtDNA-binding proteins in several yeast species, and found only one such protein per analysed species, with molecular masses ranging from 17 to 26 kDa, suggesting that phylogenetically distant yeast species tend to differ in the mode of mtDNA packaging. Abf2-like proteins have been purified from the mt-nucleoids of four species, *S. cerevisiae*, *Cluyveromyces lactis*, *Pichia jadinii* and *Williopsis mrakii* (Miyakawa *et al.*, 1995, 2000, 2003; Miyakawa & Yawata, 2007). Comparative analysis of *K. lactis* mt-nucleoid proteins indicates that Abf2p is the most divergent component of the mt-nucleoid (Nosek *et al.*, 2006).

In contrast to most fungal species, *Candida parapsilosis* mitochondria contain a linear mitochondrial genome of 30 923 bp, terminating with arrays of telomeric repeats ($n \times 738$ bp, where $n=1-12$; Nosek *et al.*, 1995, 2004). In addition to the linear mtDNA molecules, mitochondria of this yeast contain a series of circular dsDNA molecules termed telomeric circles (t-circles), which are implicated in the mitochondrial telomere transactions (Tomaska *et al.*, 2000; Nosek *et al.*, 2005). The unique molecular architecture of the *C. parapsilosis* mitochondrial genome prompted us to initiate analysis of its mt-nucleoids and to identify their conserved as well as specific features that may have contributed to the evolutionary emergence of the linear genome form in yeast mitochondria.

In this report, we experimentally identified several proteins that co-purify with mt-nucleoids from *C. parapsilosis*. In addition, we complemented the protein set with candidates detected by *in silico* analysis of the complete genome sequence of *C. parapsilosis*. Importantly, we analysed in more detail a 29 kDa protein named Gcflp and provide experimental evidence for its association with mt-nucleoids in this species. *C. parapsilosis* Gcflp is a novel member of the mitochondrial high-mobility group protein (mtHMG) family, originally identified *in silico* as putative mtDNA packaging proteins from *Candida albicans* and

Debaryomyces hansenii (Nosek *et al.*, 2006; see also Visacka *et al.*, 2009), which are distantly related to non-histone proteins from *S. cerevisiae* (Abf2p), slime mould (Glom) and human (h-mtTF1) mitochondria (Diffley & Stillman, 1991; Fisher & Clayton, 1988; Dequard-Chablat & Alland, 2002).

METHODS

Yeast strains and cultivation. *C. parapsilosis* strains CBS604^T and SR23 (*ade*⁻, *lys4*⁻) were employed in this study. The type strain CBS604^T used for isolation of mt-nucleoids was provided by H. Fukuhara (Institut Curie, Orsay, France). Cells were cultured aerobically at 30 °C to stationary phase in yeast extract-peptone-dextrose (YPD) medium that contained 1% (w/v) yeast extract (Oriental Yeast Company, Japan), 2% (w/v) peptone (Kyokuto, Japan) and 2% (w/v) glucose. *S. cerevisiae* strain YAM101 (*MATa/α*, $\Delta abf2::TRP1/ABF2$, *ade2-1/ade2-1*, *ura3-1/ura3-1*, *his3-11,15/his3-11,15*, *trp1-1/trp1-1*, *leu2-3,112/leu2-3,112*, *can1-100/can1-100*), provided by D. A. Clayton (Howard Hughes Medical Institute, Chevy Chase, MD, USA), is the parental strain of YAM101-1a and YAM101-1b, the haploids prepared by tetrad dissection of YAM101, which retained the wild-type *ABF2* allele and $\Delta abf2::TRP1$, respectively.

Isolation of mt-nucleoids from *C. parapsilosis* cells. The mt-nucleoids were isolated essentially as described previously (Miyakawa *et al.*, 1987). In brief, cells (about 40 g wet weight) were converted to spheroplasts by treatment with Zymolyase 20T (Seikagaku Kogyo). Mitochondria were prepared from disrupted spheroplasts by differential centrifugation. The mt-nucleoids were isolated from mitochondria after lysis of mitochondrial membranes with Nonidet P-40 (NP-40). Each mitochondrial pellet was suspended in NES2 buffer (0.5 M sucrose, 20 mM Tris/HCl, pH 7.6, 2 mM EDTA, 0.8 mM spermidine, 7 mM 2-mercaptoethanol, 0.4 mM PMSF) at a protein concentration of 0.25 mg ml⁻¹ (Figs 2 and 3a, lane 2), and then NP-40 was added to a final concentration of 0.5% (w/v) with gentle stirring for 10 min. The samples were centrifuged at 15 000 g for 10 min to separate supernatant (Figs 2 and 3a, lane 3) from

Fig. 1. Fluorescence photomicrographs of *C. parapsilosis* cells and the mt-nucleoids. (a, b) Vegetative cells at stationary phase, (c) spheroplasts, (d) mt-nucleoid fraction. Bar, 5 µm.

Fig. 2. SDS-PAGE and SDS-DNA PAGE of each fraction during mt-nucleoid isolation. Lanes: 1, molecular mass marker; 2, mitochondrial fraction; 3, supernatant after lysis of mitochondria by 0.5 % NP-40 and following centrifugation; 4, NP-40-insoluble materials after lysis of mitochondria by 0.5 % NP-40 and following centrifugation; 5, NP-40-soluble fraction after fractionation of the mt-nucleoids; 6, mt-nucleoid fraction; 7–11, ethidium bromide (EB) staining of lanes 2–6.

NP-40-insoluble material (Figs 2 and 3a, lane 4). The supernatant was then centrifuged at 45 000 *g* for 1 h to separate the mt-nucleoid fraction (Figs 2 and 3a, lane 6) from NP-40-soluble fraction (Figs 2 and 3a, lane 5). The mt-nucleoid fraction was suspended in 1 ml NES2 buffer and stored at -80°C until use. In this preparation, the purification step using sucrose density-gradient centrifugation was omitted.

Purification of a 29 kDa protein from the mt-nucleoid fraction.

The *C. parapsilosis* 29 kDa protein was purified by a combination of NaCl extraction of the mt-nucleoid fraction, HiTrap-SP column chromatography (Sasaki *et al.*, 2003) and DNA-cellulose chromatography. The isolated mt-nucleoids were suspended in NES2 buffer containing 0.4 M NaCl, and incubated for 30 min at 25°C . After centrifugation at 45 000 *g* for 30 min, the supernatant was dialysed overnight against HEPES buffer (50 mM HEPES, 150 mM NaCl, 1 mM DTT, pH 7.5). The dialysed fraction was loaded onto the HiTrap-SP column (HiTrap SP HP 1 ml column; Amersham Biosciences) and the column was washed with HEPES buffer. The column was eluted with an NaCl gradient of 150–500 mM in HEPES buffer. The fractions that contained the 29 kDa protein were pooled and diluted threefold with HEPES buffer, and the resulting sample was loaded onto a dsDNA-cellulose column (0.1 ml in a syringe). The column was washed with HEPES buffer and the 29 kDa protein was eluted with HEPES buffer containing 1 M NaCl.

SDS-PAGE and SDS-DNA PAGE. For SDS-PAGE, an aliquot of each sample (15 μl) was mixed with 5 μl $4\times$ SDS sample buffer and loaded on a 7.5–15 % polyacrylamide gel. SDS-DNA PAGE for detecting DNA-binding proteins was performed as described previously (Miyakawa *et al.*, 2000). The stacking and separating gels contained 10 $\mu\text{g ml}^{-1}$ native calf thymus DNA. After overnight incubation of the gels in 10 mM Tris/HCl, 1 mM EDTA, pH 7.5, 10 mM MgCl_2 and 7 mM 2-mercaptoethanol at 30°C , the gels were stained with ethidium bromide (1 $\mu\text{g ml}^{-1}$) for 30 min. Proteins were detected by the silver-staining method (Oakley *et al.*, 1980). A low-molecular-mass calibration kit (Pharmacia) was used for the estimation of molecular mass. For the determination of the N-terminal amino acid sequence, proteins were transferred to a PVDF membrane and the sequences were determined using a Protein Sequencer PPSQ-21 (Shimadzu).

Expression and purification of recombinant Gcf1 protein. The plasmid pGEX-2T-*GCF1*, expressing the recombinant version of the Gcf1 protein in fusion with glutathione *S*-transferase (GST-Gcf1p) was constructed as follows. The *GCF1* ORF was amplified by PCR from the genomic DNA of *C. parapsilosis* strain CBS604^T using two oligonucleotide primers, 5'-ATGTTGAGACTCATTGTTAACCT-3' and 5'-TAGCTGTGCTTCTTTTAGATTGTG-3'. The PCR product was then inserted into expression vector pGEX-2T (Amersham Biosciences) digested with *EcoRI* and blunt-ended with Klenow fragment of DNA polymerase I.

Escherichia coli DH5 α cells (Clontech) transformed with the plasmid pGEX-2T-*GCF1* were cultivated in LBA medium [1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.9 % (w/v) NaCl, 100 μg ampicillin ml^{-1}] until the culture reached OD_{600} 0.6, then IPTG was added to a final concentration of 1 mM and expression was induced for 3 h at 30°C . Cells were harvested by centrifugation (10 min, 4°C , 10 000 *g*), washed once with sterile ice-cold water, resuspended in 5 ml LR buffer (20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol) containing 1 mg lysozyme ml^{-1} and $1\times$ Complete protease inhibitor (Roche Molecular Biochemicals), and incubated for 20 min on ice. The suspension was sonicated for 20 s and then incubated on ice for 20 s. The sonication step was repeated five times. Subsequently, Triton X-100 to a final concentration of 0.1 % (v/v) and 50 U DNase I (Promega) were added, and the suspension was incubated for 15 min on ice and cleared by centrifugation (15 min, 16 000 *g*, 4°C). The supernatant was mixed with 300 μl glutathione agarose beads (Sigma-Aldrich) and gently agitated for 1 h at 4°C . The beads were then washed three times with 700 μl LRT buffer [20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 % (v/v) Triton X-100]. The recombinant protein was eluted with 500 μl ER1 buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 15 mM glutathione). All fractions were analysed by 10 % SDS-PAGE electrophoresis. The recombinant protein was stored at -20°C .

Electrophoretic mobility shift assay (EMSA). Purified recombinant protein GST-Gcf1 (40–100 ng) was mixed with 150 ng *C. parapsilosis* mtDNA digested with *BglII* and incubated for 1 h at room temperature. The samples were electrophoretically separated in 0.6 % agarose gels in 45 mM Tris-borate, 1 mM EDTA (0.5 $\times$ TBE) buffer and DNA was visualized by staining the gels with ethidium bromide.

(0.5 $\mu\text{g ml}^{-1}$). GST purified using the same procedure as described for GST-Gcf1 was used as a negative control.

Fluorescence microscopy. Cells and spheroplasts of *C. parapsilosis* were fixed, respectively, with 5% (v/v) glutaraldehyde in the culture medium and in 0.8 M sorbitol, 20 mM potassium phosphate buffer, pH 7.5, at room temperature for 1 h. Samples were washed twice with NS buffer (0.25 M sucrose, 20 mM Tris/HCl, pH 7.6, 1 mM EDTA, 1 mM MgCl_2 , 0.1 mM ZnSO_4 , 0.1 mM CaCl_2 , 0.8 mM PMSF, 0.05% (v/v) 2-mercaptoethanol; Miyakawa *et al.*, 1987) and mixed with an equal volume of a solution of 4',6-diamidino-2-phenylindole (DAPI; 2 $\mu\text{g ml}^{-1}$) in NS buffer on a glass slide. The isolated mitochondria and mt-nucleoids were mixed directly with an equal volume of DAPI (2 $\mu\text{g ml}^{-1}$) solution. All observations were made with an epifluorescence microscope (BHS-RFK, Olympus Optical Co.) equipped with appropriate objectives (Dplan Apo 100UVPL and 100UV, Olympus Optical Co.).

Intracellular localization of Gcf1p was done in *C. parapsilosis* SR23 cells transformed with the pBP6-GCF1 plasmid expressing Gcf1p fused with the enhanced version 3 of GFP (yEGFP3). The plasmid was constructed by insertion of the GCF1 ORF lacking the termination codon, amplified from *C. parapsilosis* CBS604^T genomic DNA using the primers 5'-ATGTTGAGACTCATTGTTAACCCT-3' and 5'-GATTGTGAATTTGACTCTTGT-3', into the pDrive cloning vector (Qiagen). Subsequently, the 771 bp *EcoRI* fragment was excised from the construct and inserted into the pBP6 vector (Kosa *et al.*, 2007) linearized with *XbaI* endonuclease. The termini of the vector and the insert molecules were blunt-ended with a Quick Blunting kit (New England Biolabs). The resulting plasmid, pBP6-GCF1, was transformed into *C. parapsilosis* SR23 (Nosek *et al.*, 2002). Transformants were selected in synthetic dextrose (SD) medium [2% (w/v) glucose, 0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% (w/v) ammonium sulfate, 0.02 mg adenine ml^{-1} , 2% (w/v) agar]. To visualize the Gcf1-yEGFP3 fusion, the transformants were cultivated overnight at 28 °C in liquid SD medium then for 6 h in synthetic galactose (SGal) medium, in which glucose was replaced by 2% (w/v) galactose. The cells were then observed using a BX50 microscope (Olympus Optical Co.).

Functional complementation of the $\Delta abf2$ mutation in *S. cerevisiae*. To demonstrate that Gcf1p is able to replace the Abf2p function(s) in *S. cerevisiae* cells, the plasmid pYES2/CT-GCF1 was constructed by insertion of GCF1, amplified from the genomic DNA of *C. parapsilosis* CBS604^T using the primers 5'-ATGTTGAGACTCATTGTTAACCCT-3' and 5'-TAGCTGTGCTTCTTTTAGATTGTG-3', into pYES2/CT vector (Invitrogen) linearized with *EcoRI* and blunt-ended with Klenow fragment of DNA polymerase I. The resulting construct, pYES2/CT-GCF1, and the vector pYES2/CT were transformed into *S. cerevisiae* YAM101-1a (*ABF2*) and YAM101-1b ($\Delta abf2$). Transformants were selected for uracil prototrophy in synthetic glycerol medium (SGlyc) [0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% (w/v) ammonium sulfate, 3% (v/v) glycerol, 2% (w/v) agar, supplemented with adenine and appropriate amino acids], and individual clones carrying either pYES2/CT or pYES2/CT-GCF1 were cultivated for 24 h in liquid synthetic media containing different carbon sources: SGlyc [3% (v/v) glycerol], SGal [2% (w/v) galactose] or SD [2% (w/v) glucose]. Stability of mtDNA in yeast cells was assayed by cultivation on yeast extract, peptone, glycerol, dextrose (YPDG) plates [1% (w/v) yeast extract, 2% (w/v) peptone, 0.1% (w/v) glucose, 3% (v/v) glycerol, 2% (w/v) agar].

In silico analyses. Amino acid sequences of *S. cerevisiae* mt-nucleoid components and their counterparts from *C. albicans* were downloaded from the *Saccharomyces* genome database (<http://www.yeastgenome.org/>) and the *Candida* genome database (<http://www.candidagenome.org/>), respectively. The coding sequences of *C. parapsilosis* mt-nucleoid proteins were identified with the TBLASTN tool at default settings using *S. cerevisiae* mt-nucleoid components or determined N-terminal sequences of *C. parapsilosis* proteins as queries in the database of assembled contigs from the *C. parapsilosis* genome sequencing project of the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/sequencing/Candida/parapsilosis>) and manually annotated. Systematic protein names were obtained from the *Candida* comparative site of the Broad Institute (http://www.broad.mit.edu/annotation/genome/candida_group/MultiHome.html).

The sequence alignments and comparison were done using the AlignX utility of the Vector NTI 10.1.1 package (Invitrogen) and GeneDoc program (Nicholas *et al.*, 1997). Sequence conservation of mt-nucleoid components between *C. albicans* versus *C. parapsilosis* (*x*) and *S. cerevisiae* versus *C. parapsilosis* (*y*) was calculated as the percentage of identical amino acids in a pair of aligned sequences. The whole set of *C. parapsilosis* mt-nucleoid proteins was visualized in a scatter plot graph in which *x* and *y* represent coordinates of individual components. Predictions of import into mitochondria were performed using MitoProt II (<http://ihg.gsf.de/ihg/mitoprot.html>; Claros & Vincens, 1996), PSORT II (<http://psort.hgc.jp/>; Nakai & Kanehisa, 1992) and MITOPRED (<http://bioinformatics.albany.edu/~mitopred/>; Guda *et al.*, 2004). The HMG boxes and coiled-coil domains were predicted using SMART (<http://dylan.embl-heidelberg.de/>; Letunic *et al.*, 2006) and COILS (http://www.ch.embnet.org/software/COILS_form.html; Lupas *et al.*, 1991), respectively.

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RESULTS

Isolation of the mt-nucleoids from stationary-phase yeast culture

C. parapsilosis cells and spheroplasts contain numerous mt-nucleoids as revealed by DAPI staining (Fig. 1a–c). In the case of *S. cerevisiae* and *K. lactis*, the mt-nucleoids were fractionated from mitochondria by sucrose density-gradient centrifugation, as reported previously (Miyakawa *et al.*, 1987, 1995, 2003). However, the mt-nucleoids of *C. parapsilosis* underwent precipitation even after NP-40 treatment of mitochondria, and the yields of the mt-nucleoids decreased significantly after the sucrose density-gradient centrifugation. Therefore, we routinely fractionated the mt-nucleoids from mitochondrial lysates using differential centrifugation as described in Methods. DAPI staining of the isolated mt-nucleoids showed that they maintained their intact size (Fig. 1d). The patterns of DNA extracted from the mt-nucleoid fraction and digested with restriction enzymes (data not shown) corresponded to mtDNA reported in our previous studies (Nosek *et al.*, 1995, 2004).

The mt-nucleoid fractions isolated from *C. parapsilosis* contained a subset of mitochondrial proteins (Fig. 2, lane 2 versus lane 6). SDS-DNA PAGE analyses of the mt-nucleoid proteins clearly detected the enrichment of a 29 kDa DNA-binding protein in the mt-nucleoids (Fig. 2, lane 11). In this assay we also detected a 37 kDa protein. This protein was abundant in the mitochondrial fraction rather than enriched in the mt-nucleoids. Similar results

were obtained when the mt-nucleoid fraction from *S. cerevisiae* was analysed and, according to the molecular mass and the requirement for Mg^{2+} ions for its appearance on the gel in the SDS-DNA PAGE assay, this protein was assumed to be the major mitochondrial nuclease Nuc1p (Miyakawa *et al.*, 2000), as SDS-DNA PAGE detects nuclease activity as well as DNA-binding proteins (Rosenthal & Lacks, 1977). However, we did not investigate the nature of the 37 kDa protein from *C. parapsilosis*.

Identification of proteins from the mt-nucleoid fraction

We compared the protein composition of the mt-nucleoids with that of the mitochondrial fraction on 7.5, 10, 12.5 and 15% gels, and 17 proteins present in the mt-nucleoid fraction were subjected to N-terminal sequence analysis as summarized in Fig. 3(a). With the exception of the 29 kDa protein, the majority of visualized proteins were not highly enriched in the mt-nucleoid fraction (see Discussion). We determined N-terminal sequences of 15 proteins, which suggested that the N-terminal amino acid was not blocked in most cases (Table 1), and subsequent TblastN searches using the peptide sequences as queries led to identification of 14 coding sequences in the *C. parapsilosis* genome sequence and, subsequently, revealed their homologues from *S. cerevisiae* and *C. albicans* (see Supplementary Tables S1 and S2). In the case of p63, we did not identify

the corresponding coding sequence, and proteins p58 and p94 were not amenable to N-terminal sequence analysis. The identified protein set includes aconitase (Aco1p), subunits of pyruvate dehydrogenase (Lat1p, Pda1p and Pdb1p), mitochondrial ribosomal proteins (Mnp1p, Mrpl4p and Mrpl6p), mitochondrial telomere-binding protein (Mtp1p/mtTBP), cytochrome *c* (Cyc1p), cytochrome *c* oxidase subunit 4 (Cox4p), mitochondrial succinate–fumarate transporter (Sfc1p), mitochondrial translation elongation factor (Tuf1p), fatty-acyl coenzyme A oxidase (Pox1p), and an HMG box-containing protein, Gcf1 (see below).

The 29 kDa protein is a putative mtDNA packaging protein

The properties of the 29 kDa protein (i.e. molecular mass, highly basic pI, relatively high abundance in mt-nucleoids and DNA-binding activity in SDS-DNA PAGE) support the hypothesis that it represents an mtDNA packaging protein in *C. parapsilosis* and may have similar function(s) to those of Abf2p, and this prompted us to investigate this protein in more detail.

Previously, we have purified an Abf2p homologue from *K. lactis* mitochondria by extraction of the mt-nucleoids by treatment with 0.2 M HCl followed by hydroxyapatite column chromatography and dsDNA–cellulose chromato-

Fig. 3. Identification of the mt-nucleoid proteins (see Methods for details). (a) Lanes: 1, molecular mass marker; 2, mitochondrial fraction; 3, supernatant after lysis of mitochondria by 0.5% NP-40 and following centrifugation; 4, NP-40-insoluble materials after lysis of mitochondria by 0.5% NP-40 and following centrifugation; 5, NP-40-soluble fraction after fractionation of the mt-nucleoids; 6, mt-nucleoid fraction. Upper and lower gels are 10 and 15% polyacrylamide gels, respectively. (b) Purification of the 29 kDa protein. Lanes: 1, marker; 2, mt-nucleoid fraction; 3, purified 29 kDa protein. The NP-40-insoluble material was resuspended in the same volume of NES2 buffer as the supernatant. Samples of 15 μ l were loaded onto the gel.

Table 1. *C. parapsilosis* proteins identified in the mt-nucleoid fraction by N-terminal sequencingUnderlined amino acid residues represent mismatches in the sequences of identified proteins annotated in the *C. parapsilosis* genome database.

Size (kDa)	N-terminal sequence	TBlastN query	Best hit using TBlastN	Identification
89	ATAINRYS(?)KVHQ ^u NLL	ATAINRYSKVHQ ^u NLL	A ^u AINR ^u DSKVHQ ^u NLL	Aco1p
76	AMAKLPW(?)F(?)VQTNKA(?)K	AMAKLPWFVQTNKAK	AMAS ^u LP ^u DFVQTNKE ^u K	Pox1p
63	M(?)IIKTKM(?)PAM(?)KN(?)FY(?)T	MIKTKMPAMKNFYT	—	—
60	SK(?)GKFPPK(?)TVIN(?)MP(?)A	SKGKFPPKTVINMPA	S ^u SGKFPPHTVIHMPA	Lat1p
44	ATAE(?)PVGA(?)GSK(?)LVS(?)I	ATAEPVGAGSKLVS ^u I	ATAEPVG ^u D ^u GS ^u DLVS ^u I	Pda1p
42	AAYYNRAKPHVNVGTI	AXYNRAKPHVNVGTI	AAYNRAKPHVNVGTI	Tuf1p
38	SNSKSGQQT(?)MTVRXA	SNSKSGQQTMTVRXA	N ^u NSKSGQQE ^u MTVRDA	Pdb1p
37	A/S(?)R(?)AKL/PLY(?)FYKLKT(?)L(?)K(?)	XRAKXLYFYKLKTLK	ARAKPL ^u K ^u FDLKT ^u IK	Mrpl4p
34	A/SA/TSTS(?)VKKQTKR/TGAT	XXSTS ^u VKKQTKXGAT	AASTE ^u VKKQTKRGAT	Sfc1p
29	STAKTTRSSKDKEAA	STAKTTRSSKDKEAA	STAKTTRSSKDKEAA	Gcf1p
28	W/(M/Q)H/(D/N)IG(?)KKP(?)IKI(?)TKA(?)VX	XXIGKKPIKITKAVX	S ^u NIGKKPIKITDGVS	Mrpl6p
20	STTTAAKSPVNPVVD/I(?)	STTTAAKSPVNPVX	N ^u TTTAAESPSPVVD	Mnp1p
18	AKAPQTTTKVKTAQV/D(?)	AKAPQTTTKVKTAQX	NKAPQTTTEVKTAQD	Cox4p
15 ¹	P/SFANGQLFGRVGNVD	XFANGQLFGRVGNVD	N ^u FANGQLFGRVGNVD	Mtp1p/mtTBP
15 ²	S/PAYYAKGSP(?)KKG/D(?)ATL	XAYYAKGSXKKXATL	PAPY ^u E ^u KGSE ^u KKGATL	Cyc1p

graphy (Miyakawa *et al.*, 2003). However, this method was not successful in the case of the 29 kDa protein of *C. parapsilosis*. On the other hand, the combination of NaCl extraction of mt-nucleoid proteins, HiTrap-SP column chromatography (Sasaki *et al.*, 2003) and dsDNA–cellulose chromatography was effective, and the 29 kDa protein was purified to near homogeneity, as indicated by the presence of a single band on SDS-PAGE (Fig. 3b). The DNA-binding activity of the purified 29 kDa protein and its enrichment in the mt-nucleoids were confirmed by SDS-DNA PAGE assay (data not shown, but see Fig. 2, lane 11).

The N-terminal amino acid sequence of the 29 kDa protein was determined to be STAKTTRSSKDKEAA (Table 1). A TBlastN search using this peptide as a query against the *C. parapsilosis* genome sequence database revealed a single ORF, CPAG_03094, with a length of 251 codons. Bioinformatic analyses of deduced amino acid sequences by MitoProt II, PSORT II and MITOPRED indicated high probabilities (0.9673, 65.2% and 99%, respectively) of its being targeted to mitochondria (Supplementary Table S1). The import signal predicted by MitoProt II (amino acids 1–28) corresponds with the experimentally determined N-terminal sequence (amino acids 34–48) of the purified protein. Analyses with the COILS and SMART utilities revealed the presence of a coiled-coil domain (amino acids 44–106) and an HMG box (amino acids 166–230), respectively. A BLASTP search against the GenBank database revealed two hits with a relatively low score; EAK92709 (*GCF1*/orf19.400/orf19.8030; E-value $1e^{-10}$) and CAG90643 (DEHA0G15059 g; E-value $2e^{-05}$) in the *C. albicans* and *Debaryomyces hansenii* genomes, respectively, which were identified in our previous *in silico* study (Nosek *et al.*, 2006) as putative mtDNA packaging proteins from the mtHMG protein family. Based on common features with *C. albicans* Gcf1p (see Visacka *et al.*, 2009), we named

the 29 kDa protein (CPAG_03094) Gcf1p (or *CpGcf1p*) and the corresponding gene *GCF1* (or *CpGCF1*). In contrast to *S. cerevisiae* Abf2p, which contains two HMG boxes involved in DNA binding, the *Candida* Gcf1 proteins apparently have only a single HMG box that corresponds to HMG box 2 of Abf2p. In addition, Gcf1 proteins have a coiled-coil domain not present in Abf2p, which could be engaged in the formation of protein dimers, possibly compensating for the absence of the second HMG box. Although the Gcf1 and Abf2 proteins exhibit structural similarities, their overall homology is weak, with only a few conserved amino acid positions (Fig. 4).

Recombinant Gcf1p is an mtDNA-binding protein that localizes to mitochondria

Analyses of the mt-nucleoid fraction as well as the purified 29 kDa protein revealed that it can bind DNA substrates in SDS-DNA PAGE assays. To confirm that the protein product of the *GCF1* gene is a bona fide mtDNA-binding protein, we analysed its properties in more detail. First, we confirmed that Gcf1p is imported into mitochondria using an in-frame fusion of the *GCF1* ORF with the sequence encoding the yEGFP3 marker. Analysis by fluorescence microscopy of *C. parapsilosis* transformants expressing the fusion protein showed a number of foci corresponding to mt-nucleoids, as revealed by DAPI staining (Fig. 5). Next, we demonstrated that recombinant GST–Gcf1p purified from overproducing *E. coli* cells, i.e. Gcf1p fused with the C terminus of GST, exhibits DNA-binding activity in EMSA. When we used purified *C. parapsilosis* mtDNA digested with *Bgl*II as a substrate, we observed that with increasing amounts of the protein all DNA fragments exhibited slower mobility, indicating that they are bound by the protein (Fig. 6). The absence of an apparent preference for any of

Fig. 4. Comparison of amino acid sequences of *C. parapsilosis* (CANPA) 29 kDa/Gcf1 protein with its counterparts from *C. albicans* (CANAL) and *D. hansenii* (DEBHA), and Abf2p from *S. cerevisiae* (SACCE). The signal sequence for mitochondrial import (determined by N-terminal sequencing of the *C. parapsilosis* protein) and the predicted coiled-coil domain and HMG box 2 are indicated above the alignment. Note that *in silico* analysis did not reveal the HMG box 1 in *C. albicans* and *C. parapsilosis*; its position in *D. hansenii* Gcf1p is indicated by a dotted line. *S. cerevisiae* Abf2p lacks the coiled-coil domain. Shading was performed using the GeneDoc utility at default settings.

the mtDNA fragments suggests that the protein binds to DNA without sequence specificity. The gradual shift of the DNA bands is probably caused by the fact that a population of DNA fragments of a given size is represented by molecules bound by different numbers of protein molecules.

C. parapsilosis GCF1 complements the $\Delta abf2$ mutation in *S. cerevisiae* cells

Yeast cells lacking functional Abf2p are able to maintain mtDNA when cultivated in media with non-fermentable

carbon sources, as Aco1p substitutes for its DNA-binding activity, but these cells quickly lose their mtDNA in glucose-containing medium when Aco1p is repressed (Diffley & Stillman, 1991, 1992; Chen *et al.*, 2005). Although Gcf1p exhibits very low sequence homology with Abf2p, amino acid alignment revealed several conserved residues, mostly within the HMG box 2. Therefore, we determined whether Gcf1p is able to substitute for Abf2p in mtDNA maintenance by complementation of a $\Delta abf2$ mutation in *S. cerevisiae* cells. We constructed the plasmid pYES2/CT-GCF1 containing the GCF1-coding sequence driven by the galactose-inducible GAL1 promoter, and introduced it into *S. cerevisiae* YAM101-1a and YAM101-1b cells. The transformants were cultivated for 24 h in liquid synthetic media containing 2 % glucose (SD) or 2 % galactose (SGal) as the sole carbon source, and then the cells were inoculated in serial (10^1 – 10^5) dilutions onto solid YPDG media (Fig. 7). In this assay, *S. cerevisiae* $\Delta abf2$ cells rapidly ceased growth due to loss of mtDNA. The expression of GCF1 clearly increased the proportion of respiratory-competent cells of the $\Delta abf2$ mutant, indicating that Gcf1p participates in the maintenance of mtDNA *in vivo*.

Fig. 5. Intracellular localization of Gcf1-yEGFP3 fusion protein. Expression of the fusion protein in *C. parapsilosis* cells transformed with pBP6-GCF1 was induced in SGal medium and observed by fluorescence microscopy (a). DAPI was used to stain cellular DNA (b). Bars, 5 µm.

Candidate components of mt-nucleoids encoded by the *C. parapsilosis* genome

To complement the set of mt-nucleoid proteins identified experimentally, we searched for other components by *in silico* analysis of the *C. parapsilosis* genome sequence. As the

Fig. 6. Gcf1p binds mtDNA in EMSA. The assay was performed using recombinant GST–Gcf1 protein and *C. parapsilosis* mtDNA digested with *Bgl*III (lanes 1–5). Purified GST was used in the control experiment (lanes 6 and 7). Digested mtDNA samples (150 ng) were incubated for 1 h at room temperature without (lane 1) or with 40 ng (lane 2), 60 ng (lane 3), 80 ng (lane 4) or 100 ng (lane 5) recombinant GST–Gcf1 protein, or 80 ng (lane 6) and 100 ng (lane 7) GST. Samples were separated by electrophoresis in a 0.6% agarose gel. λ DNA digested with *Pst*I was used as the molecular mass marker (M).

bioinformatic analysis cannot itself confirm that they are components of mt-nucleoids in *C. parapsilosis*, we consider these proteins to be candidate components for participating in the structure and/or function of these nucleic acid–protein complexes. Using amino acid sequences of 30 bona

fide mt-nucleoid proteins from *S. cerevisiae* (reviewed by Chen & Butow, 2005; Kucej & Butow, 2007) as queries in TBLASTN searches we identified almost all counterparts present in assembled contigs covering 9.2 times the *C. parapsilosis* genome (Supplementary Table S1). Our efforts did not lead to unambiguous identification of a homologue of Abf2 protein, as hits with the highest score were nuclear non-histone HMG proteins, such as Nhp6a and Nhp6b. This is in line with our hypothesis that Gcf1p represents the major mtDNA packaging protein in *C. parapsilosis*.

DISCUSSION

Proteins associated with mt-nucleoids in *C. parapsilosis*

In this study we experimentally identified 14 proteins present in the mt-nucleoid fraction prepared from *C. parapsilosis* mitochondria (Table 1, Fig. 3a). Among them, five proteins (Aco1p, Pda1p, Pdb1p, Mnp1p and Rim1p) were known to be bona fide mt-nucleoid components in *S. cerevisiae*, suggesting that at least some features of the mt-nucleoids are conserved between phylogenetically distant yeast species. The homologue of Rim1p has been previously identified in *C. parapsilosis* mt-nucleoids *in vivo* as mtTBP, which plays the dual role of single-stranded DNA-binding protein and mitochondrial telomere-binding factor (Tomaska *et al.*, 1997, 2001; Nosek *et al.*, 1999). Importantly, we detected a putative mtDNA packaging protein, Gcf1p, which is distantly related to Abf2p of *S. cerevisiae*, and an additional eight proteins (Cox4p, Cyc1p, Lat1p, Mrpl4p, Mrpl6p, Pox1p, Sfc1p and Tuf1p), whose counterparts have not been detected in *S. cerevisiae* mt-nucleoids. Moreover, we found a 37 kDa protein as a minor component of the mt-nucleoids, although its identity was not examined (Fig. 2). It remains to be

Fig. 7. *C. parapsilosis* GCF1 complements $\Delta abf2$ mutation in *S. cerevisiae*. The plasmids pYES2/CT and pYES2/CT–GCF1 were transferred into the strains YAM101-1a (*ABF2*) and YAM101-1b ($\Delta abf2$). The transformants were cultivated for 24 h in SD or SGal liquid media and then inoculated in serial dilutions onto YPDG plates (labelled as SD→YPDG or SGal→YPDG).

determined which of these proteins represent species-specific features of *C. parapsilosis* mt-nucleoids. Alternatively, proteins not found in *S. cerevisiae* mt-nucleoids may be loosely associated and therefore have escaped detection in previous studies. The latter hypothesis is supported by the presence of protein subunits from the pyruvate dehydrogenase complex (Lat1p) and mitochondrial ribosome (Mrpl4p and Mrpl6p), as other proteins from these complexes (i.e. Pda1p, Pdb1p and Mnp1p) associate with mt-nucleoids in both yeast species. The occurrence of three mitochondrial ribosomal protein subunits (Mnp1p, Mrpl4p and Mrpl6p) and a translation elongation factor Tu (Tuf1p) in the mt-nucleoid fraction is particularly interesting, as this supports a hypothesis that mitochondrial proteosynthetic machinery is closely linked with the structure and/or dynamics of mt-nucleoids, which is further strengthened, for example, by the coupling of transcription and translation in yeast mitochondria as well as the involvement of bona fide mt-nucleoid components such as Rpo41p, Sls1p and Mnp1p in these processes (Kaufman *et al.*, 2000; Bryan *et al.*, 2002; Rodeheffer & Shadel, 2003; Sato & Miyakawa, 2004). This is in line with the observation that mt-nucleoids purified from human mitochondria contain a number of mitochondrial ribosomal proteins, tRNA synthetases and several translation factors, including the elongation factor Tu, indicating that translation occurs in the peripheral region of the mt-nucleoids (Boghenagen *et al.*, 2008).

In general, it might be expected that the degree of enrichment in the mt-nucleoid fraction in the case of the proteins performing dual functions will be dependent on their distribution between the mt-nucleoid and the corresponding protein complex involved in a particular biochemical process (e.g. the Krebs cycle). For example, in *S. cerevisiae*, only a fraction of the mitochondrial enzyme aconitase (Aco1p) is associated with mt-nucleoids and participates in mtDNA maintenance (Chen *et al.*, 2005). Naturally, proteins involved solely in mt-nucleoid functions, such as Gcf1p, should be enriched by the purification. Indeed, Gcf1p is highly enriched in the mt-nucleoid fraction and represents one of its major constituents. On the other hand, we must acknowledge that our mt-nucleoid fractions were prepared without density-gradient centrifugation and therefore may contain contaminants in addition to bona fide mt-nucleoid proteins. For example, cytochrome *c* (Cyc1p) is not likely to be an mt-nucleoid protein, and the conditions employed for solubilization of the inner membrane may induce artefactual association of cytochrome *c* with the mt-nucleoids due to its basic pI. Similarly, fatty-acyl coenzyme A oxidase (Pox1p) is not a mitochondrial protein and may represent contamination from peroxisomes.

Using *in silico* analysis of the *C. parapsilosis* complete genome sequence we identified additional candidate components of the mt-nucleoids. Our results indicate that the *C. parapsilosis* genome encodes a similar set of proteins to those known to be associated with mt-nucleoids of *S.*

cerevisiae (Supplementary Table S1). In most cases, the homology between *C. parapsilosis* and *S. cerevisiae* proteins was relatively weak. Therefore, we also confirmed their identities by comparison with *C. albicans* counterparts and predicted the probability of their import into mitochondria (Supplementary Table S1). To illustrate overall conservation of the mt-nucleoid components from *C. parapsilosis*, we compared proteins identified experimentally as well as candidates detected by *in silico* analysis with their homologues from *S. cerevisiae* and *C. albicans* (Fig. 8, Supplementary Table S2). The analysis revealed that several proteins known to interact directly with mtDNA (i.e. Abf2p/Gcf1p, Mip1p, Pif1p, Rim1p/mtTBP and Rpo41p) are conserved relatively weakly. It is possible that the faster accumulation of changes in their sequences reflects the evolution of mtDNA sequences. However, this explanation may be acceptable only in certain cases, when proteins recognize specific motifs in mtDNA. For example, mtTBP has been shown to bind the terminal sequences of the linear mtDNA molecules from *C. parapsilosis* (Tomaska *et al.*, 1997, 2001; Nosek *et al.*, 1999). Similarly, Rpo41p recognizes sequence motifs of the mitochondrial promoter (Matsunaga & Jaehning, 2004), which varies among yeast species. However, other proteins, such as Gcf1p, do not

Fig. 8. Conservation of the mt-nucleoid components of *C. parapsilosis*. The set of proteins contains homologues (best hits) identified *in silico* as well as proteins identified experimentally in the mt-nucleoid fraction from *C. parapsilosis* (Supplementary Table S1). Conservation was inferred from the percentage of amino acid sequence identity between *C. albicans* versus *C. parapsilosis* [CANAL/CANPA] and *S. cerevisiae* versus *C. parapsilosis* [SACCE/CANPA], which corresponds to the *x* and *y* coordinates of each protein spot, respectively (Supplementary Table S2). Proteins known to interact directly with the mtDNA in *S. cerevisiae* are shown as open squares.

seem to recognize specific mtDNA sequences and the reason for the elevated rate of their evolution remains elusive. On the other hand, the higher degree of sequence conservation of bifunctional mt-nucleoid components (such as Aco1p and Ilv5p) may be due to a dual selection for their metabolic functions as well as their role(s) in stabilization of the mt-nucleoids. An interesting example is Mgm101p. Although overall conservation of its sequence is moderate (sequence identity with *S. cerevisiae* and *C. albicans* counterparts is 47.3 and 60.1 %, respectively), its C-terminal half is highly conserved among yeast species, indicating its important role in the processes of mtDNA replication and repair.

Gcf1p is a putative mtDNA packaging protein

Diverse proteins involved in the packaging of mtDNA molecules have been identified in phylogenetically distant organisms, ranging from slime moulds and yeasts to humans. Our results extend the list of these proteins by *C. parapsilosis* Gcf1p, a new member of the mtHMG family, distantly related to Abf2p of *S. cerevisiae*. Although sequences of Abf2p and Gcf1p are significantly diverged, their comparison revealed common structural features and several conserved amino acid positions. Most mtDNA packaging proteins identified so far (Abf2p, Glom, h-mtTFA/TFAM, *Podospora anserina* mthmg1 and *D. hansenii* Gcf1p) contain two HMG boxes. However, in contrast to Abf2p, which contains two HMG boxes, in Gcf1 homologues from *C. parapsilosis* and *C. albicans*, the SMART utility predicted a coiled-coil domain and a single HMG box corresponding to the HMG box 2 of Abf2p. As this is based only on an *in silico* prediction we cannot rule out the possibility that, in spite of sequence divergence in the region corresponding to HMG box 1, this domain has retained its function in DNA binding. Interestingly, the same analysis of *D. hansenii* Gcf1p predicts a coiled-coil domain as well as two HMG boxes. This indicates that the *D. hansenii* protein represents an intermediate type of mtDNA packaging protein from which *Candida* and *Saccharomyces* forms might have been derived by elimination of HMG box 1 and the coiled-coil domain, respectively.

Nevertheless, our results clearly demonstrate that the protein product of *GCF1* co-localizes with mtDNA-containing foci, associates with isolated mt-nucleoids, has DNA-binding activity *in vitro*, and is able to rescue the mtDNA maintenance defect in an *S. cerevisiae* $\Delta abf2$ mutant. Importantly, similar results were obtained for its homologue from *C. albicans* and, in addition, it was shown that reduced levels of CaGcf1p lead to a decreased fraction of recombination intermediates and number of mtDNA copies, which is accompanied by a reduced growth rate (see Visacka *et al.*, 2009). These data strongly indicate that the Gcf1 proteins are bona fide mt-nucleoid components implicated in mtDNA transactions. Further functional studies of Gcf1p and its counterparts from closely related

species may reveal how this protein affects the mode of mtDNA packaging and what role it plays in the maintenance of the linear mitochondrial genome.

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