Three genes for mitochondrial proteins suppress null-mutations in both Afg3 and Rca1 when over-expressed

Rep, M.; Nooy, J.; Guelin, E.J-M.; Grivell, L.A.

Published in:
Current Genetics

DOI:
10.1007/s002940050122

Citation for published version (APA):
The AFG3 gene of Saccharomyces cerevisiae encodes a mitochondrial inner membrane protein with ATP-dependent protease activity. To gain more insight into the function of this protein, multi-copy suppressors of an afg3-null mutation were isolated. Three genes were found that restored partial growth on non-fermentable carbon sources, all of which affect the biogenesis of respiratory competent mitochondria: PIM1 (LON) encodes a matrix-localized ATP-dependent protease involved in the turnover of matrix proteins; OXA1 (PET1402) encodes a putative mitochondrial inner membrane protein involved in the biogenesis of the respiratory chain; and MBA1 encodes a mitochondrial protein required for optimal respiratory growth. All three genes also suppressed a null mutation in a related gene, RCA1, as well as in the combination of afg3- and rca1-null.

Key words Multi-copy suppression · Mitochondrial membrane · Metalloprotease · S. cerevisiae

Introduction

Mitochondrial biogenesis in the yeast S. cerevisiae is an intricate process requiring the co-ordinated expression of mitochondrial and nuclear genetic systems (Grivell 1989, 1995). The latter system is quantitatively more important. For instance, with one exception (a mitoribosomal protein), all proteins necessary for mitochondrial gene expression are encoded in the nucleus and imported into the organelle. Nevertheless, mitochondrial genes encode essential components of enzyme complexes in the mitochondrial inner membrane involved in oxidative phosphorylation, making the mitochondrial genetic system essential for respiratory growth.

In yeast, several hundred nuclear genes are required for the maintenance of functional mitochondria, the so-called PET genes (Tzagoloff and Dieckmann 1991). One such gene, AFG3, was isolated previously by complementation of a mutant with a conditional pet phenotype (Guelin et al. 1994). Its translation product, Afg3p, is a protein with several interesting features. It contains the approximately 200 amino-acid domain characteristic of the AAA family of ATPases (Kunau et al. 1993; Confalonieri and Duguet 1995), together with a C-terminal domain containing the consensus sequence motif of a family of zinc metalloproteases (Campbell et al. 1994). Furthermore, hydrophobic regions in the amino-terminal domain mediate membrane association (Pajic et al. 1994). These features are shared with two other mitochondrial membrane proteins, Yme1p and Rca1p (Thorsness et al. 1993; Tzagoloff et al. 1994). Together with prokaryotic homologues, these proteins form a subfamily within the AAA family (Guélin et al. 1994). FtsH, the E. coli member of this subfamily, has been implicated in both the assembly/topogenesis of membrane proteins and the translocation of proteins across the plasma membrane (Tomoyasu et al. 1993; Akiyama et al. 1994a,b), as well as in protein degradation (Herman et al. 1993, 1995; Kihara et al. 1995; Tomoyasu et al. 1995). Similarly, Afg3p(Yta10p) is needed for rapid, ATP-dependent degradation of both prematurely terminated and complete mitochondrial translation products (Pajic et al. 1994; Guélin et al. 1996) and together with Rca1p, is required for the formation of mitochondrial inner membrane enzyme complexes (Paul and Tzagoloff 1995). The finding that inactivation of the proteolytic function of Afg3p by site-directed mutagenesis does not affect respiratory growth (Guélin et al. 1996) suggests that Afg3p may have a function besides the proteolysis of mitochondrial translation products.

One way to obtain more insight into Afg3p function is to identify other mitochondrial proteins that can (partially) bypass the need for Afg3p in mitochondrial biogenesis.
Three genes encoding such bypass proteins were identified by selecting for multi-copy suppressors of an afg3-null mutation. Interestingly, these genes also suppress an rca1-null mutation. The implications of these findings for the elucidation of Afg3p and Rca1p function are discussed.

### Materials and methods

**Strains and media.** The *S. cerevisiae* strains used in this study are listed in Table 1. WDA1 and WDA1R were derived from wild-type strains W303/1A (Muroff and Tzagoloff 1990) and W303ARCA1, respectively, by disruption of *AFG3* (see below). The following media were used for the propagation of yeast: YPD (2% glucose, 1% peptone, 1% yeast extract); YPGal (2% galactose, 2% peptone, 1% yeast extract); YPGly (2% glycerol, 2% peptone, 1% yeast extract); Lactate (1.5% lactic acid, 2% sodium lactate, 8 mM MgSO$_4$, 45 mM (NH$_4$)$_2$HPO$_4$, 0.5% yeast extract, pH 4.5); and WO [2% glucose, 0.67% yeast nitrogen base without amino acids (Difco)]. Where required, media were supplemented with nutritional requirements to the appropriate concentrations. Solid media contained 2% agar.

Inactivation of the *AFG3* gene. A 2.9-kb chromosomal PstI fragment containing almost the entire *AFG3* gene (Guélin et al. 1994) was cloned into pUC18. To create an *afg3::URA3* disruption construct, a 327-bp internal *Bgl*II fragment was replaced by a 1.2-kb *Bgl*II fragment from pFL38 containing the *URA3* gene. An *afg3::TRP1* construct was made by replacing a 1.7-kb internal *NheI*-EcoRV fragment with a 0.8-kb fragment containing the *TRP1* gene from YRp7 (Struhl et al. 1979). Linearized constructs were used for the transformation of yeast. Uracil or tryptophan prototrophs were selected and disruption of *AFG3* was checked by Southern blotting.

**Yeast genomic DNA library and construction of plasmids.** A yeast genomic DNA library in the high-copy vector YEpl3 (Nasmyth and Tatchell 1980) was used for isolation of multi-copy suppressors of *afg3-null*. Subclones were made in the high-copy LEU2 vectors YEplac181 and YEP351.

**Miscellaneous.** Standard methods were used for the manipulation of DNA, the transformation of *E. coli* (strain DH5α), and Southern blotting (Sambrook et al. 1989). Transformation of yeast cells was done according to (Chen et al. 1992); the isolation of plasmid DNA from yeast cells was as described in Hoffman and Winston (1987). DNA sequence analysis was performed by the method of Sanger et al. (1977).

### Results

**Disruption of AFG3**

The *AFG3* gene was disrupted in the wild-type strain W303/1A by transformation with an *afg3::URA3* construct (see Materials and methods). The resulting strain, WDA1, was unable to grow on the non-fermentable carbon sources ethanol, glycerol and lactate, indicating complete respiratory deficiency [as already observed by Tauer et al. (1994) for a *YTA10*(*=AFG3*)-disruption strain]. Diploids arising from a cross of WDA1 with a rho- tester strain are able to respire, excluding loss or mutation of mtDNA. Cells without (functional) mtDNA (rho+/rho-) do, however, accumulate to about 10–20% in stationary phase cultures of WDA1 grown on glucose or galactose. *AFG3*-disruption mutants without mitochondrial introns show less rho- induction and grow very slowly on non-fermentable carbon sources (see Fig. 4A). This is probably related to a deficiency in mitochondrial intron splicing in *afg3* mutants (Rep, M., unpublished observations).

Isolation of multi-copy suppressors of an *afg3-null* mutant

To learn more about *AFG3* function we set out to identify genes which, when over-expressed, can compensate for the absence of functional *AFG3*. For this purpose, WDA1 was transformed with a yeast genomic DNA library on a multi-copy plasmid and the transformants were screened for growth on glycerol. From about 40 000 transformants, 27 fast-growing and 21 slow-growing clones were identified. Plasmids isolated from the fast-growing clones all exhibited a restriction pattern typical of the *AFG3* locus (data not shown) and were not examined further. Upon re-transformation of the plasmids isolated from the slow-growing clones into WDA1, 14 were able to induce a slow-growth phenotype on glycerol, thus confirming the presence of suppressor genes on these plasmids. By restriction mapping, the inserts in these plasmids were divided into three classes. Class A consisted of only one insert (Fig. 1), class B of five indistinguishable inserts (Fig. 2), and class C of eight inserts grouped into three overlapping sets (Fig. 3).

The chromosomal regions from which the three classes of inserts were derived were identified by sequencing into the borders of the inserts in YEpl13/34 (class A), YEpl13/36 (class B), and pJN-C, a plasmid containing the region of overlap between the inserts of class C (Fig. 3). For the first two inserts, the sequences obtained were present in the EMBL database, and the restriction maps obtained corresponded to those predicted for the region between each set of inserts.

### Table 1 Genotypes and sources of the *S. cerevisiae* strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nuclear genotype</th>
<th>Mitochondrial genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WDA1</td>
<td>ade2-1, his3-11,-15, leu2-3,-112, ura3-1, trpl-1, can1-100, <em>afg3::URA3</em></td>
<td>(W303)</td>
<td>This study</td>
</tr>
<tr>
<td>W303ARCA1</td>
<td>ade2-1, his3-11,-15, leu2-3,-112, ura3-1, <em>rca1::URA3</em></td>
<td>(W303)</td>
<td>Tzagoloff et al. (1994)</td>
</tr>
<tr>
<td>WDA1R</td>
<td>ade2-1, his3-11,-15, leu2-3,-112, ura3-1, trpl-1, can1-100, <em>rca1::URA3, afg3::TRP1</em></td>
<td>(W303)</td>
<td>This study</td>
</tr>
<tr>
<td>DAY6</td>
<td>his3-11,-15, leu2-3,-112, ura3-1, <em>afg3::URA3</em></td>
<td>(167)</td>
<td>Guélin et al. (1996)</td>
</tr>
</tbody>
</table>

*(167) Mitochondrial DNA is from KAR(167) and contains no introns (Séraphin et al. 1987)*
of sequences. Subsequent deletion analysis localized the suppressor activities to single open reading frames (ORFs) on each DNA-fragment (Figs. 1 and 2). Both ORFs were characterized earlier and encode a mitochondrial ATP-dependent protease, Pim1p or Lon (Suzuki et al. 1994; Van Dyck et al. 1994), and a putative mitochondrial membrane protein, Oxa1p or Pet1402p (Bauer et al. 1994; Bonnefoy et al. 1994), respectively. The third insert was hybridized to a chromosome blot, and found to be homologous to sequences on chromosome II (data not shown). The sequence of the insert of plasmid pJN-C1 was kindly provided by M. Jacquet and was later published as a contribution to the chromosome-II sequencing project (Demolis et al. 1994).

As shown in Fig. 3, deletion analysis identified YBR1307 as the ORF responsible for suppression. The new gene, termed MBA1, for (Multi-copy By-pass of AFG3), encodes a putative 278 amino-acid protein whose N-terminus displays features characteristic of mitochondrial targeting sequences (Hartl et al. 1989). We have indeed found that MBA1 encodes a mitochondrial protein that is necessary for optimal mitochondrial function (Rep and Grivell 1996).

Suppression of rca1-null

Afg3p belongs to the FtsH subfamily of the AAA family which includes both mitochondrial and prokaryotic members (Confalonieri and Duguet 1995). Yeast mitochondria contain two additional members of this subfamily, Yme1p(Yta11p) and Rca1p(Yta12p). Of these, Rca1p is the most similar to Afg3p in sequence and structure. Disruption of RCA1 and AFG3 leads to similar phenotypes (see Discussion), and both proteins probably share the same topology in the mitochondrial inner membrane (Guélin et al. 1994; Schnall et al. 1994). The list of these similarities is here extended by the observation that the three multi-copy suppressors of afg3-null suppress an rca1-null mutation to a similar extent (Fig. 4 A). However, Afg3p and Rca1p cannot replace each other, as witnessed by the pet phenotype of the respective disruption strains. Even over-expression of either gene cannot overcome the res-
piratory defect caused by the absence of the other (data not shown). One interpretation of these observations is that both proteins are required for a single activity. If this is true, the three multi-copy suppressors should also suppress an \textit{afg3-null} / \textit{rca1-null} double disruption. This is indeed the case (Fig. 4B).

\section*{Discussion}

A search for multi-copy suppressors of an \textit{afg3-null} mutation has been employed to obtain clues to the primary defect in mitochondrial function caused by deletion of \textit{AFG3} and its homologue \textit{RCA1}. This defect manifests itself in the strong reduction in the amount and activity of respiratory chain complexes (Tauer et al. 1994; Tzagoloff et al. 1994) and a deficiency in the assembly of ATP synthase (Paul and Tzagoloff 1995). Clearly, the assembly of the mitochondrial inner membrane complexes does not pro-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Restriction map and subcloning of the class-C inserts. The three overlapping sets of inserts isolated from the screen for multi-copy suppressors of \textit{afg3-null} are indicated by (i), (ii) and (iii). The area of overlap was subcloned from a type (iii) insert using BamHI, creating pJN-C1 (the left BamHI site is not shown; it was generated by ligation of the genomic DNA fragment into YEpl3 during construction of the DNA library). This subclone has \textit{afg3-null} suppressor activity and contains three complete ORFs. \textit{YBR1305} and \textit{YBR1306} are named according to Demolis et al. (1994). In that reference, \textit{MBA1} is termed \textit{YBR1307}. Subclones that allowed identification of \textit{MBA1} as the gene responsible for the suppression of \textit{afg3-null} are shown. See legend of Fig. 1 for explanation of symbols.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{A, B \textit{PIM1}, \textit{OXA1} and \textit{MBA1} suppress \textit{afg3-null}, \textit{rca1-null} and \textit{afg3-rca1-null} mutations. Strains WDA1 (\textit{ΔAFG3}), \textit{ΔAY6 [ΔAFG3 (Δintrons)]}, \textit{W303ΔRCA1 (ΔRCA1)} and WDAR1 (\textit{ΔAFG3-ΔRCA1}) were transformed with the high-copy plasmid YEplac181 (181), a low-copy plasmid containing the \textit{AFG3} gene (\textit{AFG3}), a low-copy plasmid containing the \textit{RCA1} gene (\textit{RCA1}), pJN-A3 (\textit{PIM}), pMR-B2 (\textit{OXA}) or pJN-C4 (\textit{MBA}), grown in glucose media selective for plasmid-containing cells and spotted on either minimal glucose plates (WO-Glu) or rich glycerol plates (Gly). Photographs were taken after 3 (WO-Glu) and 10 (Gly) days. A Suppression of \textit{afg3-null} and \textit{rca1-null}; B Comparison of suppression of single and double disruptions of \textit{AFG3} and \textit{RCA1}.}
\end{figure}
ceed normally in afg3 and rca1 mutants. This fits well with the proposed function of the E. coli homologue of these genes, FtsH, in the assembly and/or topology of plasma membrane proteins (Tomoyasu et al. 1993; Akiyama et al. 1994 a, b).

On the other hand, mutations in FtsH have been found to stabilize different cellular proteins (Herman et al. 1993, 1995; Kihara et al. 1995), and disruption of AFG3 (YTA10) leads to stabilization of otherwise rapidly degraded incomplete (Pajic et al. 1994) and complete (Guélin et al. 1996) mitochondrial translation products. Although involvement of FtsH in protein degradation has been suggested to be indirect, by presenting target proteins to a protease (Herman et al. 1993; Kihara et al. 1995), a protease activity of the members of the FtsH-subfamily is supported by their conserved metallopeptase active-site motif (Campbell et al. 1994), combined with the dependence of turnover of mitochondrial translation products on divalent metal-ions (Nakai et al. 1994; Yasuhara et al. 1994). Moreover, degradation of the heat-shock transcription factor sigma32 by purified FtsH has recently been demonstrated in vitro (Tomoyasu et al. 1995). These observations have led some investigators to conclude that all defects in ftsH cells will eventually be shown to result from the defective turnover of different cellular proteins (Herman et al. 1995).

Such an interpretation seems less likely for explaining the pet phenotype of yeast cells upon disruption of AFG3, since specific mutational inactivation of the protease activity of Afg3p does not affect respiratory growth (Guélin et al. 1996). Rather, Afg3p may be directly involved in complex assembly. The identification of OXA1 as one of the suppressors supports this notion, as it also has a role in the assembly of the inner membrane complexes. Disruption of OXA1 results in a complete absence of cytochrome aa3 and reduction of cytochrome b, while mitochondrial translation products are present (Bonnefoy et al. 1994). Other researchers have isolated the same gene as PET1402 by a screen for mutants defective in the processing of CoxII (Bauer et al. 1994). In addition to the CoxII-processing defect, a general reduction of mitochondrial protein synthesis was observed in pet1402 mutants. Finally, F1F0-ATPase assembly is also affected in oxa1 cells (Altamura et al. 1996). Whatever the exact function of Oxa1p, it is not specific for the assembly of a mitochondrial-type respiratory chain since OXA1 homologues have also been found in bacteria (Bonnefoy et al. 1994). It will be interesting to see if these homologues (or OXA1 itself) can suppress mutations in FtsH.

Preliminary data indicate that the second suppressor, MBA1, also affects the assembly of the respiratory chain, although to a lesser extent than AFG3, RCA1 or OXA1 (data not shown). However, the finding that PIM1 (LON) is one of the suppressors apparently disturbs the notion of assembly being the primary defect in afg3-null cells. PIM1 encodes an ATP-dependent protease of the mitochondrial matrix which until now has only been implicated in the maintenance of mitochondrial DNA and the turnover of proteins localized to the matrix (Suzuki et al. 1994; Van Dyck et al. 1994; Wagner et al. 1994). It is therefore tentative to assume that Pim1p can take over a degradation function of Afg3p when present in high amounts. However, preliminary data indicate that suppression by PIM1 may be independent of its protease activity (Rep et al. 1996).

Our observation that the three multi-copy suppressors act on both afg3-null and rca1-null mutations, as well as on a combination of these, even though AFG3 and RCA1 have non-overlapping functions, suggests that Afg3p and Rca1p are both needed for a single activity which is essential for the biogenesis of respiratory competent mitochondria. This is also supported by the similar assembly defects seen in the single- and double-disruption mutants (Paul and Tzagoloff 1995). One obvious possibility is that they are part of a heteromultimeric complex. Complex formation has been suggested before for Yme1p (Thorsness and Fox 1993; Thorsness et al. 1993; Nakai et al. 1995) and recently demonstrated for FtsH (Akiyama et al. 1995). Moreover, formation of (ring-shaped) multimers appears to be common among members of the AAA family (Peters et al. 1990, 1993; Whiteheart et al. 1995; Frolich et al. 1995).

In conclusion, this study shows that the roles of OXA1 and AFG3/RCA1 in the assembly of mitochondrial inner membrane complexes are partially overlapping, and identifies two new actors, PIM1 and MBA1, in the same process. The main question that we are now addressing is how these proteins affect the assembly process at the molecular level.

Acknowledgements We thank Dr. Alex Tzagoloff for kindly providing us with ΔRCA1 strains, Nathalie Bonnefoy for communicating results prior to publication, Dr. Y. de Steensma for a yeast chromosomal blot, Corien Maat for technical assistance, and Dr. Hans van der Spek for critical reading of the manuscript. This work was supported by grants to L.A. Grivell from the Netherlands Organization for the Advancement of Science (NWO) and the EU under contract no. CHRX-CT94 0520 of the Human Capital and Mobility Programme.

References


Demolis N, Mallet L, Jacquet M (1994) A 12.5-kb fragment of the yeast chromosome II contains two adjacent genes encoding ribosomal proteins and six putative new genes, one of which encodes a putative transcriptional factor. Yeast 10:1511–1525

Fröhlich KU, Fries HW, Peters JM, Mcke De (1995) The ATPase activity of purified CDC48 from Saccharomyces cerevisiae shows complex dependence on ATP-, ADP-, and NADH-concentrations and is completely inhibited by NEM. Biochim Biophys Acta 1253:25–32


Muroff I, Tzagoloff A (1990) CBP7 codes for a co-factor required in conjunction with a mitochondrial matrue for splicing of its cognate intervening sequence. EMBO J 9:2765–2773


Thorsness PE, Fox TD (1993) Nuclear mutations in Saccharomyces cerevisiae that affect the escape of DNA from mitochondria to the nucleus. Genetics 134:21–28


Van Dyck L, Porcell DA, Sherman F (1994) PIM1 encodes a mito- chondrial ATP-dependent protease that is required for mitochondrial function in the yeast Saccharomyces cerevisiae. J Biol Chem 269:238–242

