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The transcriptional activator *HAP4* is a high copy suppressor of an *oxa1* yeast mutation

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Abstract

Oxa1p is a key component of the machinery for the insertion of membrane proteins in mitochondria, and in the yeast *Saccharomyces cerevisiae*, the deletion of *OXA1* impairs the biogenesis of the three respiratory complexes of dual genetic origin. Oxa1p is formed from three domains located in the intermembrane space, the inner membrane and the mitochondrial matrix. We have isolated a high copy suppressor able to partially compensate for the respiratory deficiency caused by a large deletion of the matrix domain. We show that the suppressor gene corresponds to the nuclear transcriptional activator Hap4p which is known to regulate respiratory functions.

Keywords: Mitochondria; Respiratory complexes; *Saccharomyces cerevisiae*; Cytochrome oxidase; Membrane insertion

1. Introduction

The biogenesis of membrane oligomeric complexes of the mitochondrial respiratory chain is an intricate process that requires the co-assembly of mitochondrial and nuclear encoded subunits and the participation of nuclear-encoded factors that are not intrinsic components of the complex. Most of these proteins are conserved through evolution and several human neurodegenerative pathologies are due to respiratory complex assembly defects.

Oxa1p is functionally conserved from bacteria to eukaryotic organelles and is a key component of the insertion machinery of membrane subunits. In the yeast *Saccharomyces cerevisiae*, the deletion of *OXA1* impairs the biogenesis of the three respiratory complexes of dual genetic origin (complex III, IV and V) and the membrane subunits are rapidly degraded (Bonnefoy et al., 1994; Altamura et al., 1996; Lemaire et al., 2000). Moreover, Oxa1p seems essential for the translocation of the hydrophilic domain of the complex IV subunit, Cox2p (He and Fox, 1997; Hell et al., 1997; Herrmann and Bonnefoy, 2004). Oxa1p interacts with nascent mitochondrial polypeptides (Hell et al., 2001) and its C-terminal tail located in the mitochondrial matrix binds the mitochondrial ribosome. This interaction has been proposed to mediate the co-translational insertion of mitochondrially encoded subunits (Szyrach et al., 2003; Jia et al., 2003). Oxa1p show an N-out C-in topology (Herrmann et al., 1997) with three domains located in the intermembrane space, the inner membrane and the matrix. In order to clarify the precise role of these domains, we have constructed point and deletion mutants (Lemaire et al., 2004) and we have undertaken a systematic search for suppressors able to compensate for the respiratory deficiency due to various *oxa1* mutations.

We have already isolated two classes of extragenic suppressors able to compensate the respiratory deficiency of *oxa1* mutants. First, suppressor mutations located in the

Abbreviations: PCR, polymerase chain reaction; ORF, open reading frame; TM, trans-membrane domain.

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trans-membrane (TM) domains of two subunits of complex III, Cyt1p and Qcr9p, are able to compensate for the absence of Oxa1p (*Δoxa1*). We have proposed that these mutated TM domains of Cyt1p or Qcr9p could interact with the TM domains of other respiratory complex subunits leading to an insertion process independent of Oxa1p (Hamel et al., 1998; Saint-Georges et al., 2001). Second, the high copy suppressor gene, *OMS1*, encoding a methyl-transferase like protein, is specific for some *oxa1* alleles. The over-expression of *OMS1* increases the steady-state level of Oxa1p and thus facilitates the membrane insertion of respiratory subunits. This stabilization of Oxa1p could result from modifications of Oxa1p residues or of phospholipids (Lemaire et al., 2004). Thus, the suppression mechanisms are both acting at the assembly level but appear completely different in both cases.

In this paper, we describe the characterization of a new high copy suppressor of an *oxa1* mutation. We show that the suppressor gene corresponds to the transcriptional activator Hap4p which is known to regulate respiratory functions.

2. Materials and methods

2.1. Strains, media and genetic techniques

All strains were derived from the W303 nuclear background *MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*. The *oxa1* mutants were described in Lemaire et al. (2004). The *Δafg3* strain (*afg3::HIS3*) and the *Δhap4* (*hap4::G418*) strain are from A. Tzagoloff and B. Guiard. Yeast genetic methods and media used for *S. cerevisiae* have been described in Dujardin et al. (1980). Glucose or galactose were used as fermentable carbon sources and glycerol as a respiratory carbon source. Yeast cells were transformed by the lithium acetate procedure of Schiestl and Gietz (1989) for library screening.

2.2. High copy suppressor isolation

The *oxa1* mutant was transformed with a high copy library constructed by F. Lacroute into the *URA3* 2μ vector pFL44L. [Ura^+] clones were selected and replica-plated onto glycerol medium at 36 °C. Total yeast genomic DNA was extracted from fast or slow-growing co-segregating [Gly+] clones and used to transform *Escherichia coli* cells to recover the plasmids. Molecular analysis by restriction enzymes and sequencing identified the chromosomal fragments present in each plasmid.

2.3. Epitope tagging of Oxa1p

Oxa1p was tagged at its C-terminus with a six histidine epitope using the *Saccharomyces pombe HIS5* marker gene as described in Longtine et al. (1998). The PCR fragment was used to transform the starting strain to histidine prototrophy in order to fuse the tag to the end of the *OXA1* ORF in the yeast genome. Correct integration was confirmed by PCR amplification and sequencing. The strain CWOXA expressing the tagged protein is respiratory competent at 28 °C and 36 °C showing that the tagged protein is fully functional.

2.4. Isolation of mitochondria, Western blotting

Mitochondria were purified following the differential centrifugation procedures after digestion of cell walls by Zymoliase-100T (Kermorgant et al., 1997). The mitochondrial protein concentration was determined using the Bio-Rad assay. Mitochondrial proteins were separated on 12% acrylamide gels and blotted onto nitrocellulose membranes. Western blots were probed with various antibodies using the standard chemi-luminescence method.

3. Results and discussion

3.1. Over-expression of HAP4 compensates for the respiratory deficiency of oxa1-ΔL1-K332* mutant

In the search for genetic interactions involving the *OXA1* gene, we decided to search for high copy suppressor genes able to alleviate the respiratory defect of *oxa1* mutants. In *oxa1-ΔL1-K332**, the Oxa1p variant presents a large deletion of the first loop (L1) and a premature stop codon at position 332 in the C-terminal tail; both these domains protrude into the matrix. This mutant presents a tight respiratory deficiency associated to a strong defect in complex IV assembly and activity and a much weaker effect on complexes III and V (Lemaire et al., 2004). Three plasmids carrying overlapping fragments of chromosome XI (see Fig. 1A and B) were able to compensate for the respiratory deficiency of the mutation *oxa1-ΔL1-K332**. By subcloning the larger 7-kb insert of YEpSu1 (YEPSu1A and YEPSu1B), we demonstrated that the *HAP4* gene was responsible for the suppression. Surprisingly, the 4-kb fragment present in YEpSu8 and YEpSu22 exhibits a truncated *HAP4* gene encoding a 471-residue protein lacking the last 83 amino acids when compared to the full-length Hap4p. However, the over-expression of this truncated gene (*HAP4-S* allele) is as active in suppression as the complete *HAP4* gene.

Hap4p is responsible for the transcriptional activation capability of the Hap2/3/4/5 complex that positively regulates many of the genes involved in respiratory functions (Forsburg and Guarente, 1989). According to Bourgarel et al. (1999) and Stebbins and Triezenberg (2004), there is, in the C-terminal part of Hap4p, an activation domain that extends from the positions 450 to 471. In order to determine if the last 83 amino acids were required for the Hap4p activity, we have transformed the *Δhap4* strain with the plasmid carrying the truncated *HAP4-S* gene. As shown in Fig. 2, the *HAP4-S* gene is able to fully complement the respiratory defect of the *Δhap4* strain.

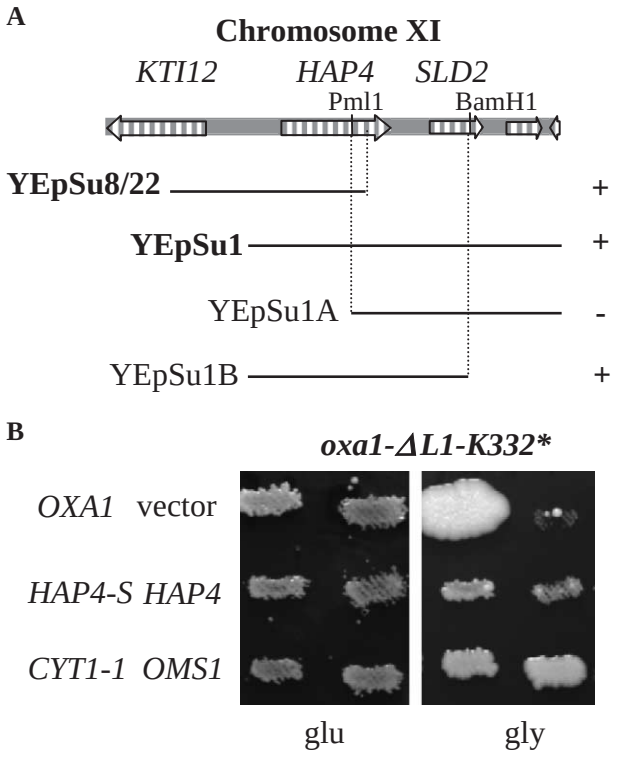

Fig. 1. The *HAP4* gene is a high copy suppressor of the *oxa1-ΔL1-K332** mutant. (A) The thick grey bar represents the region of chromosome XI carrying the suppressor gene. The arrows indicate the direction of transcription of the different ORFs present in this region. The genomic fragments inserted in the plasmids are indicated by thin straight lines. YEpSu1, YEpSu8 and YepSu22 were obtained after transformation of the *oxa1* mutant by the high copy library in pFL44 (see Materials and methods); YEpSu22 is identical to YEpSu8. YEpSu1A and YEpSu1B were obtained by sub-cloning YEpSu1 using the sites BamH1 and Pml1. The mutant cells were transformed with these different plasmids and the respiratory growth of transformants was tested as described in panel (B).+ respiratory growth; – no growth. (B) The *oxa1-ΔL1-K332** mutant was transformed with six plasmids : YEp30 carrying the *OXA1* gene, the empty control vector pFL44, the YEpSu1 and YEpSu8 plasmids expressing the truncated (*HAP4-S*) and full-length *HAP4* gene, YEpPH66 carrying the *CYT1-1* genetic suppressor (Hamel et al., 1998) and pNB212 carrying the high copy suppressor *OMS1* (Lemaire et al., 2004), respectively. The cells were patched on minimal glucose medium lacking uracil (Glu), replica-plated on non-fermentable substrate (Gly) and incubated at 28 °C for 10 days. The respiratory competent papillae correspond to genetic revertants.

Thus, the last 83 amino acids of Hap4p appear dispensable for both suppression and complementation.

3.2. Specificity of the suppression by over-expression of HAP4

We have compared the suppression efficiency of *HAP4* to the previously isolated suppressors (see Fig. 1B). The suppressor mutation *CYT1-1* is able to compensate for the total absence of Oxa1p while the over-expression of *OMS1* does not compensate for the absence of Oxa1p but suppresses partial *oxa1* defects (Lemaire et al., 2004). The over-expression of *HAP4* is able to partially restore the growth of the *oxa1-ΔL1-K332** mutant on respiratory substrates but cannot compensate for the respiratory defects observed in the null allele, *Δoxa1* (not shown). We have also tested the missense mutation *oxa1-WW>AA* which is clearly suppressed by *CYT1-1* and *OMS1*. In this mutant, a pair of tryptophan residues (positions 128–129) in or close to the first trans-membrane segment TM1, are replaced by a pair of alanine residues. The mutant *oxa1-WW>AA* exhibits a slight growth delay on respiratory medium at 28 °C and a tight respiratory deficiency at 36 °C associated to a strong reduction in complex IV activity (Lemaire et al., 2004). The over-expression of *HAP4* suppresses the growth delay at 28 °C but does not restore the respiratory capacity at 36 °C, even after extended incubation. Interestingly, the suppression of *oxa1-ΔL1-K332** by *HAP4* is also much weaker at 36 °C than at 28 °C (not shown). Thus, *HAP4* is a high copy suppressor of partial oxa1 defects and this suppression appears more efficient at 28 °C. As Oxa1p is involved in the insertion of the membrane subunits of respiratory complexes, this effect of temperature could be due to temperature-dependent modifications of the mitochondrial membrane or protein folding.

3.3. HAP4 over-expression strongly increases the steady-state level of Cox2p in the oxa1-ΔL1-K332* mutant

Lascaris et al. (2003) have shown that cells over-expressing *HAP4* up-regulate genes involved in mitochondrial biogenesis and activity. In particular, in a genome-wide expression experiment, *OXA1* mRNA appears induced about twofold by *HAP4* over-expression even though the *OXA1* promoter does not contain the consensus regulatory sequence of the Hap2/3/4 complex. As we have previously shown that increasing the level of mutated Oxa1p can compensate for its own defect (Lemaire et al., 2004), *HAP4* over-expression could be also responsible for an increase in the steady-state level of Oxa1p. In order to test this hypothesis, we have used a strain with a tagged form of Oxa1p (see Materials and methods) and transformed this strain with the high copy plasmid carrying the *HAP4* gene (YEpSu1) or the empty plasmid (pFL44L) and purified

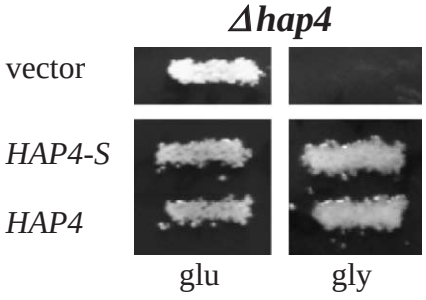

Fig. 2. The truncated *HAP4* gene fully complements the absence of Hap4p. The *Δhap4* mutant was transformed with the plasmids carrying the truncated (YEpSu8, HAP4-S) and full-length HAP4 gene as well as with the empty control vector. The respiratory growth was tested as in Fig. 1, panel B.

mitochondria from these transformants. As shown in Fig. 3A, we were not able to detect, in the strain over-expressing *HAP4*, any significant increase in the state-level of Oxa1p as compared to Arg8p. Arg8p is a mitochondrial protein not involved in respiratory function and is not induced by *HAP4* over-expression according to Lascaris et al. (2003). In parallel, we have also transformed a mutant lacking the mitochondrial matrix protease Afg3p, with the plasmids carrying either *HAP4*, *OXA1* or *OMS1*. Indeed, it is known that over-expression of *OXA1* can restore growth of the *Δafg3* mutant on respiratory substrates (Rep et al., 1996; Lemaire et al., 2004). As shown in Fig. 3B, the *Δafg3* mutant is not suppressed by the over-expression of *HAP4* while over-expression of *OXA1* or *OMS1* which increases the Oxa1p level (Lemaire et al., 2004) clearly restores respiratory growth under the same conditions. Altogether, these experiments suggest that the suppression via *HAP4* is not due to an increased steady-state level of Oxa1p.

Hap4p positively regulates the expression of several genes involved in mitochondrial respiratory functions (Forsburg and Guarente, 1989). Interestingly enough, Northern analyses have shown that the over-expression of

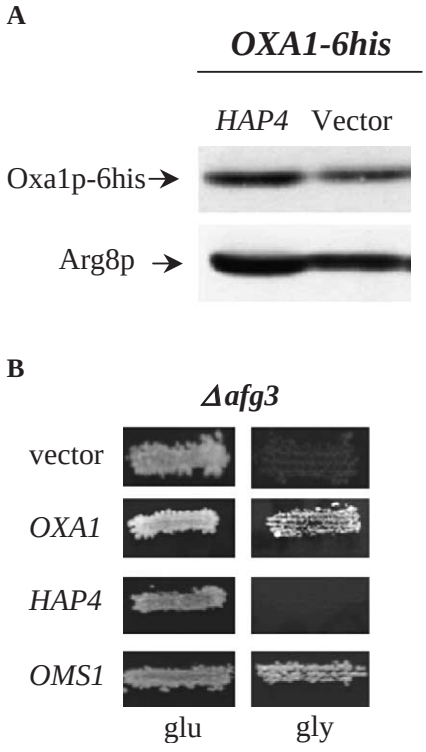

Fig. 3. *HAP4* over-expression does not lead to a significant increase in Oxa1p accumulation. (A) The CWOXA strain expressing a histidine tagged form of Oxa1p (see Materials and methods) was transformed with the YEpSu1 plasmid over-expressing HAP4 and the empty vector. Mitochondria were purified from transformants grown in minimal galactose medium for plasmid selection. Mitochondrial proteins (80 μg) were analyzed by Western blotting (12% acrylamide gel) using the anti-his (Tetrahis, Quiagen) and the anti-Arg8 (T.D. Fox) antibodies. B) The *Δafg3* mutant was transformed with high copy plasmids carrying the *OXA1*, *HAP4* or *OMS1* genes or with the empty vector and the respiratory growth was tested as in panel B of Fig. 1.

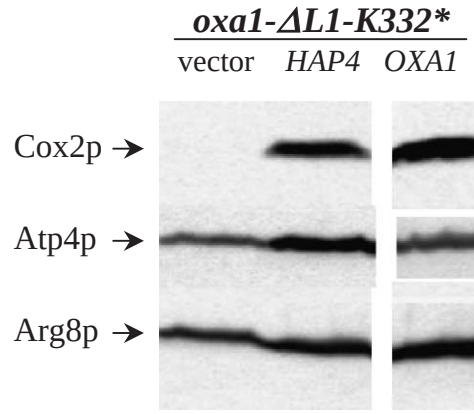

Fig. 4. Effect of *HAP4* over-expression on the steady-state level of respiratory complex subunits in the *oxa1-ΔL1-K332** mutant. The *oxa1-ΔL1-K332** cells transformed with the empty control vector, the YEpSu1 or YEp30 plasmids over-expressing *HAP4* or *OXA1*, respectively, were grown in minimal galactose medium for selecting the plasmid and mitochondria were purified. Mitochondrial proteins (80 μg) were analyzed by Western blotting (12% acrylamide gel) using the anti-Cox2p (Molecular Probes), the anti-Atp4p (J. Velours), anti-Arg8p (T.D. Fox) antibodies.

HAP4 significantly increases the steady-state level of *COX2* mRNA (Lascaris et al., 2003). The *oxa1-ΔL1-K332** mutant exhibits a pleiotropic defect in respiratory complex assembly and activity. In particular, the assembly of complex IV and the steady-state level of Cox2p are strongly decreased (Lemaire et al., 2004). Thus, the *HAP4* effect on *COX2* could be responsible for the suppression effect. We have analysed the level of Cox2p in the *oxa1-ΔL1-K332** mutant transformed either with the high copy plasmid carrying the *HAP4* or the *OXA1* gene. As shown in Fig. 4, the steady-state level of Cox2p is very strongly increased by *HAP4* over-expression in the *oxa1-ΔL1-K332** mutant as compared to the effect on the level of Arg8p. The steady-state level of Atp4p is also slightly but reproducibly increased (150%) by *HAP4* over-expression.

4. Conclusion

Whilst it is clear that the over-expression of a wild-type or truncated *HAP4* gene is able to suppress the respiratory defect of the *oxa1-ΔL1-K332** mutant but does not compensate for the absence of Oxa1p, the mechanism of this suppression remains to be elucidated. The over-expression of *HAP4* does not seem to increase the steady-state level of Oxa1p but leads to a strong increase in the level of Cox2p in the *oxa1-ΔL1-K332* mutant. This increase could explain the restoration of respiratory functions as a reduction in complex IV assembly and activity is the principal defect in the *oxa1-ΔL1-K332** mutant. As Cox2p is encoded in the mitochondrial genome, it is probable that the effect of Hap4p is indirect. The increase in the level of Cox2p may be sufficient to explain the suppressor effect; alternatively, the suppression may be due to a combination of the increase of Cox2p and an increase in

the level of other mitochondrial proteins whose transcription is controlled by Hap4p.

This suppression clearly differs from those previously reported for the *CYT1-1* and *OMS1* suppressors that act at a post-translational level of respiratory complex biogenesis. Tight interactions exist between the synthesis and assembly of respiratory complex subunits in mitochondria and are essential for the biogenesis of mitochondrial respiratory complexes. They are mediated through membrane-bound translational activators (Sanchirico et al., 1998) and the matrix C-terminal part of Oxa1p that was shown to interact with ribosomes (Szyrach et al., 2003; Jia et al., 2003). Over-expression of *HAP4* suppresses the *oxa1-ΔL1-K332** mutant that exhibits a large deletion of the matrix domain of Oxa1p. Thus, it is tempting to propose that the increase in the expression of respiratory subunits compensate for the defect in co-translational membrane insertion of these subunits due to the *oxa1* mutation.

Acknowledgments

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