Overexpression of yeast karyopherin Pse1p/Kap121p stimulates the mitochondrial import of hydrophobic proteins in vivo

M. Corral-Debrinski,¹ N. Belgareh,² C. Blugeon,¹ M. G. Claros,³ V. Doye² and C. Jacq¹*¹
¹Ecole Normale Supérieure, Laboratoire de Génétique Moléculaire URA CNRS 1302, 46 Rue d’Ulm, 75230 Paris Cedex 05, France.
²Institut Curie, CNRS UMR 144, 26 Rue d’Ulm, 75248 Paris Cedex 05, France.
³Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Instituto Andaluz de Biotecnología, Universidad de Málaga, E-29071 Málaga, Spain.

Summary
During evolution, cellular processes leading to the transfer of genetic information failed to send all the mitochondrial genes into the nuclear genome. Two mitochondrial genes are still exclusively located in the mitochondrial genome of all living organisms. They code for two highly hydrophobic proteins: the apocytochrome b and the subunit I of cytochrome oxidase. Assuming that the translocation machinery could not efficiently transport long hydrophobic fragments, we searched for multicopy suppressors of this physical blockage. We demonstrated that overexpression of Pse1p/Kap121p or Kap123p, which belong to the superfamily of karyopherin β proteins, facilitates the translocation of chimeric proteins containing several stretches of apocytochrome b fused to a reporter mitochondrial gene. The effect of PSE1/KAP121 overexpression (in which PSE1 is protein secretion enhancer 1) on mitochondrial import of the chimera is correlated with an enrichment of the corresponding transcript in cytoplasmic ribosomes associated with mitochondria. PSE1/KAP121 overexpression also improves the import of the hydrophobic protein Atm1p, an ABC transporter of the mitochondrial inner membrane. These results suggest that in vivo PSE1/KAP121 overexpression facilitates, either directly or indirectly, the co-translational import of hydrophobic proteins into mitochondria.

Introduction
The majority of mitochondrial proteins are encoded by the nuclear genome, translated in the cytoplasm, and translocated into the organelle. However, all the mitochondrial genomes known so far always contain two genes, coding for apocytochrome b and for the subunit I of cytochrome oxidase. This specific feature suggests that the mitochondrial import machinery cannot cope with some properties shared by these two proteins. Genetic engineering of the yeast apocytochrome b coding sequence has shown that two or more of its transmembrane segments can not be imported easily to mitochondria. Therefore, a combination of hydrophobicity characteristics, designated as mesohydrophobic, distinguishes proteins that cannot be mitochondrialy imported (Claros et al., 1995). Usually, proteins delivered to mitochondria have at their amino terminal region a mitochondrial targeting sequence (mts) characterized by positive charges and amphiphilicity, which is related to the folding tightness of the attached passenger protein. Hydrophobic proteins, such as the ATPase subunit 9 of Neurospora crassa (F₀-9), have a particularly long mts spanning the two mitochondrial membranes, a feature that accelerates the unfolding of the precursor (Matouschek et al., 1997). Early recognition of the precursor via mts identification is certainly a critical step in the import process (Cartwright et al., 1997; George et al., 1998). Cytosolic factors interacting directly with the mts are involved in the delivery of the protein to the mitochondrial surface. The precursor is subsequently recognized by the translocase of the outer mitochondrial membrane (TOM), which, together with the translocase of the inner mitochondrial membrane (TIM), accomplishes transportation across both membranes. These mechanisms have been the focus of intensive research, resulting in an elaborate picture of the process as a whole (for reviews, see Schatz, 1996; Neupert, 1997). In vitro, a very tight coupling between precursor synthesis and membrane transport was observed when the translation of several precursors was performed in the presence of mitochondria, indicating a co-translational import mechanism (Fujiki and Verner, 1991; 1993; Verner, 1993). In addition, it has been reported that ribosomes are specifically associated with the mitochondrial surface (Kellens and Butow, 1974; Kellens et al., 1974; 1975; Pon et al.,
1989), where they can actively translate mitochondrial proteins and be involved in the co-translational transport of nascent precursors (Ades and Butow, 1980; Suissa and Schatz, 1982; Pon et al., 1989).

To test whether cellular sorting of highly hydrophobic proteins could reveal new elements of the mitochondrial import machinery, we initiated studies to identify proteins that when overproduced would permit the mitochondrial import of a reporter protein fused to transmembrane domains of the apocytochrome b. Unexpectedly, we found that two multicopy suppressors encode Pse1p/Kap121p and Kap123p. These highly similar proteins belong to the karyopherin β family, which carries proteins and/or RNAs through the nuclear pore complexes (NPC) (Görlich, 1997; Rout et al., 1997; Schlenstedt et al., 1997; Seedorf and Silver, 1997). We propose that, either directly or indirectly, PSE1/KAP121 overexpression facilitates the co-translational import of hydrophobic proteins.

Results

Respiration deficiency of CW02 cells can be corrected by the overexpression of PSE1/KAP121

To study the inhibitory effects of protein hydrophobicity on the mitochondrial import process, we previously constructed a universal code version of apocytochrome b (Claros et al., 1995). Several chimeric proteins were designed to be cytoplasmically translated and targeted to mitochondria. They were named according to the presence of the N. crassa ATPase subunit 9 mts (S) and to the presence of the different apocytochrome b transmembrane helices (1–8) and the bi4 RNA maturase (M). This last protein acts as a reporter to complement the bi4 intron deletion and is involved in apocytochrome b and cytochrome oxidase subunit I (COXI) mRNA maturation (Banroques et al., 1986; 1987). We previously demonstrated that CW02 cells expressing either S12345M or S678M chimeric proteins are unable to grow on glycerol medium, indicating that these proteins were not imported into the mitochondria to fulfill the maturase function (Claros et al., 1995). To isolate multicopy suppressors of this mitochondrial import defect, CW02 cells producing these chimeras were transformed with a genomic library constructed with a multicopy vector (Claros et al., 1996). One of the most active suppressor genes turned out to code for a C-terminal truncated version of the 1089-amino-acid Pse1p/Kap121p protein, a member of the karyopherin β family (Rout et al., 1997; Seedorf and Silver, 1997). This truncated version, which contains the 600 N-terminal amino acids of Pse1p, will be referred to hereafter as PSE1-t. The entire PSE1 gene was obtained by PCR and tested for its ability to restore CW02 cell respiration in the presence of S1234M, S12345M, S56M or S678M chimeras. As shown in Fig. 1A for the S12345M and S678M constructs, the 600 N-terminal amino-acid form of Pse1p/Kap121p (PSE1-t 600) and the full-length protein (PSE1) were equally able to restore CW02 cell respiration. To determine whether shorter versions of Pse1p/Kap121p remained active in this assay, we constructed different truncated versions of PSE1/KAP121. We observed that a protein composed of the 453 N-terminal amino acids is still active (PSE1-t 453), whereas a shorter protein with only the 265 N-terminal amino acids (PSE1-t 265) is not able to act as a multicopy suppressor (Fig. 1A). To assess the exact role of the mitochondrial targeting sequence (mts) in the in vivo assays, we constructed mts-less versions of some of the chimeric genes. When the chimeric constructions S678M or S56M were deleted for the presequence (S), no restoration of respiration for the CW02 strain was observed upon overexpression of PSE1 or PSE1-t (Fig. 1B). This result indicates that the PSE1-driven translocation of the cytoplasmically made chimeras requires the presence of a mts and is likely to be dependent on the major mitochondrial import system.

The C-terminal truncated form of Pse1p/Kap121p does not complement the lethal phenotype of the pse1 disruption and is not associated with the NPCs

Pse1p/Kap121p is essential for yeast growth (Seedorf and Silver, 1997). When heterozygous diploids, in which one copy of PSE1 was replaced by TRP1, were transformed with the PSE1-t plasmid, only two viable Trp− spores were obtained per tetrad after asci dissection, indicating that the C-terminal region of Pse1p is required for its essential function. Both full-length and truncated Pse1p were tagged with the green fluorescent protein (GFP). GFP-Pse1p rescues the lethal phenotype of the PSE1 disruption. In addition, GFP-PSE1 or GFP-PSE1-t, when overexpressed, restore CW02 cell respiration, and thus the fusion proteins behave as the untagged proteins (data not shown). Western blot analysis revealed that both fusion proteins migrated at the expected sizes and were expressed at similar levels (Fig. 2A). Subcellular localization of GFP-Pse1p, examined by fluorescence microscopy, revealed a perinuclear labelling together with some cytoplasmic and intranuclear staining. When expressed in nup133Δ cells that display a constitutive NPC clustering phenotype (Doye et al., 1994), GFP-Pse1p was localized to one or only a few clusters around the nuclear envelope, indicating that Pse1p/Kap121p interacts with nuclear pore proteins. In contrast, GFP-Pse1p-t did not accumulate at the nuclear periphery in wild-type cells and was not localized to NPC clusters in nup133 cells (Fig. 2B). Thus, the C-terminal region of Pse1p, which is dispensable in our mitochondrial assay, is required for both its essential function and subcellular localization.

© 1999 Blackwell Science Ltd, Molecular Microbiology, 31, 1499–1511
Fig. 1. Cytoplasmically made bI4 RNA maturase fused to apocytochrome b fragments, with a functional mts, complement mitochondrial deficiencies when PSE1/KAP121 is overexpressed.
A. The nomenclature of the set of chimeric proteins (upper part) is: S1...8M, in which S refers to the mts of the subunit 9 of the ATPase of N. crassa (Fo9-ts), 1...8 to the corresponding transmembrane helices of apocytochrome b, and M to the bI4 RNA maturase. CW02 cells transformed with multicopy vectors directing the expression of the S12345M (left) or the S678M (right) chimera do not grow on glycerol (control). Overexpression of the entire PSE1 gene (PSE1) or its truncated versions PSE1-t 600 and PSE1-t 453, which code, respectively, for 600 and 453 amino-acid N-terminal forms of Pse1p, restores the ability of CW02 to grow on glycerol. In contrast, the 265 N-terminal amino-acid form of Pse1p/Kap121p (PSE1-t 265) is unable to rescue CW02 cell respiration.
B. PSE1-t represents the construct encoding the 600 N-terminal amino acids of Pse1p/Kap121p. CW02 strains overexpressing PSE1 or PSE1-t and transformed with multicopy vectors directing the expression of the S56M or the S678M chimera are able to grow on glycerol. In contrast, overexpression of the 56M or 678M mts-less sequences does not rescue CW02 respiration. Transformant cells were grown on synthetic complete medium containing 2% glucose until an OD600 of 2. Two independent clones were serially diluted (1:5) and spotted either on synthetic medium containing 2% glucose (glucose) or on 2% glycerol medium (glycerol). The plates were incubated at 30°C for 2 days (glucose) or 8 days (glycerol).

Fig. 2. Expression and localization of GFP–Pse1p fusion proteins.
A. Whole cell extract from BMA41 cells expressing GFP–Pse1p and GFP–Pse1p-t proteins were analysed by Western blotting using antibodies directed against GFP and against Nup133p. Both GFP–Pse1p and GFP–Pse1p-t are produced at similar levels and migrated at their expected sizes.
B. Fluorescence microscopy of BMA41 and nup133Δ living cells expressing GFP–Pse1p and GFP–Pse1p-t proteins showed that the full-length Pse1p fusion protein interacts with the NPC, whereas the truncated GFP–Pse1p-t is mainly cytosolic. Cells were also photographed with Nomarski optics.
Overproduction of Kap123p but not Kap95p/Kap60p facilitates mitochondrial import of hydrophobic proteins

To determine whether the ability to restore CW02 cell respiration in the presence of S12345M or S678M is restricted to Pse1p/Kap121p, we tested two other karyopherin β family members, Kap95p and Kap123p. These proteins have in common a role in the trafficking of molecules across the nuclear pores and the ability to bind Ran, an essential element of all the nucleocytoplasmic transport processes studied so far (Görlich, 1997; Görlich et al., 1997). Kap95p, known as karyopherin β1, is a soluble transport protein that interacts with the nuclear localization signal (NLS) receptor, Kap60p, to allow the translocation of nuclear proteins across the NPC (for reviews, see Görlich, 1997; Nigg, 1997). KAP123, the closest homologue of PSE1/KAP121, was recently shown to be involved in the nuclear import of ribosomal proteins, and its association to mRNA export was also suggested (Rout et al., 1997; Schlenstedt et al., 1997; Seedorf and Silver, 1997). A recent report showed that Pse1p/Kap121p is also involved in the nuclear import of the transcription factor Pho4p (Kaffman et al., 1998). Figure 3 shows the respiration ability of CW02 cells expressing the S12345M or S678M constructs, and transformed with high-copy number plasmids carrying KAP123 or KAP95 expressed under the control of their endogenous promoters. We also transformed CW02 cells with a high-copy number vector carrying KAP60 alone or in combination with the KAP95 plasmid. We observed that Kap95p overproduction did not allow CW02 cells to grow on glycerol medium, even when KAP60 was overexpressed at the same time. Interestingly, Kap123p, like Pse1p/Kap121p, restores CW02 respiration for all the chimeric constructs tested, indicating that overexpression of only a subset of karyopherin β proteins interferes with the mitochondrial phenotype described in this study.

Overproduction of Pse1p/Kap121p can usher an excess of the ABC transporter Atm1p into mitochondria

Restoration of CW02 cell respiration by overexpression of PSE1 and apocytochrome b domains fused to the maturase implies the complete translocation of the chimera into the mitochondrial matrix. It was therefore important to determine the impact of PSE1 overexpression on the transport of hydrophobic proteins which are naturally imported into mitochondria. For that purpose, we chose Atm1p, a recently described ABC transporter of the mitochondrial inner membrane (Leighton and Schatz, 1997). Atm1p contains six putative transmembrane fragments, and its mesohydrophobicity level is very high although still compatible with an in vivo mitochondrial import (Fig. 4A). To confirm the mitochondrial localization of Atm1p, we purified mitochondria from cells expressing a C-terminal myc-tagged version of Atm1p produced from a multicopy vector (Fig. 4B). Purified mitochondria and mitoplasts were enriched for Atm1p as they were for the mitochondrial Abf2 protein (Caron et al., 1979; Difflay and Stillman, 1991), in contrast they lacked the nucleoporin Nup133p (Doye et al., 1994).
To assess PSE1 overexpression effect on Atm1p mitochondrial translocation, purified mitochondria from cells producing an excess of Atm1p alone (control) or in combination with Pse1p (PSE1) or its truncated form (PSE1-t) were treated with proteinase K (PK) and submitted to Western blot analyses (Fig. 4C). The amounts of Cox2p and Abf2p were successively determined and used to normalize the Atm1p-myc signal for each mitochondrial preparation. Cox2p, encoded by the mitochondrial genome, is highly hydrophobic, whereas Abf2p is very hydrophilic. Both Atm1p and Abf2p are encoded by the nuclear DNA whereas Cox1p and Cytb are encoded by the mitochondrial genome.
analyses using the TINA software were performed to calculate the ratio between m56HA1 and Cox2p signals in intact mitochondria (±PK) or in (upper panel), monoclonal antibody against Cox2p (middle panel) and polyclonal antibody against Abf2p (lower panel). Right: densitometric out in the presence of 1% Triton X-100 (Triton). The following antibodies were used: monoclonal antibody 12CA5 against the HA1 epitope

‡

for a better definition of the effect of transmembrane domains

b

mitochondrial translocation of apocytochrome PSE1/KAP121 overexpression also facilitates the efficiency of this translocation is significantly improved.

PSE1/KAP121 overexpression also facilitates the mitochondrial translocation of apocytochrome b transmembrane domains

For a better definition of the effect of PSE1/KAP121 overexpression on the mitochondrial import of highly hydrophobic proteins, we purified mitochondria from cells expressing the chimeric protein S56HA1. The precursor (pS56HA1) and the mature form of the chimera (m56HA1) were equally enriched in crude mitochondria and in mitochondria purified on sucrose density gradients (Fig. 5A). The presence of m56HA1 polypeptide, obtained after the cleavage of the presequence by the matrix processing peptidase (MPP), indicates that the precursor recognizes the TOM complex and engages in the translocation process. Both pS56HA1 and m56HA1 polypeptides were equally abundant in mitochondria from wild-type cells (control) and from cells overproducing either Pse1p (PSE1) or its truncated form (PSE1-t). This is true for chimeras expressed from a multicopy vector (Fig. 5B) or from a low-copy number vector (Fig. 5C). Thus, the steady-state levels of the precursor and its ability to recognize the TOM complex do not depend on PSE1 overexpression. When mitochondria were treated with proteinase K (PK) (Fig. 5B and C), we clearly observed that m56HA1 was much less accessible to proteolysis in mitochondria purified from cells overproducing Pse1p (PSE1) or its truncated version (PSE1-t) compared with control cells, as observed for the Atm1p-myc protein. The amounts of Cox2p and Abf2p were determined and used to normalize the m56HA1 signal for each mitochondrial preparation. In contrast to m56HA1, we did not observe any difference in the proportion of Abf2p in PK-treated mitochondria when Pse1p/Kap121p or its truncated form were overproduced (Fig. 5B and C). Densitometric analyses clearly showed that PK-treated mitochondria from cells overexpressing PSE1 or its truncated form accumulate five- to sevenfold more of the m56HA1 protein than PK-treated mitochondria from control cells, independently of the vector used to direct S56HA1 synthesis (Fig. 5B and C). These results indicate that in wild-type cells most of the chimera undergoes the initial steps of mitochondrial import, but ~90% gets stuck across the membranes. In this blocked situation, the most hydrophobic part of the protein remains in the cytoplasm, whereas the mts is cleaved by the MPP in the mitochondrial matrix (Fig. 5D, l). When Pse1p or its truncated form is overexpressed, the import...
PSE1 overexpression improves mitochondrial protein delivery

A

B

C

D

© 1999 Blackwell Science Ltd, Molecular Microbiology, 31, 1499–1511
process is successful for more than 50% of the precursor, as measured by the amount of the mature form resistant to proteolysis (Fig. 5D, II).

PSE1/KAP121 overexpression leads to an enrichment of the S56M transcript in the ribosome population associated with mitochondria

It was previously shown that mitochondria-associated ribosomes can be recovered by isolating mitochondria in the presence of Mg2+ and cycloheximide (Kellems et al., 1975; Ades and Butow, 1980; Suissa and Schatz, 1982). These ribosomes were found to be enriched in mRNA species coding for imported mitochondrial proteins, and it has been suggested that they were probably bound to mitochondria by the nascent chains of these proteins (Ades and Butow, 1980; Suissa and Schatz, 1982; Pon et al., 1989; Cartwright et al., 1997; George et al., 1998). The process controlling this asymmetric distribution is, for the time being, largely speculative. In connection with these observations, we studied the influence of PSE1/KAP121 overexpression on the specific localization of the S56M mRNA coding for the hydrophobic chimeric protein. We first observed that the steady-state level of the S56M mRNA was not modified upon PSE1/KAP121 overexpression (Fig. 6A). We then purified free cytoplasmic ribosomes (Fig. 6B, R) and mitochondria-bound ribosomes (Fig. 6B, M) to analyse their respective mRNA contents. ACT1 mRNA and cytochrome oxidase subunit III (COXIII) mRNA were considered as internal controls for free cytoplasmic ribosomes and mitochondria-bound ribosomes fractions respectively. The 1.8 kb S56M mRNA was mainly found in mitochondria-bound ribosomes, as expected for a mitochondrially targeted protein (Ades and Butow, 1980; Suissa and Schatz, 1982). This transcript is poorly represented in the free cytoplasmic ribosome fraction and only detectable by RT-PCR (data not shown). When PSE1 or its truncated form was overexpressed, we consistently observed a three- to fourfold increase of the S56M mRNA level in mitochondria-bound ribosomes (Fig. 6, B and C). These data suggest that PSE1/KAP121 overexpression might, somehow, enhance the asymmetric subcellular localization of a mRNA coding for a highly hydrophobic protein fused to a mitochondrial targeting sequence. Further studies are required to assess the generality of this phenomenon.

Discussion

Stuck hydrophobic precursors can be driven into mitochondria when PSE1/KAP121 is overexpressed

We have previously studied the inhibitory effects on mitochondrial import of diverse combinations of apocytochrome b transmembrane helices (1–8). When these hydrophobic domains were fused to the mts of F0 subunit 9 of N. crassa (S) and maturase (M) to create chimeric proteins such as S12345M, S678M or S56M, the ability to complement a mitochondrial maturase deficiency was totally abolished (Claros et al., 1995). Here, we demonstrated that very little of the S56M hybrid is actually delivered to the mitochondrial matrix because the precursor

Fig. 6. S56M mRNA level is increased in mitochondria-bound ribosomes from cells overexpressing PSE1 or PSE1-t.
A. Northern blots were performed with 15 µg of total RNAs prepared from CW04 cells co-transformed with the S56M low-copy number vector and pFL44L (control) or PSE1 (PSE1) or PSE1-t (PSE1-t) plasmids. Filters were stained with a methylene blue solution (top) and successively probed with the maturase oligonucleotide and the ACT1 coding sequence.
B. RNAs were purified from mitochondria-bound ribosomes (M) and from free cytoplasmic ribosomes (R) fractions obtained from wild-type cells (CW04). CW04 cells co-transformed with the S56M low-copy number vector and pFL44L (control) or with either PSE1 (PSE1) or PSE1-t (PSE1-t) plasmids. Before hybridization, membranes were stained with a methylene blue solution (top). Northern blot analyses were performed using successively ACT1, COXIII and maturase probes. Maturase probe revealed a 1.8 kb species corresponding to the S56M mRNA. Longer exposure times revealed the signal for the apocytochrome b 3.6 kb RNA precursor, which contains the bI4 intron (not shown).
C. Densitometric analyses of three independent experiments were performed using phosphorimagery and TINA software systems. Ratios between the S56M and COXIII signals are represented.
gets stuck between both membranes. This transport block does not preclude the N-terminal cleavage of the precursor by the matrix processing protease MPP. Such intermediates, spanning both mitochondrial membranes, have been widely used in vitro to study the mitochondrial import machinery and they have also been detected in vivo (Pon et al., 1989; Schulke et al., 1997). Our genetic screen revealed that overproduction of either Pse1p/Kap121p or its C-terminal truncated versions improves the ability of several combinations of apocytochrome b fragments fused to the mature to complement mitochondrial matrix deficiency. Biochemical analyses demonstrated that this effect is unambiguously due to a more efficient delivery of the chimeric protein into the mitochondrial matrix. The fact that the Pse1p/Kap121p's effect entirely relies on the presence of the mts indicates that its overproduction acts in collaboration with the classical TOM/TIM mitochondrial import machinery (for reviews, see Neupert, 1997; Schatz, 1996). Moreover, PSE1/KAP121 overexpression can stimulate mitochondrial translocation of Atm1p, a recently characterized hydrophobic protein (Leighton and Schatz, 1997). These data strongly suggest that PSE1/KAP121 overexpression actually improves, directly or indirectly, a limiting step in the mitochondrial delivery of hydrophobic proteins.

Connections between karyopherins β and the mitochondrial import process

Structurally, Pse1p/Kap121p belongs to the karyopherin β superfamily of proteins which bind Ran (Görlich et al., 1997; Rout et al., 1997; Schlenstedt et al., 1997; Seedorf and Silver, 1997). All of these proteins share the same set of motifs in their amino-terminal regions: the CRIME motifs, the acidic domain and the ARM1–ARM2 domains (Fornerod et al., 1997; Schlenstedt et al., 1997). Among the karyopherins, Kap123p shares the closest homology with Pse1p/Kap121p and showed similar effects on the mitochondrial import of hydrophobic proteins. In contrast, overproduction of Kap95p alone or in combination with Kap60p did not have any effect on mitochondrial import, confirming that this phenotype is restricted to a subset of karyopherins β. In the movement of macromolecules in and out of the nucleus, two different functions have been proposed for Pse1p/Kap121p and Kap123p. These karyopherins could either control the nuclear import of ribosomal proteins (Görlich et al., 1997; Rout et al., 1997; Schlenstedt et al., 1997) or be involved in the nuclear export of mRNAs (Seedorf and Silver, 1997). A recent report showed that Pse1p/Kap121p is also involved in the nuclear import of the transcription factor Pho4p (Kaffman et al., 1998). How could PSE1/KAP121 overexpression modify the nucleocytoplasmic traffic? It seems that karyopherins might act in dynamic competition to transport more or less distinct classes of substrates (Rout et al., 1997). In that respect, amino-terminal deletions of karyopherin β1(Kutay et al., 1997) and Kap123p (Schlenstedt et al., 1997) were shown to block bidirectional traffic across the NPC. In our system, a dynamic competition might be influenced by Pse1p/Kap121p overproduction and specific pathways of the nucleocytoplasmic traffic may be modified, particularly when the truncated form, poorly localized to the nuclear envelope, is overexpressed.

PSE1 overexpression might improve the co-translational import of hydrophobic proteins

Because we observed that PSE1/KAP1211 overexpression does not quantitatively modify the steady-state levels of either the hybrid mRNA or the corresponding protein, we can discard the possibility that Pse1p/Kap121p could control the transcription or translation of the chimera. Instead, high levels of Pse1p/Kap121p lead to a successful translocation of the hydrophobic protein into the mitochondria. It is then possible that translation might be modified in a way that prevents aggregation of the apocytochrome b helices, beneficially maintaining unfolded structures required for the import. This is reminiscent of a long-standing hypothesis in which mitochondrial import could be a co-translational process (Ades and Butow, 1980; Suissa and Schatz, 1982; Verner, 1993). Although controversial, this process is generally envisaged either for subclasses of mitochondrially imported proteins (Neupert, 1997) or during special growth conditions (Reid and Schatz, 1982; Schatz and Butow, 1983). Recent reports strengthen this hypothesis because both the nascent polypeptide-associated complex (NAC) and Mtt52p, a protein that binds both the presequence and Tom20p, co-operate in initiating mitochondrial protein targeting in vivo (Cartwright et al., 1997; George et al., 1998). Therefore, hydrophobic proteins delivery to the mitochondria could actually rely on a tight coupling between both translation and import processes. It has been shown that mRNAs for different imported mitochondrial polypeptides are enriched in mitochondria-bound polysomes fractions (Ades and Butow, 1980; Suissa and Schatz, 1982). In our hands, mitochondria-bound ribosomes purified from cells overproducing Pse1p/Kap121p are actually enriched in the mRNA coding for the hydrophobic protein S56M. Further studies are required to generalize this phenomenon to additional hydrophobic protein messengers. Nevertheless, we postulate that the enrichment of mRNA species coding for hydrophobic proteins in mitochondria-bound ribosomes could favour the co-translational import of this kind of protein into the organelle.

Considering the possible role of mRNA binding proteins in the transport of mRNAs through the cytosol, some relationships between mRNA export and mitochondrial protein...
transport were proposed (Lithgow et al., 1997). In this respect, it is worth remembering that a mutation in Npl3p, an essential protein involved in mRNA trafficking between the nucleus and the cytoplasm (Bossie et al., 1992; Flach et al., 1994; Singleton et al., 1995), restores the mitochondrial delivery of the ATPase subunit β, in which the targeting signal was corrupted (Ellis and Reid, 1993). Interestingly, the mammalian ATPase β mRNA was located in the vicinity of mitochondria. A role for this localization in fast tracking the sorting of the precursor protein to its mitochondrial destination has been suggested (Egea et al., 1997; Izquierdo and Cuezva, 1997; Lithgow et al., 1997).

We therefore favour the hypothesis that PSE1/KAP121 overexpression can, either directly or indirectly, improve the coupling between mRNA-specific localization/translocation and mitochondrial import processes. Studies aimed at generalizing mRNA-specific delivery and translation in the vicinity of mitochondria for other hydrophobic proteins and at characterizing sequences responsible for the asymmetric distribution of mitochondrial transcripts are currently in progress.

Experimental procedures

Yeast strains and growth conditions

All of the S. cerevisiae strains used in this study are isonuclear with the strain W303-1B (MATa, leu2-3; 3; ura3-1; trp1-1, ade2-1, his3-11, can1-100). CW02 and CW04 strains were obtained as described previously (Conde and Fink, 1976; Dujardin et al., 1980). CW02 cells do not possess the b14 maturase activity and are therefore unable to respire because the splicing of the aI4 intron in the precursor of COXI messenger does not occur. CW04 cells used for mitochondria purification possess a functional b14 RNA maturase.

Table 1. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5′ Primer 3′</th>
<th>PCR product length (bp)</th>
<th>AUG position (bp)</th>
<th>STOP position (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSE1/KAP121</td>
<td>gataaggatcaatagcctcataaggatcaatagcctc</td>
<td>3970</td>
<td>+365</td>
<td>3550</td>
</tr>
<tr>
<td>KAP123/YRB4</td>
<td>gcctaacgtaatagcctcataaggatcaatagcctc</td>
<td>4370</td>
<td>+405</td>
<td>3744</td>
</tr>
<tr>
<td>KAP95</td>
<td>gggggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttgggccagttggttggccc</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
described (Claros et al., 1996). The sequence coding for the 453 N-terminal amino acids of Pse1p/Kap121p was cloned using the unique Nael site present in the original pFL44L-PSE1-t vector. Gene sequences coding for full-length Pse1p, its 265 N-terminal amino-acid form, Kap123p, Kap60p and Kap95p were obtained by a 16 cycle PCR amplification, using as template yeast genomic DNA and the tRth DNA polymerase XL (Perkin Elmer Cetus) (Barnes, 1994). The DNA from PCR products was cloned in both high-copy number (pFL44L) and low-copy number (pFL38) vectors (Bonneaud et al., 1991).

**PSE1 gene disruption and subcellular localization of the corresponding protein**

Two kilobases of the PSE1 coding sequence, including the AUG codon, was removed and replaced by the TRP1 gene. This sequence was used to disrupt the PSE1 chromosomal locus of the BMA41 strain. Tryptophan prototrophs obtained were checked by Southern blot and PCR analyses to confirm that PSE1 was correctly disrupted. The MCD01 strain obtained was sporulated and dissected. Each tetrad yielded only two viable spores, which were trp1−. To obtain the GFP-PSE1 and GFP-PSE1-t fusion genes, the GFP-coding sequence of pEGFP-N3 (Clonetech Laboratories) was amplified by PCR using the Pfu protein and cloned in frame at the unique PmlI site of the PSE1 and PSE1-t vectors, located 27 nucleotides upstream of the PSE1 AUG codon. Therefore, the initiation codon for these fusion genes is the GFP one, PSE1 ORF being in frame with the last amino acid of the GFP and nine additional amino acids before the authentic PSE1 AUG (AIARTVTTSI).

Living cells expressing the GFP-tagged proteins were observed by fluorescence with a chilled CCD camera (Hamatsu Photonics) as previously described (Doye et al., 1994). Whole cell extracts from BMA41 strains expressing the GFP-Pse1p or GFP-Pse1p-t proteins were prepared using glass beads. Twenty micrograms of protein was applied on 8% SDS-polyacrylamide gels. Immune sera were used at dilutions 1:250 for affinity-purified anti-Nup133p (Doye et al., 1991). Pse1p or GFP±Pse1p-t proteins were prepared using glass beads. Twenty micrograms of protein was applied on 8% denaturing formaldehyde agarose gels, after transfer nylon membranes were stained with methylene blue solution (Sambrook et al., 1989). Filters were probed with the following antibodies: mouse monoclonal antibody 12CA5 against the HA1 epitope (1:5000); mouse monoclonal antibody 9E10 against the myc epitope (1:5000); rabbit polyclonal antibody against Abf2p (1:10000); mouse monoclonal antibody 4B12-A5 against Cox2p (1:10000). Immuno reactive bands were visualized with either anti-mouse or anti-rabbit IgGs coupled to horseradish peroxidase followed by ECL detection (Amersham International) according to the manufacturer’s instructions. Densitometric analyses were performed on independent mitochondrial preparations. Specific signals for m56HA1 and Atm1p-myc were normalized for each sample, using the Cox2p or the Abf2p signals (TINA software). In all the experiments performed, both normalized results were very similar, suggesting that Abf2p and Cox2p quantities were equivalent in all the mitochondria preparations tested. The amounts of m56HA1 and Atm1p-myc tagged protein resistant to proteolysis in mitochondria from wild-type cells or from cells overproducing Pse1p were compared with a paired Student’s t-test using a confidence level of 95%.

**Purification of free and mitochondria-bound ribosomes**

Purification of mitochondria-bound ribosomes and free cytoplasmic ribosomes was performed as described previously (Ades and Butow, 1980; Suissa and Schatz, 1982). Total RNA from both fractions and from whole cells were obtained by a hot-phenol method (Schmitt et al., 1990). Northern blots were performed using 15 μg of RNA electrophoresed through denaturing formaldehyde agarose gels, after transfer nylon membranes were stained with methylene blue solution (Sam brook et al., 1989). Probes used were the 1 kb EcoRI±HindIII fragment of the ACT1 ORF, COXIII and Maturase oligonucleotides shown in Table 1, which revealed, respectively, ACT1 mRNA, COXIII mRNA and the S56M hybrid transcript. Phosphoimager system and TINA software were used to compare the relative abundance of the S56M hybrid mRNA.

**Acknowledgements**

We would like to thank A. Delahodde, Suzanne Joyce and E. Fabre for friendly discussions and critical reading of the manuscript. We are grateful to J. P. diRago for the gift of the BMA41 strain and to Dr G. Schatz for the gift of the ATM1-c-myc vector. This work was supported by CNRS (URA1302) by the École Normale Supérieure and by a grant from the AFM (French Association for Myopathies, MNV 96).

**References**


