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RESEARCH ARTICLE

**Udo Krause-Buchholz · Kathrin Barth
Cora Dombrowski · Gerhard Rödel**

Saccharomyces cerevisiae translational activator Cbs2p is associated with mitochondrial ribosomes

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Abstract A characteristic feature of the mitochondrial expression system in *Saccharomyces cerevisiae* is the requirement for gene-specific translational activator proteins. Translation of mitochondrial apocytochrome *b* mRNA requires the nucleus-encoded proteins Cbs1p and Cbs2p. These proteins are thought to tether cytochrome *b* mRNA to the mitochondrial inner membrane via binding to the 5′ untranslated mRNA leader. Here, we demonstrate by the use of affinity chromatography and coimmunoprecipitation that Cbs2p interacts with the mitoribosomes. We further provide evidence that the C-terminus of Cbs2p is important for ribosome association, while the N-terminal portion is essential for the formation of homomeric structures.

Keywords *Saccharomyces cerevisiae* · Translational activator proteins · Cbs2p · Mitochondrial ribosomes

Introduction

Most mitochondrial (mt) proteins are encoded by nuclear genes and are imported into the organelle after translation on cytosolic ribosomes. A few key components, however, are products of the mtDNA. With the exception of Var1p, a protein of the small mt ribosomal (mitoribosomal) subunit, all these proteins are subunits of the oxidative phosphorylation (OXPHOS) system. The mtDNA-encoded proteins are synthesized on mitoribosomes, which are preferentially detected at the matrix side of the inner mt membrane (Watson 1972). In the yeast *Saccharomyces cerevisiae*, genetic data led to the identification of specific translational activator proteins which are essential for the translation of individual mt transcripts. In the absence of a functional activator the protein is not synthesized, despite the presence of its target mRNA. When translation of intron-encoded RNA-maturases is necessary for RNA maturation [as in the case of apocytochrome *b* (*COB*) RNA], a lack of translational activator proteins results in the accumulation of unspliced precursor RNAs. Analysis of mt suppressor mutations suggested that the 5′ untranslated RNA leader sequences (5′UTL) are the target sites of the translational activator proteins (for a review, see Fox 1996). For example, translation of Cox3p is no longer dependent on its activator Pet494p, when the *COX3* gene is fused to the 5′UTL of *COB* mRNA due to a rearrangement of mtDNA (Rödel and Fox 1987). Translation of this novel chimeric gene requires the *COB*-specific activators Cbs1p and Cbs2p (Rödel 1997). Similarly, mutations affecting Cbs1p or Cbs2p can be overcome by mt mutations which replace the *COB* 5′UTL by the *ATP9* 5′UTL (Rödel 1986). The idea that 5′UTLs directly interact with activator proteins is strongly supported by the finding that alterations or overproduction of genes encoding translational activators were able to bypass *COX2* and *COX3* 5′UTL mutations, respectively (Mulero and Fox 1993; Wiesenberger et al. 1995).

All translational activator proteins studied so far are firmly bound or at least associated with the inner mt membrane (Michaelis et al. 1991; McMullin and Fox 1993). Because of this association, it was proposed that the activators act by tethering their target RNA to specific sites at the inner membrane to promote translation and concomitant assembly of the nascent proteins into the OXPHOS complexes (Michaelis et al. 1991). Strong experimental evidence for this view was obtained by Sanchirico et al. (1998). Those authors showed that

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U. Krause-Buchholz (✉) · K. Barth · C. Dombrowski · G. Rödel
Institut für Genetik, Technische Universität Dresden,
01062 Dresden, Germany
E-mail: ukrause@rcs.urz.tu-dresden.de
Tel.: +49-351-46335960
Fax: +49-351-46337725

Present address: K. Barth
Institut für Anatomie,
Medizinische Fakultät Carl Gustav Carus,
01307 Dresden, Germany

chimeric mRNAs consisting of the *COX2* and *COX3* coding sequence, respectively, and the 5′UTL of the *VAR1* gene are translated, but not assembled. Further evidence comes from recent studies on Oxa1p, which was shown to mediate the membrane association of mt ribosomes (Jia et al. 2003; Szyrach et al. 2003). Szyrach et al. (2003) proposed a model in which the membrane-bound translational activators recruit transcripts to the matrix face of the inner membrane, with subsequent initiation of translation. The emerging nascent polypeptide chain and the close proximity of the ribosomes allows their binding to the Oxa1p translocase. Alternatively, the authors suggested that the ribosomes might already be associated with Oxa1p prior to translational initiation.

In one case, genetic studies suggested a direct interaction between an activator protein and the mitoribosome: starting with a truncated form of Pet122p, which results in respiratory deficiency due to lack of translation of Cox3p, Fox and coworkers were able to isolate suppressor mutations in the genes *MRP1*, *MRP17* and *PET127* (Haffter et al. 1991; Haffter and Fox 1992), which all encode proteins of the small mitoribosomal subunit. Data concerning an interaction of cytochrome *b* activator proteins with mitoribosomes are only preliminary. Western blot analysis of isolated ribosomal subunits hints at an association of Cbs2p with the small mitoribosomal subunit (Michaelis et al. 1991). However, lack of information concerning the purity of the ribosomal preparation in this study and the cross-reactivity of the antibodies with an unknown mt protein renders this result uncertain.

In this paper, we address the question whether Cbs2p is associated with high-molecular-weight complexes. We show that Cbs2p is able to form homomeric complexes and we provide evidence for an interaction of Cbs2p with mitoribosomes. Analysis of a mutant form of Cbs2p indicates that ribosome interaction is dependent on the C-terminal portion of Cbs2p.

Materials and methods

Strains and media

The *Escherichia coli*strains used in this work were DH5α (BRL) and BL21-CodonPlus RIL (Stratagene). The *S. cerevisiae* strains are listed in Table 1. *E. coli* and yeast media were as described by Sambrook et al. (1989) and Kaiser et al. (1994). Growth on a non-fermentable substrate was tested on YPGly-plates, which contained 2% glycerol as the sole carbon source.

Epitope tagging

Hemagglutinin (HA)-tagged Cbs2p and N-terminally truncated Cbs2 mutant proteins were created as described by Tzschoppe et al. (2000). Fusion of Cbs2p, Cbs2p(delC) and Mrp13p with the cMyc tag or the TAP tag was achieved by homologous recombination of the corresponding integration cassettes in the chromosomal loci (Knop et al. 1999) of strains BY4741 or BY4742, respectively (Table 1). Integration cassettes for

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
DBY747	*MATa*, *his3-1*, *leu2-3*, *leu2-112*, *trp1-289*, *ura3-52*	ATCC 44774
KT1	*MATa*, *his3-1*, *leu2-3*, *leu2-112*, *trp1-289*, *ura3-52*, *cbs2::Sphis5*$^{+}$	Tzschoppe et al. (2000)
KT4	*MATa*, *his3-1*, *leu2-3*, *leu2-112*, *trp1-289*, *ura3-52*, *cbs2::Sphis5*$^{+}$, *mrp13::CaURA3*, *leu2::YIp351MRP13-3HA-LEU2*	This work
KT5	*MATa*, *his3-1*, *leu2-3*, *leu2-112*, *trp1-289*, *ura3-52*, *leu2::YIp351MRP13-3HA-LEU2*	This work
BY4741	*MATa*, *his3*Δ1, *leu2*Δ, *met15*Δ0, *ura3*Δ0	Euroscarf accession Y00000
CDO4	*MATa*, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0, *CBS2::*(*CBS2-TAP-tag-URA3*), *MRP13::*(*MRP13-cMyc-tag-Sphis5*$^{+}$)	This work
BY4742	*MATα*, *his3*Δ1, *leu2*Δ0, *lys2*Δ0, *ura3*Δ0	Euroscarf accession Y10000
CDO7	*MATα*, *his3*Δ1, *leu2*Δ0, *lys2*Δ0, *ura3*Δ0, *CBS2::*(*CBS2-cMyc-Sphis5*$^{+}$)	This work
CDO9	*MATα*, *his3*Δ1, *leu2*Δ0, *lys2*Δ0, *ura3*Δ0, *MRP13::*(*MRP13-cMyc-Sphis5*$^{+}$)	This work
UKB3	*MATα*, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, CBS2::(*cbs2*(*1-385*)-*cMyc-Sphis5*$^{+}$)	This work
CDO11	*MAT a*/α, *ADE2*/*ade2*, *ARG4*/*arg4*, *leu2-3,112*/*leu2*Δ0, *TRP1*/*trp1-289*, *ura3-52*/*ura3*Δ0, *HIS3*/*his3*Δ, *LYS2*/*lys2*Δ, *MRP4*/*MRP4::*(*MRP4-TAP-tag-URA3*), *CBS2*/*CBS2::*(*CBS2-cMyc-HIS5*)	This work
SC1069	*MAT a*, *ade2*, *arg4*, *leu2-3,112*, *trp1-289*, *ura3-52* *MRP4::*(*MRP4-TAP-tag-URA3*)	CellZome AG (Heidelberg, Germany)

TAP-tagging and cMyc-tagging were PCR-amplified from vector pBS1539 (Rigaut et al. 1999) and pGA2254 (containing nine tandem repeats of the cMyc epitope, a kind gift of W. Zachariae, MPI-CBG Dresden), respectively. For construction of strain UKB3, expressing a C-terminally truncated cMyc-tagged derivative of Cbs2p [Cbs2p(delC), lacking amino acids (aa) 386–389], the corresponding codons of the deleted aa were omitted from the primer sequence used for amplification of the integration cassette. *MRP13-3HA* was cloned into YIp351 (Hill et al. 1986) and the linarized vector was transformed into DBY747 and KT1 to yield strains KT5 and KT4 (Table 1), respectively. Strain CDO11 was obtained by mating SC1069 with CDO7.

Cloning, expression and purification of glutathione-*S*-transferase fusion proteins

Genomic DNA of strain DBY747 was used as template with primer A (5′-TATATAGGATCCATGTCAAGCTCAATACCTAGAG-3′) and primer B (5′-TATATAGCGGCCG CTCACAGGTAATGATAATCTAGTGC-3′) to PCR-amplify the *CBS2* coding sequence. Mutant *cbs2* alleles were created by amplification with primer A and one of the following indicated primers: for *cbs2-M2* (Y386Q, Y388Q) primer C (5′-TATATAGCGGCCGCTTAT CACAGCTGATGTT GATCTAGTGCTTTTTG-3′), for *cbs2-M9* (Y388V) primer D (5′-TATATAGCGGCCGC TTATCACAGAACATGATAATCTAGT GCTTTTTG-3′), for *cbs2-M10* (Y386V) primer E (5′-TATATAGC GGCCGCTTATCACAG GTAATG AACATC TAGTGCTTTTTG-3′) and for *cbs2-M11* (Y386V, Y388V) primer F (5′-TATATAGCGG CCGCTT ATCACAGAACATGAACATCTAG TGCTTTTTG-3′). The PCR products were cut with *Bam*HI and *Not*I and cloned into vector pGEX-4T3 (Amersham Pharmacia Biotech) to yield pGEX-4T3-CBS2, pGEX-4T3-*cbs2-M2*, pGEX-4T3-*cbs2-M9*, pGEX-4T3-*cbs2-M10* and pGEX-4T3-*cbs2-M11*. Correct in-frame fusion was verified by DNA sequencing. Plasmids were transformed into *E. coli* strain BL21. Expression and purification of the glutathione (GSH)-*S*-transferase (GST) fusion proteins were done as described by Lode et al. (2000).

Isolation and fractionation of mitochondria

Yeast cells were grown to early stationary phase in lactate medium or supplemented minimal medium containing 2% raffinose as sole carbon source. Isolation and fractionation of mitochondria was carried out as described by Daum et al. (1982).

GST pull-down experiments

Mitochondria were isolated and 300 μg mt protein were lysed on ice for 1 h in 200 μl lysis buffer [30 mM $MgSO_4$, 20% glycerol, 0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4, 1% digitonin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 units RNasin]. The supernatant obtained after centrifugation at 18,000 *g*and 4°C for 10 min was used for GSH affinity chromatography.

Blue native polyacrylamide gel electrophoresis

For blue native polyacrylamide gel electrophoresis (BN-PAGE; Schägger 2001), 200 μg mt protein were sedimented by centrifugation (12,000 *g*, 10 min, 4°C), resuspended in 20 μl low-salt buffer (50 mM NaCl, 5 mM ϵ-aminocaproic acid, 50 mM imidazol/HCl, pH 7.0, 1 mM PMSF, 1× proteinase inhibitor cocktail; Roche) with (0.5 μl) or without RNasin (Promega) containing digitonin (Sigma; detergent/protein = 4:1) and incubated on ice for 10 min. Insoluble material was removed by centrifugation (20,000*g*, 20 min). Immediately after the addition of 4 μl Coomassie-free loading buffer (10% glycerol, 0.01% Ponceau S), the samples were loaded onto a 5–13% gradient gel. A high-molecular-weight gel filtration calibration kit (Amersham Pharmacia Biotech) was used to determine the apparent molecular weights (thyroglobulin monomer/dimer at 669/1,300 kDa, ferritin at 440 kDa, aldolase at158 kDa, bovine albumin at 66 kDa).

Coimmunoprecipitation

Coimmunoprecipitation (Co-IP) was carried out essentially as described by Lode et al. (2000). Mitochondria were isolated from a strain coexpressing Mrp13p-3HA and Cbs2p-3HA; and 200 μg mt protein were solubilized for 1 h at 4°C in lysis buffer [50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM PMSF] containing 0.5% Triton X-100. Insoluble material was removed by centrifugation (20,000 *g*, 20 min) and the supernatant was incubated with Nam9p-antibodies (a kind gift of A. Chacinska, Universität Freiburg) coupled to protein A-agarose.

Tandem affinity purification method

The tandem affinity purification (TAP) method was performed essentially as described by Rigaut et al. (1999) on an analytical scale: 500 μg mt protein of strain CDO4 (expressing Cbs2p-TAP and Mrp13p-cMyc) or CDO11 (expressing Mrp4p-TAP and Cbs2p-cMyc) were lysed for 1 h with 1% digitonin in 500 μl lysis buffer (30 mM $MgSO_4$, 20% glycerol, 0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4, 40 units RNasin). The extract was cleared by centrifugation for 10 min at 18,000 *g* and incubated with an IgG matrix (Sigma) for 2 h. TEV cleavage (AcTEV, Invitrogen) was performed without EDTA in lysis buffer with 1 mM DTT for 1 h at 23°C. To the supernatant were added $CaCl_2$ and imidazole (final concentrations 2 mM

and 1 mM, respectively) and 10 mM β-mercaptoethanol before the incubation with a calmodulin matrix (Stratagene) at 4°C in the presence of 0.1% digitonin for 1 h. Aliquots from each step of the TAP procedure were collected for SDS-PAGE.

Sucrose gradient centrifugation

Mitochondria (500 μg protein) were solubilized in 100 μl lysis buffer [1% digitonin, 20 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 40 units RNasin] for 30 min at 4°C. Following a clarifying spin (18,000 *g*, 10 min), the supernatant was loaded on top of a linear 20–40% sucrose gradient (5 ml, in 0.1% digitonin, 20 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 1 mM AEBSF) and subjected to ultracentifugation at 33,000 rpm (Beckman MLS50 rotor, Optima Max; 16 h, 4°C). Fractions of 500 μl were collected from the bottom of the gradient. Proteins were precipitated with trichloroacetic acid (TCA) and quantitatively loaded on a 12% SDS gel.

Western blot analysis

Protein electrophoresis in the presence of SDS was carried out according to Laemmli (1970). Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore), probed with primary antibodies and detected with horse raddish peroxidase-conjugated secondary antibodies and the ECL-Plus Kit (Amersham Pharmacia Biotech). Primary antibodies were directed against HA (Roche), cMyc (Roche), Cox2p (Molecular Probes), Cox3p (Molecular Probes), *E. coli* Atp5p (Molecular Probes; an antibody which cross-reacts with *S. cerevisiae*Atp2p), Mrp51p (Green-Willms et al. 1998; a kind gift of N. Bonnefoy, CNRS, Gif sur Yvette, France) and MrpL36p (a kind gift of J.M. Herrmann, Universität München, Germany).

Miscellaneous procedures

Standard DNA techniques were as described by Sambrook et al. (1989). Yeast cells were transformed by the lithium acetate method (Schiestl and Gietz 1989). Genomed columns were used for the isolation of DNA from agarose gels. DNA sequencing was carried out with the dideoxy chain termination method (Sanger et al. 1977), using 5′ IRD800-labeled primers (MWG-Biotech).

Results

Molecular organization of Cbs2p

To decipher the molecular organization of Cbs2p we performed BN-PAGE with mitochondria of strain CDO7 expressing the cMyc-tagged version of Cbs2p. This epitope tag does not interfere with Cbs2p function, as the strain is respiratory-competent (data not shown). Digitonin was chosen for solubilization because of its ability to maintain weak and/or lipid-mediated protein interactions (Cruciat et al. 2000). Mitochondrial proteins were separated in a two-dimensional gel as described in the Materials and methods. Cbs2p-cMyc (with a calculated molecular mass of 57 kDa) was mainly detected as a bipartite signal in the range of about 50–120 kDa (Fig. 1). The signal of about 50 kDa could reflect the monomeric form, whereas the ca. 120-kDa signal could represent a homomeric form of Cbs2p.

Cbs2p forms homomeric structures in vitro

We next tested whether Cbs2p is able to form homomeric structures in vitro. A GST-Cbs2p fusion protein was expressed in *E. coli* and coupled to GSH-sepharose beads (see Materials and methods). As a control, GST was bound to GSH-sepharose. Homomer formation was tested by incubation of the sepharose beads with mt lysate of KT1 transformants expressing Cbs2p-3HA. This Cbs2p variant is functional, as it confers respiratory competence to the *cbs2* null mutant strain KT1 (data not shown). Figure 2a shows that Cbs2p-3HA is bound by GST-Cbs2p (lane 2), but not by GST alone (lane 1). Specificity of binding was demonstrated by the observation that Cox3p is not retained on the resin (see also Fig. 5). This result confirms the conclusion of the BN-PAGE experiments, that Cbs2p forms homomeric structures.

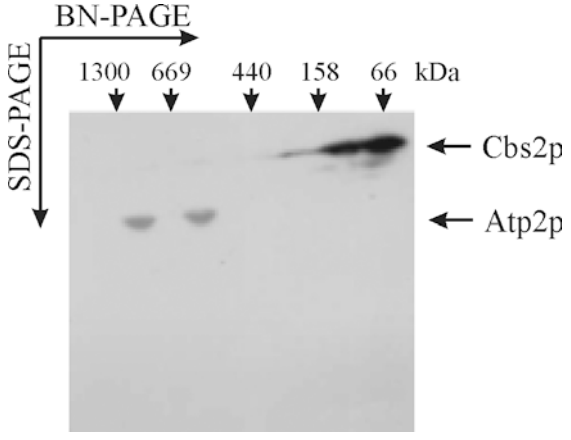

Fig. 1 Molecular organization of Cbs2p. Mitochondria from the Cbs2p-cMyc expressing strain CDO7 were solubilized with digitonin and analyzed by two-dimensional SDS/BN-PAGE. Cbs2p-cMyc was detected in a Western blot analysis with cMyc antibodies. In the first dimension, molecular weights of protein standards are indicated. The monomeric (500 kDa) and the dimeric (1,000 kDa) forms of ATPase were detected by an antibody directed against subunit Atp2p

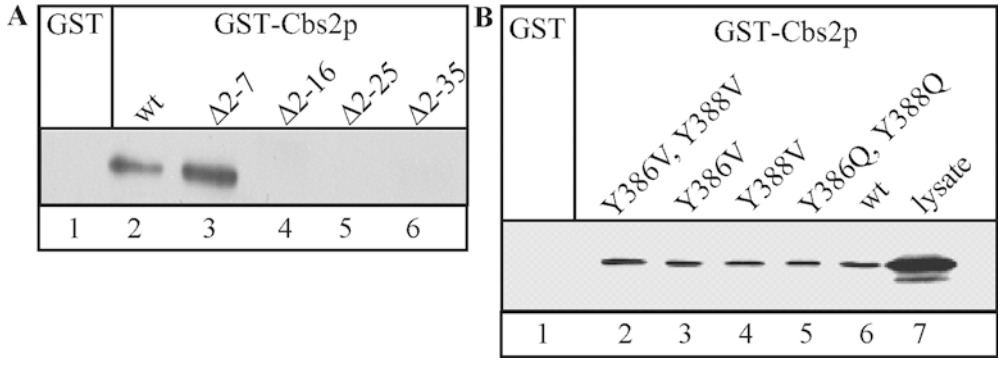

Fig. 2 a,b The N-terminal, but not the C-terminal part of Cbs2p is essential for homomerization. **a** Mitochondria of KT1 transformants expressing either Cbs2p or the indicated mutant Cbs2 proteins lacking six or more of the N-terminal aa were lysed and incubated with immobilized GST-Cbs2p (*lanes 2–6*). As a control, the wild-type (*wt*) lysate was incubated with immobilized GST (*lane 1*). **b** Immobilized GST-fusion proteins of full-length Cbs2p (wt, *lane 6*) and the indicated Cbs2p mutant proteins (*lanes 2–5*) were incubated with Cbs2p-3HA-containing lysates of KT1 transformants. Following extensive washing, bound proteins were separated on a 12% SDS-polyacrylamide gel, blotted and detected with HA antibodies

The N-terminal portion of Cbs2p is necessary for dimerization

Recently, we reported that the N-terminal part of Cbs2p is required for proper functioning of the protein (Tzschoppe et al. 2000). Mutant proteins lacking six or more aa at the N-terminus are imported into mitochondria, but are no longer able to complement the *cbs2* deletion. We addressed the question whether the N-terminal portion of Cbs2p might be necessary for homomer formation. A series of N-terminally truncated HA-tagged Cbs2p mutant proteins [Cbs2p(Δ2-7)-3HA, Cbs2p(Δ2-16)-3HA, Cbs2p(Δ2-25)-3HA, Cbs2p(Δ2-35)-3HA] were expressed in strain KT1 and the mt proteins of the respective transformants were incubated with GST-Cbs2p coupled to GSH-sepharose beads. Besides Cbs2p-3HA (Fig. 2a, lane 2) only Cbs2p(Δ2-7)-3HA (lane 3) was able to interact with the immobilized Cbs2p. Cbs2p derivatives lacking aa 15, 24 or 34 of the N-terminus (lanes 4–6) were not able to bind to GST-Cbs2p. Again, specificity of binding was controlled by GST alone and by detection of Cox3p (see also Fig. 5). This result shows that the formation of homomeric structures in vitro depends on the N-terminal portion of Cbs2p.

The C-terminal portion of Cbs2p is not essential for homomerization

We recently showed that truncations and some point mutations (affecting Y386 and/or Y388) in the C-terminal region of Cbs2p render the protein non-functional (Tzschoppe et al. 1999). To test whether the C-terminal part is required for homomer formation, we incubated immobilized GST-fusion proteins of full-length Cbs2p and Cbs2p mutant proteins with Cbs2p-3HA-containing lysates (Fig. 2b, lane 7) of KT1 transformants (see Materials and methods). Like the wild-type protein (Fig. 2b, lane 6), all mutant proteins (lanes 2–5), but not GST alone (lane 1) were able to bind Cbs2p-3HA. This result shows that all tested versions of the protein can interact with Cbs2p in vitro. The loss of function of the mutant proteins due to the C-terminal mutations is therefore not caused by the inability to form homomeric structures.

In the presence of RNase inhibitor, Cbs2p is associated with a high-molecular-weight complex

As outlined in the Introduction, genetic studies indicate that the 5′UTL of *COB* mRNA is the target of Cbs2p. It is not known whether the postulated interaction of Cbs2p with the RNA is direct or indirect. Michaelis et al. (1991) showed that a salt concentration of ca. 200 mM NaCl is necessary to release Cbs2p from mt membranes, indicating a weak interaction with membrane and/or membrane-associated components via electrostatic forces. To test whether the association of Cbs2p with high-molecular-weight complexes might depend on the presence of RNA, we isolated mitochondria of strain CDO7 (expressing Cbs2p-cMyc) and performed a BN-PAGE analysis with the RNase inhibitor, RNasin (Promega). In the absence of RNasin, Cbs2p-cMyc can be detected as a bipartite signal in the low-molecular-weight range, possibly reflecting its monomeric and dimeric state (Fig. 1). Despite the gentle conditions used for lysis and BN-PAGE, no Cbs2p signal could be observed in the high-molecular-weight range. Interestingly, a different result was obtained when RNasin was present during the preparation and lysis of mitochondria. Again, the Cbs2p signal is mainly observed as a bipartite signal in the low-molecular-weight range (Fig. 3). In addition, however, the protein can be detected in a tail-shaped signal spreading from > 1 MDa to the low-molecular-weight signals. This altered distribution is specific for Cbs2p, because the β-subunit of ATPase, which served as a control, can be detected in the form of two clear signals [corresponding to the monomer (500 kDa) and dimer (1,000 kDa) of ATPase],

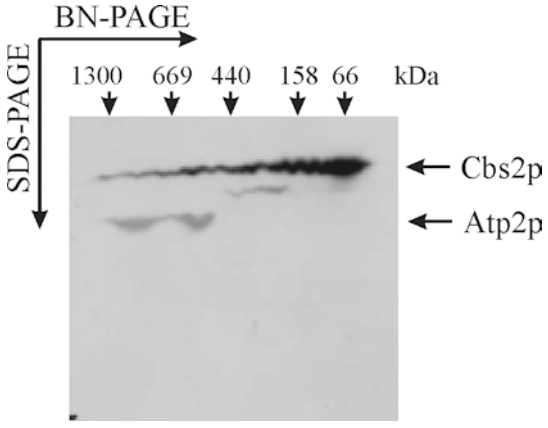

Fig. 3 In the presence of RNasin, Cbs2p is associated with a high-molecular-weight complex. Mitochondria of strain CDO7 were isolated, lysed and analyzed by BN-PAGE in the presence of RNasin. Proteins were analyzed by Western blotting with cMyc and Atp2p antibodies, respectively. Positions of protein standards are indicated

irrespective of the presence of RNase inhibitor. The tail-shaped signal of Cbs2p originates at the interface of the stacking gel and separating gel. Because complexes larger than 1.5 MDa can not enter the separating gel (Nijtmans et al. 2002), one may speculate that Cbs2p is partially associated with such a huge complex. Removal of RNase inhibitor during electrophoresis leading to increased destabilization of RNA and/or abolishment of weak physical interactions might result in a continuous release of Cbs2p from a high-molecular-weight complex.

Our data hint at a possible involvement of RNA in stabilizing or mediating the association of Cbs2p with a high-molecular-weight complex. Candidates for such a complex are the mitoribosomes, which—due to their size—are likely not to enter the separating gel or even the stacking gel in BN-PAGE and which have been suggested to be associated with translational activator proteins (see the Introduction).

Cbs2p co-migrates with mitoribosomal proteins on sucrose gradients

To assess the association of Cbs2p with mt ribosomes, we tested whether Cbs2p co-migrates with known constituents of the mt ribosomes on sucrose gradients. To this end, mitochondria of lactate-grown transformants expressing Cbs2p-cMyc were lysed with 1% digitonin in the presence of 10 mM Mg^{2+} and RNasin and then subjected to velocity centrifugation in a sucrose gradient. The distribution of Cbs2p-cMyc, the ribosomal proteins Mrp51p (small subunit) and MrpL36p (large subunit) and the matrix protein aconitase (Aco1p) in the fractions was monitored by immunological detection (Fig. 4). Cbs2p shows a bipartite distribution in the gradient, with the majority of the protein towards the top of the gradient. A small percentage of Cbs2p fractionates at higher sucrose densities (fractions 9, 10).

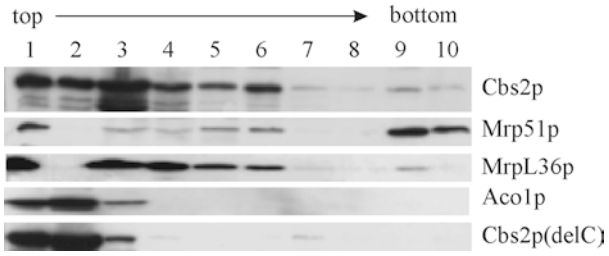

Fig. 4 Cbs2p co-sediments with mitoribosomal proteins on sucrose gradients. Mitochondria of cells expressing Cbs2p-cMyc or Cbs2p(delC) were solubilized in 1% digitonin. The lysate was loaded onto a 20–40% sucrose gradient after a clarifying spin at 18,000 *g* for 10 min and ultracentrifuged. Fractions of 500 µl were collected from the bottom of the gradient, precipitated with TCA and quantitatively subjected to SDS-PAGE. The distribution of Cbs2p, Cbs2p(delC), the ribosomal proteins Mrp51p, MrpL36p and the matrix enzyme Aco1p was analyzed by immunoblotting

Both ribosomal proteins Mrp51p and MrpL36p possess an almost identical fractionation profile. The presence of their respective signals on the bottom of the gradient (fractions 9, 10) indicates the fractionation of intact ribosomes, whereas the accumulation in fractions 3–6 of Mrp51p and MrpL36p may specify the fractions containing free small and large ribosomal subunits, respectively. In contrast, Aco1p was detected exclusively in the upper fractions of the gradient. We conclude from the co-sedimentation of Cbs2p with the ribosomal proteins that a small pool of Cbs2p is associated with mitoribosomes.

Cbs2p is associated with mt ribosomes

To confirm the interaction of Cbs2p with mt ribosomes, we performed GST-pulldown experiments using sepharose-immobilized GST-Cbs2p fusion protein. Mitochondria were prepared from wild-type cells and lysed with 1% digitonin in the presence of RNasin. The clarified extract (Fig. 5, lane 1; 2% of the total)

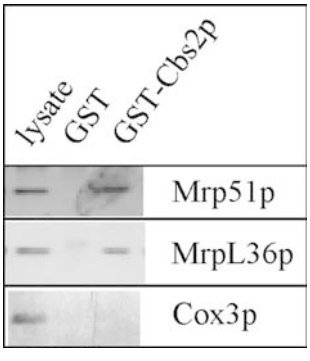

Fig. 5 Cbs2p interacts in vitro with mitoribosomal proteins. Immobilized GST or GST-Cbs2p was incubated with mt lysate from wild-type cells. The affinity matrices were extensively washed, bound proteins were resolved by SDS-PAGE, blotted and detected with antibodies against Mrp51p, MrpL36 and—as a control—Cox3p

was then applied to the immobilized proteins. After extensive washing, the presence of the ribosomal proteins Mrp51p, MrpL36p and the mt membrane protein Cox3p in the precipitated material was analyzed by immunoblotting. Neither of the proteins was precipitated in the control experiment by GST alone (Fig. 5, lane 2). In contrast, both Mrp51p and MrpL36p were bound by GST-Cbs2p (Fig. 5, lane 3). The specificity of the interaction was confirmed by the finding that the hydrophobic Cox3p was not precipitated by GST-Cbs2p. These results are in line with those of the sucrose gradient centrifugation and confirm the association of the translational activator Cbs2p with mitoribosomes.

A third line of evidence for the observed interaction comes from co-IP experiments. Antibodies directed against Nam9p, another component of the small mitoribosomal subunit, were immobilized on protein A-agarose and incubated with mt lysates of cells expressing Cbs2p-3HA and Mrp13p-3HA. Figure 6a, lane 4 shows that both proteins are concomitantly immunoprecipitated, again indicating that Cbs2p is associated with mitoribosomes. Although we cannot completely exclude the possibility that the faster-migrating protein may result from degradation of Cbs2p-3HA, this seems to be unlikely as we do not observe a protein band of comparable mobility in a control experiment, where the mt lysate of a Cbs2p-3HA over-expressing strain was analyzed (lane 5). The specificity of Cbs2p binding was documented by the finding that antibodies directed against the mt membrane proteins Cox2p and Cox3p yielded signals in the co-IP supernatant, but not in the precipitate (Fig. 6a, lane 4).

Further support for an interaction of Cbs2p with mt ribosomes in vivo was obtained by application of the TAP method (Rigaut et al. 1999). For that approach, the TAP tag was C-terminally fused to *CBS2* via homologous recombination. cMyc-tagged Mrp13p was used to detect the small mt ribosomal subunit. The fused tags do not interfere with the protein's function, as growth of the resulting strain (CDO4) on non-fermentable carbon source (glycerol) is indistinguishable from that of the corresponding wild-type strain (data not shown). Mitochondria of cells from strain CDO4 coexpressing Cbs2p-TAP and Mrp13p-cMyc were isolated, lysed with 1% digitonin (Fig. 6b, lane 1, 2% of the total) and subjected to the two consecutive affinity steps of the TAP procedure on an IgG matrix (lane 2, 10% of the total) and a Calmodulin matrix (lane 3, quantitatively applied). Detection of Mrp13p-cMyc and MrpL36p, but not Cox3p, on both matrices documents the association of Cbs2p with mt ribosomes. This finding was further corroborated by a reverse-TAP procedure with a respiratory-competent strain coexpressing TAP-tagged Mrp4p, a constituent of the small mitoribosomal subunit, and cMyc-tagged Cbs2p. Again, Cbs2p but not Cox3p was precipitated by the affinity matrices (Fig. 6c, lanes 2, 3). The presence of intact ribosomes was confirmed by detection of MrpL36.

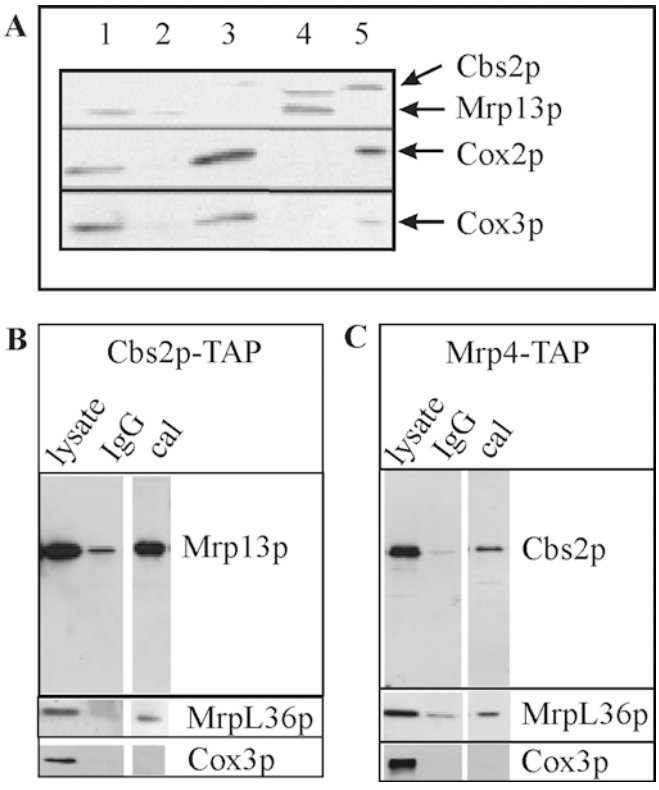

Fig. 6 a–c Cbs2p interacts in vivo with mitoribosomes. **a** Mitochondria from cells expressing Cbs2p-3HA and Mrp13p-3HA (*lane 1*, 30 μg) were lysed, insoluble material was removed by centrifugation (*lane 2*) and the lysate was incubated with Nam9p antibodies immobilized on protein A-agarose. Unbound (*lane 3*) and bound proteins (*lane 4*) were analyzed. Due to its low expression, Cbs2p was not detected in the lysate (*lane 1*). To mark the position of Cbs2p, mitochondria of a strain expressing Cbs2p from a high-copy plasmid were applied to the gel (*lane 5*). **b**,**c** Cbs2p-associated proteins were purified from mt lysates (2% of the total, *lane 1*) of strains expressing either Cbs2p-TAP and Mrp13p-cMyc (**b**) or Mrp4-TAP and Cbs2p-cMyc (**c**) by the TAP method. The bound proteins of the IgG matrix (10% of the total, *lane 2*) and the calmodulin matrix (*cal*, quantitatively applied, *lane 2*) were separated by SDS-PAGE. Immunological detection was performed with cMyc , MrpL36p and Cox3p antibodies

The C-terminus of Cbs2p is important for association with mt ribosomes

As mentioned above, truncations and some point mutations in the C-terminal region of Cbs2p render the protein non-functional (Tzschoppe et al. 1999). We could also show that the failure of these mutants to respire is not caused by the inhibition of homomer formation (see Fig. 2). Here, we tested whether the C-terminal portion of Cbs2p is required for association with mt ribosomes. For this reason, the non-functional Cbs2p mutant protein lacking the last four C-terminal aa [Cbs2p(delC)] was fused to the cMyc tag. Mitochondria were lysed by digitonin and the proteins were fractionated by density centrifugation (Fig. 4).

In contrast to the wild-type protein, the C-terminally truncated version of Cbs2p fractionates almost exclusively in the upper fractions of the gradient, as is the case

for Aco1p. This indicates that the C-terminus of Cbs2p may be essential for the association with ribosomes. In conclusion, the respiratory deficiency of mutations affecting the extreme C-terminus of Cbs2p can be explained by the loss of the ability to interact with mt ribosomes.

Discussion

The data presented in this paper provide strong evidence that Cbs2p, one of the translational activator proteins for cytochrome *b* synthesis, is associated with the mitoribosomes. The previous observation that Cbs2p was detected in a Western blot analysis of the proteins of the small, but not the large mitoribosomal subunit (Michaelis et al. 1991), may indicate an exclusive or preferential association with the small subunit. In the experiments of this paper, we identified both small and large subunit proteins in the affinity experiments, suggesting that Cbs2p may interact with intact ribosomes. Our data recall the system of translational activation of Cox3p with the three activator proteins Pet122p, Pet494p and Pet54p, of which Pet122p seems to be associated with the small mitoribosomal subunit. In that case, genetic data strongly supported the view that all three activators interact in a complex, whereas we are not able to prove such an interaction for the translational activators Cbs1p and Cbs2p (unpublished data).

Our data are in line with the previous proteome analysis of mitoribosomes, which did not detect Cbs2p among the mitoribosomal proteins (Gan et al. 2002). Instead, only a small pool of Cbs2p seems to be associated with a sub-population of mitoribosomes. Under the conditions of our experiments, only a small percentage of Cbs2p was associated with mitoribosomes. Because the concentration of Cbs2p was reported to be low (Michaelis 1991), it is extremely unlikely that this result is due to a limiting number of ribosomes. However, only a subpopulation of ribosomes may be in a competent state which allows binding of Cbs2p. If one assumes that at least five, but possibly eight, translation-activating complexes are present in yeast mitochondria and that a single ribosome can recruit only one of these, the number of interacting ribosomes may be much lower than the actual number of ribosomes and indeed may become the limiting factor. In addition, however, the interaction between Cbs2p and the mitoribosomes may be very weak or only transient. This last interpretation could also account for the finding of the BN-PAGE analysis that Cbs2p can be detected in the high-molecular-weight range only in the presence of RNase inhibitor. On one hand, the maintenance of RNA is crucial for the integrity of the ribosomal subunits and therefore may also be essential for the protein-mediated association of Cbs2p with the mitoribosomes. On the other hand, this interaction could be mediated by the ribosome-bound mRNA. Perhaps the contact between Cbs2p and mitoribosomes exists only prior to the elongation phase, when the translation machinery is recruited on the *COB* mRNA for translational initiation. Mitoribosomes are probably not able to bind in an unassisted way to mRNAs and to find the AUG initiation codon. Cytosolic ribosomes of eukaryotes find the translation start site by scanning the 5′UTL region, starting at the 5′ cap. Such a mode is unlikely to operate in yeast mitochondria, not only because mRNAs lack a cap structure, but especially because the long 5′UTL sequences are predicted to form stable secondary structures and contain a number of upstream AUG codons and short open reading frames. Similarly, there is no evidence for the prokaryotic mode of translation: a Shine–Dalgarno (SD)-like sequence (which was initially postulated to interact with the 3′ end of the 15S rRNA) is unlikely to exist. Fox and coworkers were able to show that a chimeric mRNA lacking the putative SD-like sequence was translated normally. Therefore, other mechanisms of start-site selection seem to operate in yeast mitochondria. Especially, the experiments of Mulero and Fox (1994) and Bonnefoy and Fox (2000) led to the conclusion that finding the proper initiation site by ribosomes requires some sequence or structural information in addition to the AUG codon. Perhaps the translational activator proteins are involved in the correct positioning of ribosomes. In this scenario, by interacting with the mitoribosomes, the translational activator system would not only position the mRNAs at the inner mt membrane, but in addition would assist the translation machinery in finding the correct start site. By transient binding to different translational activators, yeast mitoribosomes could acquire the ability to translate the various mt mRNAs.

Perhaps the activator proteins fulfill additional functions, e.g., they could interact with components of the inner mt membrane in order to define the exact site where the synthesis and assembly of a given mt translation product has to occur. In the case of cytochrome *b* synthesis, Abc1p (whose over-expression can suppress a mutant *cbs2* allele; Bousquet et al. 1991), could be a candidate for such an interaction. However, the possible function of translational activators in defining the site of synthesis cannot be essential, because cells bearing chimeric genes (in which the coding sequence of a hydrophobic mt translation product is fused to the 5′UTL of another mt gene encoding a membrane protein) are respiratory-competent. Therefore, at least a substantial amount of the proteins which are synthesized at a "wrong" localization on the membrane would find their way to the correct site of assembly. Notable exceptions are fusions bearing the *VAR1*5′UTL sequence, which are translated but rapidly degraded (Fiori et al. 2003). Although Var1p is the only soluble mt translation product in *S. cerevisiae*, it seems to be translated via the action of an as-yet-unidentified translational activator in close proximity to the inner membrane, possibly at a putative site of ribosome assembly (Fiori et al. 2003). Therefore tethering mRNAs to the membrane may be an important, yet not sufficient function of the activators.

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