Yeast mitochondrial F_1F_0-ATPase: the novel subunit e is identical to Tim11

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Received 23 May 1997

Abstract We report here the identification of the novel subunit of the mitochondrial F_1F_0-ATPase from *Saccharomyces cerevisiae*, ATPase subunit e. Yeast ATPase subunit e displays significant similarities in both amino acid sequence, properties (hydropathy and predicted coiled-coil structure) and orientation in the inner membrane, with previously identified mammalian ATPase subunit e proteins. Estimation of its native molecular mass and ability to be co-immunoprecipitated with α subunit of the F_1-ATPase, demonstrate that subunit e is a subunit of the F_1F_0-ATPase. Stable expression of subunit e requires the presence of the mitochondrially encoded subunits of the F_0-ATPase. Subunit e had been previously identified as Tim11 and was proposed to be involved in the process of sorting of proteins to the mitochondrial inner membrane.

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Key words: Mitochondrion; F_1F_0-ATPase; Tim11; Intramitochondrial sorting

1. Introduction

The mitochondrial F_1F_0-ATPase (ATP-synthetase EC 3.6.1.3) plays a pivotal role in maintaining cellular ATP levels in eukaryotic organisms. The composition and the assembly of this enzyme complex in a number of organisms has been the focus of research for many years. Like other H^+-ATPases, the mitochondrial ATPase can be divided functionally into distinct domains, the F_1 domain which contains the catalytic site for the hydrolysis and synthesis of ATP and the F_0 part which is embedded in the inner membrane and forms a proton channel linking the proton gradient across the inner membrane to the synthesis of ATP by the F_1 domain. The F_1 and F_0 parts are linked together through a stalk known as the F_A sector [1–3].

In the yeast *Saccharomyces cerevisiae*, 11 subunits of the mitochondrial F_1F_0-ATPase have been identified to date [2]. The F_1 domain is comprised of α, β, γ, δ, and ε subunits, the F_A is made up by b, OSCP and d subunits, whilst the F_0 section is comprised of subunits 6, 8 and 9. With the exception of the three subunits of the F_0 which are encoded by the mitochondrial genome, all the remaining subunits of the F_1F_0-ATPase are nuclear encoded. Hence the biogenesis of this complex involves the co-ordinate action of both the nuclear and mitochondrial genomes.

Analysis of the mammalian mitochondrial F_1F_0-ATPases has indicated that it contains four further subunits, e, f, g and F_6 [1,4–6]. Evidence for the identification of a homolog of subunit g of the F_1F_0-ATPases from the yeast *Saccharomyces cerevisiae* has been recently reported [7]. Homologs of the other subunits, however, have not been found in yeast to date. We present evidence here that the recently described Tim11 protein [8] represents a yeast homolog of the ATPase subunit e and its steady state levels are strongly affected by the presence of the F_0 sector.

2. Materials and methods

2.1. Yeast strains and growth conditions

Mitochondria isolated from the yeast strain D273-10B grown in lactate medium [9] were used for the biochemical analysis of Tim11, namely, the submitochondrial fractionation, detergent extraction, gel filtration and co-immunoprecipitation experiments. The rho° strain analyzed in this study was YNR5c (*MATa mdj1::URA3*) transformed with a CEN plasmid pMDJ315 containing the MDJ1 gene [10]. The isogenic wild-type YNR3 yeast strain was used for direct comparison, both strains were grown in lactate medium supplemented with 1% galactose.

The TIM11 gene was cloned after the GAL10 promoter together with the LEU2 gene (see Fig. 3). The resulting linearized construct (YMFB2) was transformed into the yeast strain W334-*a* (*MATa, leu2, ura3-52*) as described below, thus replacing of the endogenous TIM11 promoter by the GAL10 one. Mitochondria were isolated from resulting yeast strain grown in SD-lactate medium with uracil (24 mg/ml) and with either 0.1% galactose (for the down-regulation of Tim11 expression, Tim11↓) or 1% galactose (for the induction of Tim11 expression, Tim11↑).

2.2. Detergent solubilisation of Tim11 and gel-filtration analysis

Isolated mitochondria (1 mg protein) were lysed in 200 μl digitonin buffer (1% (w/v) digitonin, 150 mM K-acetate, 30 mM HEPES pH 7.4, 1 mM PMSF, 0.1 mg/ml α_2-macroglobulin, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 30 min on ice. Following a clarifying spin (60 min, 226 000×*g*), the supernatant was loaded onto a Superose 6 column equilibrated with the same digitonin buffer. Fractions (0.5 ml) were collected, precipitated by adding TCA to a final concentration of 12.5% (w/v) and analysed by SDS-PAGE. Tim11, $F_1\alpha$, $F_1\beta$, Tim22 and Tim23 were detected in the eluate fractions by immunoblotting.

2.3. Antibody production

Antisera against the C-terminal region of Tim11 was raised in rabbits against the chemically synthesized peptide CVILNAVESLKEAS which had been coupled to activated ovalbumin (Pierce).

2.4. Co-immunoprecipitation of Tim11 and $F_1\alpha$

Mitochondria (200 μg protein) were resuspended at a concentration of 0.2 mg/ml in the digitonin buffer described above and lysed on ice for 30 min. Following a clarifying spin at 226 000×*g* for 60 min, the supernatant was incubated under gentle shaking for 60 min at 4°C with the $F_1\alpha$ antiserum or preimmune serum, as indicated, that had been bound to protein A-Sepharose. The protein A-Sepharose beads were then washed twice with 1 ml of digitonin buffer. The immunocomplexes were dissociated in SDS-sample buffer by shaking for 20 min at 4°C. The immunoprecipitates were analysed by SDS-PAGE and immunostaining.

2.5. Miscellaneous

Hypotonic swelling and carbonate extraction of mitochondria were performed as previously described [11,12]. Protein determination and

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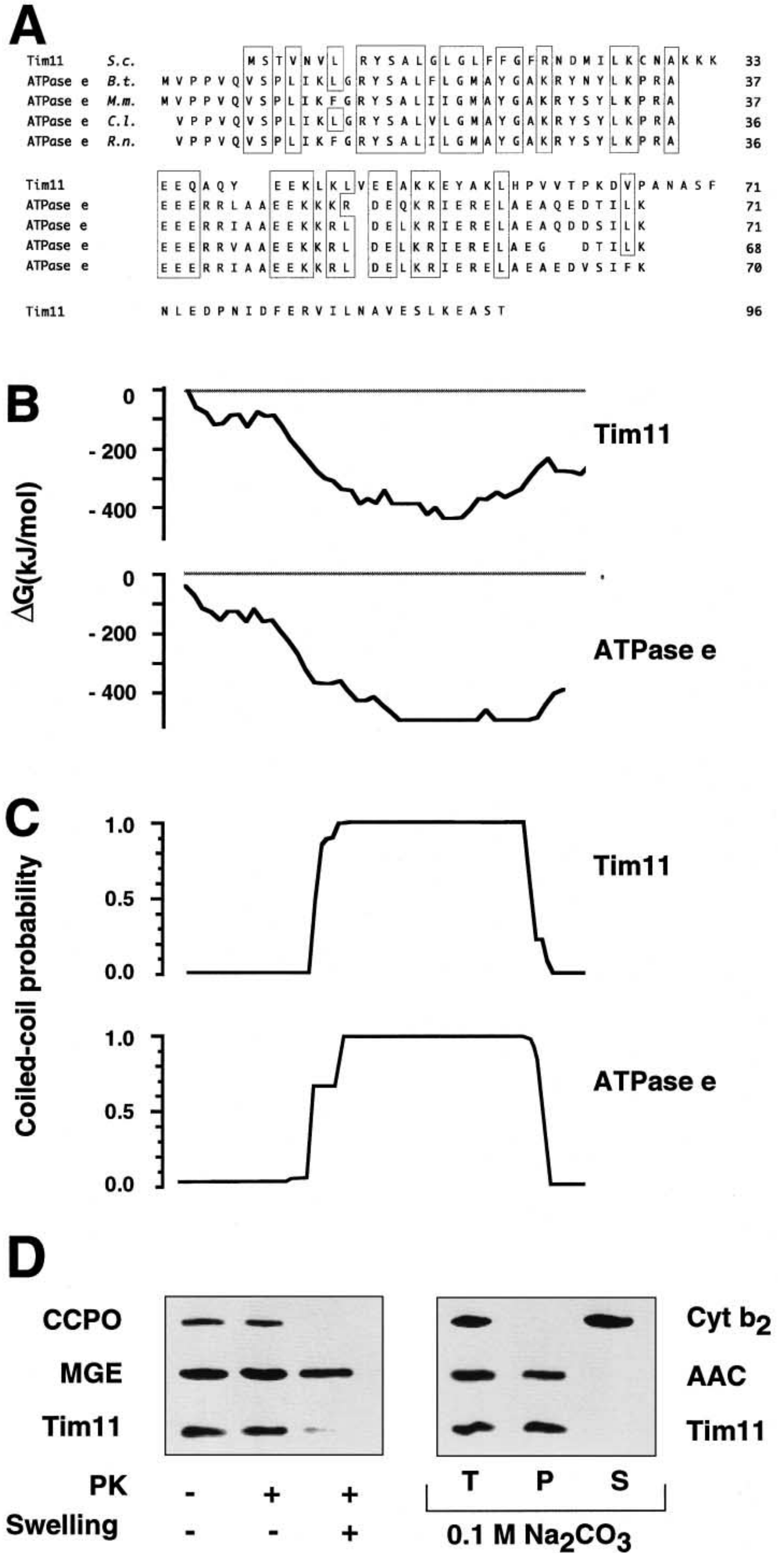
PII S0014-5793(97)00691-1



SDS-PAGE were performed according to the published methods of Bradford [13] and Laemmli [14], respectively. The detection of proteins after blotting onto nitrocellulose was performed using the ECL detection system (Amersham).

Fig. 1. Amino acid sequence comparison of Tim11 and mammalian ATPase subunit e proteins, transfer energy profile, coiled-coil prediction and submitochondrial localization of Tim11. (A) The conserved amino acid residues between Tim11 from *Saccharomyces cerevisiae* (S.c.) [8] and the mammalian ATPase subunit e proteins from bovine (*Bos taurus*) (M64751, EMBL), mouse (*Mus musculus*) (S52977, EMBL), hamster (*Cricetulus longicaudatus*) (M22350, EMBL) and rat (*Rattus norvegicus*) (D13121, EMBL) are indicated by boxes. (B) The transfer energy profile, ΔG (kJ/mol) and (C) the probability to form coiled-coil structures as calculated by Coiled-coil Prediction Program from ISREC Bioinformatics Group at Swiss Institute for Experimental Cancer Research [18] are depicted for Tim11 (amino acid residues 1–71) and bovine ATPase subunit e (complete amino acid sequence). (D) Submitochondrial localization of Tim11. Mitochondria and mitoplasts, generated by hypotonic swelling, were incubated for 30 min at 4°C in the presence or absence of 40 μg/ml proteinase K (PK), as indicated. Mitochondria were subjected to alkaline extraction (0.1 M Na_2CO_3, pH 11.5), divided and one half was directly TCA precipitated (total, T) and the other was separated by centrifugation (60 min at 226 000 × *g*) into pellet (P) and supernatant fractions (S), prior to TCA precipitation. All mitochondrial fractions were analysed by SDS-PAGE and Western blot analysis, using specific antisera for cytochrome *c* peroxidase (CCPO), cytochrome b_2, both soluble proteins of the intermembrane space; MGE, a matrix localised protein; the ADP/ATP translocase (AAC) an integral protein of the inner membrane; and Tim11.

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3. Results

3.1. *Similarity of Tim11 to mammalian ATPase subunit e proteins*

The Tim11 protein has been recently identified in *S. cerevisiae* in search for proteinaceous components involved in the process of protein sorting to the mitochondrial inner membrane. In attempt to identify further potential homologs of Tim11, we performed a data base search using the complete amino acid sequence of Tim11. The result indicated that Tim11 contained a significant similarity to all known mammalian subunit e proteins of the mitochondrial F_1F_0-ATPase complex. The similarity extended over the whole sequence of the proteins, with the exception of the C-terminal region, which is slightly longer in the case of Tim11 (Fig. 1A). The transfer energy profile of Tim11 also closely resembles that of the mammalian ATPase e subunits (as depicted for bovine ATPase subunit e, Fig. 1B) where the N-termini of both proteins display the potential to partition into a lipid bilayer. Furthermore, the sequence of Tim11 predicts the ability to form a coiled-coil structure (Fig. 1C), as has also been described for the mammalian ATPase e subunits [6].

In order to address whether Tim11 represents a novel yeast homolog of ATPase subunit e, we initially raised antibodies against Tim11. For this purpose we used a Tim11-specific peptide corresponding to the C-terminal region of the protein. The resulting antibody could detect Tim11 by Western blotting from as little as 5 μg mitochondrial protein after the first bleed, suggesting that Tim11 is quite an abundant protein (results not shown). Tim11 was inaccessible to exogenously added protease in intact isolated mitochondria. However, subfractionation of the mitochondria by hypotonic swelling (disrupts the outer membrane whilst leaving the inner membrane intact) rendered Tim11 accessible to the added protease (Fig. 1D). Furthermore, Tim11 is an integral membrane protein as it was resistant to alkaline extraction of the mitochondria (Fig. 1D). We conclude therefore that Tim11 is anchored to the inner mitochondrial membrane by its transmembrane domain at its N-terminus and adopts an N_{in}-C_{out} topology. A similar topology had been described for the bovine ATPase subunit e [6].

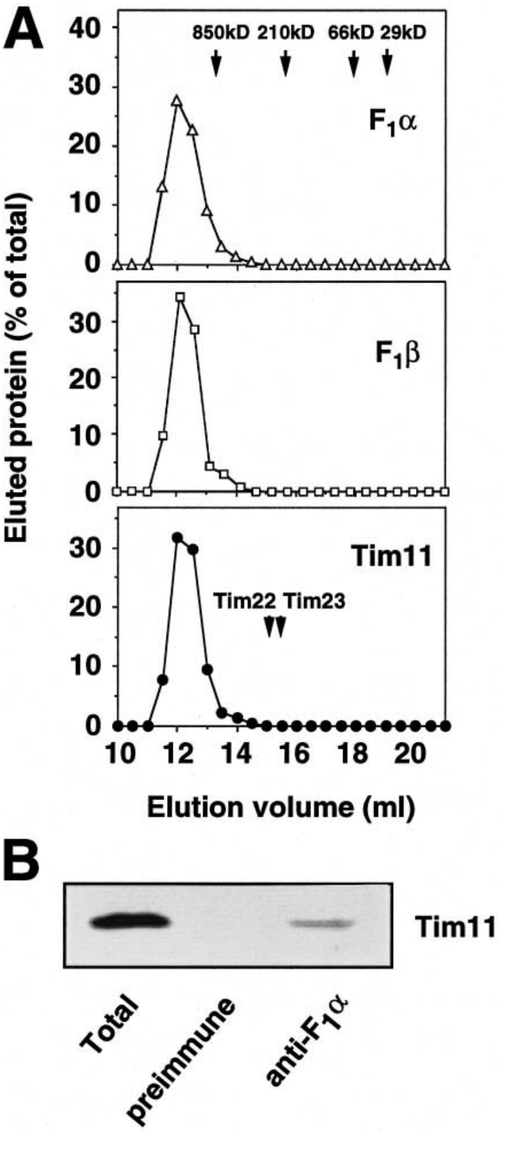

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Fig. 2. Tim11 is a subunit of the F_1F_0-ATPase. (A) Gel-filtration analysis of the Tim11 protein. Isolated wild-type mitochondria were solubilized with digitonin, as described in Materials and methods. Superose 6 chromatography of detergent extracts of mitochondria was performed. $F_1\alpha$, $F_1\beta$, Tim22, Tim23 and Tim11 were detected in the eluate fractions, as indicated, by Western blot analysis, using the enhanced chemiluminescence system. Protein amounts, determined by densitometry, as given as a percentage of the respective proteins in the eluate ('total'). (B) Co-immunoprecipitation of Tim11 and $F_1\alpha$. Isolated mitochondria were solubilised in digitonin, as described. After the clarifying spin the supernatant was divided into three aliquots. One was precipitated with TCA (total) and the remaining portions were subjected to co-immunoprecipitation with preimmune or $F_1\alpha$-specific antibodies (anti-$F_1\alpha$). Precipitated proteins were analysed by SDS-PAGE and immunostaining, using the antiserum directed against the C-terminus of Tim11.

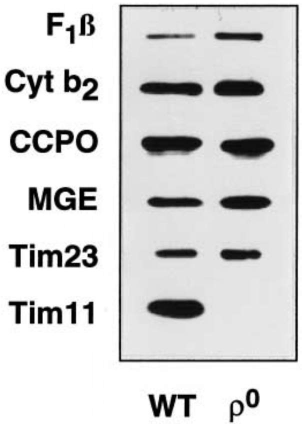

Fig. 3. Tim11 is absent in rho° mitochondria. Mitochondria (50 μg protein) isolated from a rho° strain (ρ°) and its isogenic wild-type (WT) were subjected to SDS-PAGE and analysed by Western blotting for the presence of marker proteins, as indicated. Abbreviations, as in Fig. 1.

3.2. Tim11 co-fractionates with F_1-ATPase subunits α and β upon gel filtration and can be co-immunoprecipitated with $F_1\alpha$

Isolated wild-type mitochondria were solubilized with the detergent digitonin. The resulting protein extract was subjected to gel filtration chromatography in order to determine the native molecular mass of the Tim11 protein. The eluate was analysed by Western blotting using the C-terminal specific Tim11 polyclonal antiserum (Fig. 2A). Tim11 was exclusively recovered from the column in fractions corresponding to apparent native molecular masses > 850 kDa. Further analysis of the eluate fractions by immunoblotting revealed that F_1-ATPase subunits α and β ($F_1\alpha$ and $F_1\beta$), were almost exclusively recovered in these fractions also. From its size we conclude that the F_1F_0-ATPase has remained intact under the experimental conditions used here. Immunoblotting of the eluate fractions with antibodies specific for Tim22 and Tim23, indicated that Tim11 did not co-fractionate with the other known components of the inner membrane translocases (Fig. 2A). It appears unlikely therefore that Tim11 is a subunit of the known TIM complexes.

A direct interaction of Tim11 with components of the F_1F_0-ATPase complex was investigated. Isolated mitochondria were solubilized with digitonin under conditions where the F_1F_0-ATPase remained intact, as described above. An interaction of Tim11 with the other subunits of the F_1F_0-ATPase was demonstrated by co-immunoprecipitation analysis using an

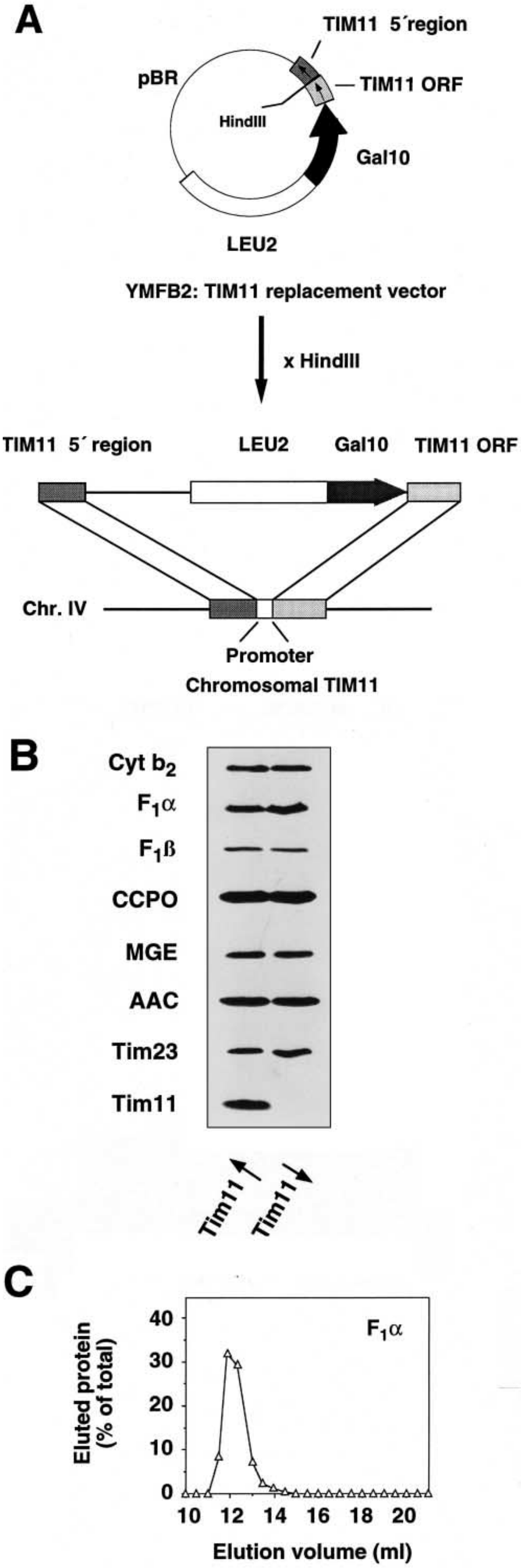

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Fig. 4. Regulated expression of the Tim11. (A) The promoter region of the TIM11 gene in chromosomal IV was replaced by the galactose inducible Gal10 promoter (YMFB2:TIM11). (B) Mitochondria were isolated from this yeast strain which had been either grown in the presence (Tim11↑) or absence (Tim11↓) of galactose. The resulting mitochondria (50 μg protein) were subjected to SDS-PAGE and analysed by Western blotting for the presence of marker proteins, as indicated. (C) Isolated Tim11↓ mitochondria were lysed with digitonin and resulting protein extract was subjected to Superose 6 chromatography. The resulting eluate fractions were analysed by SDS-PAGE and Western blotting with antibodies specific for $F_1\alpha$, as described in Fig. 2A.

antibodies specific for $F_1\alpha$ (Fig. 2B). The amount of Tim11 co-immunoprecipitated with $F_1\alpha$ was approximately 15% of the total Tim11 species. As the efficiency of immunoprecipitation of the $F_1\alpha$ protein was in the same range (results not shown), it appears that Tim11 is largely or completely in association with $F_1\alpha$.

We conclude therefore that Tim11 is associated with the F_1F_0-ATPase complex.

3.3. The levels of Tim11 are influenced by the presence of the F_0-ATPase sector

We next addressed if the steady state levels of Tim11 were influenced by the presence of the F_0 subunits of the F_1F_0-ATPase. As these subunits are encoded by the mitochondrial genome, we analysed a rho° yeast strain which lacks mitochondrial DNA and hence a functional F_0-ATPase, for the presence of Tim11. Mitochondria isolated from a rho° yeast strain and its corresponding wild-type strain were analysed by Western blotting. Immunodecoration of the resulting blot with the Tim11 C-terminal specific antiserum indicated the near to complete absence of Tim11 in the rho° mitochondria, in contrast to the wild-type. Furthermore immunoblotting of $F_1\alpha$ and $F_1\beta$ and a number of other control mitochondrial proteins indicated that they were not significantly down-regulated in the absence of the intact F_0-ATPase (Fig. 3).

We conclude therefore that Tim11 requires the presence of the mitochondrially encoded subunits of the F_0-ATPase for its stability in the mitochondrial inner membrane.

3.4. Down regulation of Tim11 does not influence the assembly of the F_1F_0-ATPase complex

In order to gain more insight into the function of Tim11 we cloned the Gal10 promoter into the chromosome in front of the TIM11 gene, thus enabling us to regulate the expression of Tim11 (Fig. 4A). Mitochondria were prepared from the Tim11-(Gal10) cells grown in the presence (Tim11$\uparrow$) and absence of galactose (Tim11$\downarrow$). They were analysed by Western blotting for various mitochondrial proteins. In the presence of galactose Tim11 was expressed, however, when the cells were shifted to galactose-free medium, the levels of Tim11 were strongly reduced (Fig. 4B). Expression levels of other mitochondrial marker proteins including $F_1\alpha$ and $F_1\beta$ and cytochrome b_2 were not affected by the absence of Tim11 (Fig. 4B).

Gel filtration analysis of the digitonin solubilized Tim11$\downarrow$ mitochondria indicated that $F_1\alpha$ eluted in a fraction corresponding to the native molecular mass of the assembled F_1F_0-ATPase sector (Fig. 4C). We conclude therefore that the assembly and the stability of the F_1F_0-ATPase is not influenced by the absence of the Tim11.

4. Discussion

Homologs of a number of the subunits of the mammalian F_1F_0-ATPases have not been identified to date in yeast. We present evidence here that the recently described Tim11 protein, a mitochondrial protein from *Saccharomyces cerevisiae*, is F_1F_0-ATPase subunit e. The following data support this conclusion: First, the Tim11 amino acid sequence displays significant similarity to the mammalian e subunits. Second, the hydropathy profile, membrane orientation and predicted coiled-coil interactions are conserved between Tim11 and the ATPase e subunits. Third, Tim11 is assembled in the F_1F_0-ATPase complex. The native molecular mass, as judged by gel filtration of Tim11 coincides with that of α and β subunits of the F_1-ATPase. Tim11 can be specifically immunoprecipitated with $F_1\alpha$, demonstrating that these two proteins exist together in the same oligomeric complex.

Tim11 may correspond to the uncharacterised 10 kDa protein observed in a recent analysis of the isolated F_1F_0-ATPase complex from yeast mitochondria [15]. Tim11 is not an essential protein, disruption of the TIM11 gene causes slowed growth and diminished mitochondrial respiration [8]. Tim11 thus does not play a catalytic role in the yeast mitochondrial F_1F_0-ATPase complex, as has been suggested for the mammalian subunit e proteins [3].

Interestingly Tim11 amino acid sequence also displayed homology to an SH3 domain binding protein, mouse 3BP1 protein (EMBL X87671). Binding to the SH3 domains is mediated by a common PXXP amino acid sequence present on all ligands, specificity involves other interactions, often ones including arginine [16,17]. For example Src-SH3 specific binding uses a seven amino acid residue consensus sequence of RPLPXXP. A potential SH3 domain, PLPLVP, is found in the subunit 6 of the F_0-ATPase (ATPase6). It is tempting to speculate that the conserved SH3 domain binding sequence of Tim11 reflects a direct interaction of Tim11 with ATPase6.

In summary, we conclude that Tim11 is subunit e of the yeast F_1F_0-ATPase. Tim11 was originally proposed to be a component of the protein import system of the inner membrane of mitochondria (the TIM complexes), and was suggested to be specifically involved in the sorting of cytochrome b_2 to the intermembrane space [8]. We show here that Tim11 is not found associated with the known TIM complexes. Although it appears not to be essential for cytochrome b_2 sorting, the interesting question of whether Tim11 has a dual function, in protein sorting and as subunit of the F_1F_0-ATPase, remains.

Acknowledgements: We would like to thank Jens Fuhrmann, Chung-Bum Shin, and Christiane Kotthoff for their excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft Sonderforschungsbereich 184 (Teilprojekte B2 and B18).

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