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Tim18p Is a New Component of the Tim54p-Tim22p Translocon in the Mitochondrial Inner Membrane

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The mitochondrial inner membrane contains two separate translocons: one required for the translocation of matrix-targeted proteins (the Tim23p-Tim17p complex) and one for the insertion of polytopic proteins into the mitochondrial inner membrane (the Tim54p-Tim22p complex). To identify new members of the Tim54p-Tim22p complex, we screened for high-copy suppressors of the temperature-sensitive tim54-1 mutant. We identified a new gene, TIM18, that encodes an integral protein of the inner membrane. The following genetic and biochemical observations suggest that the Tim18 protein is part of the Tim54p-Tim22p complex in the inner membrane: multiple copies of TIM18 suppress the tim54-1 growth defect; the tim18::HIS3 disruption is synthetically lethal with tim54-1; Tim54p and Tim22p can be coimmunoprecipitated with the Tim18 protein; and Tim18p, along with Tim54p and Tim22p, is detected in an ~300-kDa complex after blue native electrophoresis. We propose that Tim18p is a new component of the Tim54p-Tim22p machinery that facilitates insertion of polytopic proteins into the mitochondrial inner membrane.

INTRODUCTION

Mitochondrial function requires the import of hundreds of proteins synthesized in the cytosol (for review, see Ryan and Jensen, 1995; Pfanner, 1998; Ryan et al., 1998; Rassow et al., 1999). In the yeast *Saccharomyces cerevisiae*, mitochondrial protein import uses multiple subunit translocons found in the mitochondrial outer membrane (called the TOM complex) and in the inner membrane (called the TIM complexes). Most proteins destined for mitochondria carry amino-terminal targeting signals called presequences. Presequences vary in length and primary amino acid sequence, yet they share a common motif of a positively charged amphipathic helix (Allison and Schatz, 1986; Roise et al., 1986, 1988; Roise, 1992). Once in the matrix, the presequence is removed by a two-plex) and in the inner membrane (called the TIM complexes). The mitochondrial IM translocases, Tim23p, Tim22p, and Tim17p (Sirrenberg et al., 1996; Kerscher et al., 1997), are required to translocate proteins across the IM into the matrix (Enz et al., 1993; Maarse et al., 1994; Ryan et al., 1994; Lohret et al., 1997). Tim44p and mt-Hsp70 provide the pulling force for this import (Pfanner et al., 1994; Rassow et al., 1994; Stuart et al., 1994; Berthold et al., 1995; Glick, 1995). Precursors that carry positively charged presequences are targeted to Tim23p-Tim17p after their translocation across the OM (Moro et al., 1999). The Tim23p-Tim17p complex also plays a role in the insertion of some proteins into the IM.

A second translocon in the IM is the Tim54p-Tim22p complex (Sirrenberg et al., 1996; Kerscher et al., 1997). Tim54p and Tim22p are required for the insertion of many polytopic proteins into the IM. Substrates for the Tim54p-Tim22p complex do not carry cleavable, amino-terminal presequences. These include members of the mitochondrial carrier family, such as the phosphate carrier (PiC) and the ADP/ATP carrier, as well as the membrane components of the IM translocases, Tim23p, Tim22p, and Tim17p (Sirren-
Plasmid Constructions

2µ-HIS3 plasmid pOK9 carries TIM54 on a 3-kbp ClaI fragment inserted in pRS423 ( Sikorski and Hieter, 1989) and was constructed by homologous recombination in yeast (Oldenburg et al., 1997) between a PouII fragment derived from pOK31 (Kerscher et al., 1997) and Xhol-EcoRI–digested pRS423. Plasmid pH203 (Holler, unpublished data) was constructed by insertion of an EcoRI fragment containing TIM22 from ~392 to 863 into pRS423. 2µ- YLR164w plasmid pOK90 was constructed by amplifying yeast genomic DNA with the use of oligonucleotides 234 (5′-GGAATTCCCTGATTCGAGAAGGATCTCTAGG-3′) and 235 (5′-GGAATTCCGATCCACGAGTTGTGGGAGG-3′) and PCR (Saiki et al., 1985). After EcoRI digestion, the PCR product was inserted into pRS426 (Sikorski and Hieter, 1989), forming plasmid pOK90. 2µ-SDH4 plasmid pOK91 was constructed by isolating a PouI fragment containing SDH4 from plasmid SDH4-17 (a gift from B. Lemire, University of Alberta, Edmonton, Alberta, Canada) (Bullis and Lemire, 1994) and using this fragment for recombination in yeast with Xhol-BamHI–digested pRS425 (Sikorski and Hieter, 1989).

Gene Disruption of TIM18 and TIM54

A complete null mutation in the chromosomal copy of TIM18 was constructed with the use of a PCR-based gene disruption method (Lorenz et al., 1995). Briefly, a HIS3 DNA fragment was amplified with the use of oligonucleotides 221 (CTTGTAAAGTCCGAAAAAGAAAGAGATAGGGTGAGTGCAATGAGAGTGCAC) and 222 (ATGCGAGTGCAACACTGGAATTTAATACCTTGTGACTGCGATTTTGACC) and plasmid pRS302 (Sikorski and Hieter, 1989) as the template. The amplified HIS3 DNA fragment, flanked by 40 base pairs (bp) of TIM18 sequences immediately upstream and downstream of the ORF, was transformed into his3/3is4 diploid strains 605 and 1082. His+ diplotype transformants were isolated and shown by PCR analysis to have one of two copies of TIM18 disturbed (tim18::HIS3). MATa tim18::HIS3 strain 992, MATa tim18::HIS3 strain 994, MATa TIM54 strain 993, and MATa TIM54 strain 995 are the meiotic products from tim18::HIS3/TIM54 diploid strain 987 (strain 605 background). tim54::KAN strain 1078, which lacks the TIM46 ORF, was constructed by PCR-mediated gene replacement into diploid strain 605 with the use of oligonucleotides 382 (CTTGTAAAGTCCGAAATCAGCTTCTCAAAAAGAAAGAGATAGGGTGAGTGCAATGAGAGTGCAC) and 383 (CGTCGATGCGATGCAATGAAACATATTATATATATACCAACGGGATGTACAGCC) and plasmid pRS500 (Brachmann et al., 1998). G418-resistant transformants were then transformed with TIM54-URA3 plasmid pOK30 (Kerscher et al., 1997). After sporulation and meiotic analysis, haploid strain 1078, which carries tim54::KAN and pOK30, was isolated.

Construction of a Hemagglutinin Epitope-tagged Version of the TIM18 Protein

pOK70, which contains TIM18 with a NotI site proximal to the stop codon, was constructed as follows. An ~1-kbp PCR product containing the TIM18 ORF was amplified from plasmid pOK66 with the use of oligonucleotides 229 (GGAATTTCGTGTTAATG) and 230 (GGAATTCCCTGATTCGAGAAGGATCTCTAGG) and inserted into Bluescript SK II+ (Stratagene, La Jolla, CA). A 114-bp NotI fragment containing three copies of the influenza hemagglutinin (HA) epitope (Field et al., 1988; Tyers et al., 1992) was inserted into pOK70, yielding pOK73. pOK73 was digested with AatII and SaeI, and an AatII-SaeI fragment from pOK48 (Kerscher et al., 1997) containing the 3′-untranslated region of TIM23 was inserted into pOK70, yielding pOK74. TIM18-HA was isolated from pOK74 as a 2.2-kbp PouII plasmid pRS315 (Sikorski and Hieter, 1989). After homologous recombination in yeast (Oldenburg et al., 1997), forming pOK1032, pOK1032 was transformed into MATa tim18::HIS3 strain 992 (making strain 1032). 2µ-TIM18-HA plasmid pOK1032 was constructed as described.
for pOK302 except that a 2.2-kbp PvuI fragment containing TIM18-HA from pOK74 was inserted into the LEU2-2a plasmid pRS425 (Sikorski and Hieter, 1989).

Subcellular and Submitochondrial Fractionation
Yeast cells were grown to OD600 of ~1.0 in YEP medium containing glycerol and ethanol. Cells were converted to spheroplasts and homogenized in breaking buffer (0.6 M mannitol, 20 mM HEPES/KOH, pH 7.4), and a crude cytosolic fraction and a mitochondrial pellet were isolated by centrifugation at 9600 × g for 10 min as described (Daum et al., 1982). Osmotic disruption of the mitochondrial OM was accomplished by resuspending mitochondrial pellets in 20 mM HEPES-KOH, pH 7.4, at a protein concentration of 1 mg/ml. For protease digestions, 100 µg of mitochondria at 1 mg/ml was treated with 50 µg/ml proteinase K for 20 min on ice, followed by the addition of 1 mM PMSF (Sigma Chemical, St. Louis, MO). Separation of mitochondrial OM and IM vesicles on sucrose gradients was performed as described (Emtage and Jensen, 1993). To show that Tim18p is an integral membrane protein, mitochondria were treated with either 0.1 M NaC104, pH 11, or 1 M NaCl and centrifuged for 30 min at ~30 pounds per square inch in a Beckman (Fullerton, CA) Airfuge. For analysis, proteins were separated on SDS-polyacrylamide gels (Laemmli, 1970; Haid and Suisa, 1983) and transferred (Haid and Suisa, 1983) to Immobilon P membranes (Millipore, Bedford, MA). Membranes were decorated with antibodies (Haid and Suisa, 1983), and immune complexes were detected by chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL).

Imports into Isolated Mitochondria
For in vitro transcription, TIM18 was placed behind the SP6 promoter as follows. The TIM18 ORF was amplified with the use of PCR and primers 219 (GAATTCATGGAAGCACTG) and 220 (CCGCTCGAGAGGTGCAACAACTG) (Promega, Madison, WI), forming plasmid pOK67. pOK68, which expresses a truncated version of TIM18 lacking the first 43 amino acids, was produced with the use of the purified protein (Sigma Chemical, Berkeley, CA). Antiserum to hexokinase (Davis, unpublished data) was raised to PiC by expressing the protein in bacteria from plasmid pNYHM131 as described (Wohlrab and Briggs, 1994). Inclusion bodies containing PiC were isolated (Harlow and Lane, 1988) and used to immunize rabbits (Covance, Denver, PA). Antibodies to the HA epitope (Niman et al., 1983) were isolated from ascites fluid prepared with the use of the 12CA5 cell line (BABCO, Berkeley, CA). Antiserum to hexokinase (Davis, unpublished data) was produced with the use of the purified protein (Sigma Chemical). Antiserum to Tim54p (Kerscher et al., 1997), Tim22p (Kerscher et al., 1997), Tim23p (Emtage and Jensen, 1993), Tim17p (Ryan et al., 1998), OM45p (Yaffe et al., 1989), and Cox4p (Jensen et al., 1992) have been described.

RESULTS
TIM18 Encodes a Multiple Copy Suppressor of the tim54–1 Mutant
Part of the evidence that Tim22p interacts with Tim54p came from our previous observation that multiple copies of the TIM22 gene suppressed the temperature-sensitive growth defect of a tim54–1 mutant (Kerscher et al., 1997). To identify additional components of the Tim54p-Tim22p import complex, we screened a yeast genomic library for new high-copy suppressors of tim54–1. We transformed tim54–1:URA3 trp1 leu2 strain 735, which contains tim54–1 on plasmid pOK24, with a genomic library carried in the 2µ-LEU2 vector Yep13 (Nasmyth and Tatchell, 1980). Plasmids carrying the 2µ origin of replication are present in 10–40 copies per cell, resulting in overexpression of genes carried on these plasmids (Armstrong et al., 1989). Approximately 5000 Leu+ transformants were isolated at 24°C and tested for their ability to grow at 34°C. We initially identified 36 colonies that grew at 34°C, but the temperature-resistant phenotype was shown to be plasmid dependent for only 7 transformants. PCR analysis showed that three of the plasmids carried TIM22 and one of the plasmids contained TIM54. The remaining three plasmids were partially sequenced. Two plasmids contained overlapping inserts of DNA from chromosome XV, and the tim54–1-suspressing activity was localized to an ~1.26-kbp EcoRI fragment containing a single...
Tim18p Is Required for Wild-Type Cell Growth

To examine the function of the Tim18 protein, we constructed a complete disruption of TIM18 coding sequences with HIS3. We identified transformants in which one of the two copies of TIM18 was replaced by the tim18::HIS3 disruption. His\(^+\) diploid cells were sporulated, and the meiotic progeny were allowed to grow at 24, 30, or 34°C on rich medium containing glucose as the carbon source (Figure 1A). When spores were grown at 34°C, all four meiotic progeny germinated and formed colonies of approximately equal size. In each tetrad, two of the colonies were His\(^+\) whereas the other two colonies were His\(^-\) and thus carried the wild-type TIM18 gene. At 30°C, tim18::HIS3 cells formed colonies that were His\(^+\) and two of the colonies were His\(^-\) and two were His\(^+\). The His\(^+\) colonies grew at 34°C. Multiple copies of either TIM18 or TIM22 suppressed the growth defect of the tim54-1 mutant. In contrast, TIM18 did not allow tim23-1 cells to grow at 34°C. Our results raise the possibility that Tim18p is part of the Tim54p-Tim22p pathway.

Although multiple copies of TIM18 allow the temperature-sensitive tim54-1 mutant to grow at 34°C, high levels of Tim18p do not completely bypass the requirement for Tim54p or Tim22p. As shown in Figure 1B, the 2\(\mu\)-TIM18 plasmid did not allow strains carrying a tim54::KAN or a tim22::TRP1 disruption to grow in the absence of TIM54 or TIM22, respectively.

Figure 1. (A) Multiple copies of TIM18 suppress the growth defect of tim54-1 mutants but not tim23-1 mutants. tim54-1 his3 strain 735 was transformed with one of the following high-copy plasmids: 2\(\mu\)-TIM18 plasmid pOK66, 2\(\mu\)-TIM54 plasmid pOK99, 2\(\mu\)-TIM22 plasmid pH203, or the empty vector pRS426 (Sikorski and Hieter, 1989). tim23-1 leu2 ura3 strain 574 was transformed with 2\(\mu\)-TIM18 plasmid pOK66, 2\(\mu\)-TIM23 plasmid pKR21 (Ryan et al., 1998), 2\(\mu\)-TIM17 plasmid pKR7 (Ryan et al., 1994), or the empty vector pRS426 (Sikorski and Hieter, 1989). Transformants were streaked onto YEPD medium containing 5-FOA to select for loss of the TIM22::URA3 or TIM54::URA3 plasmids.

ORF, YOR297c (Poirey et al., 1997). As described below, we found that the protein encoded by YOR297c is located in the mitochondrial IM and is part of the Tim54p-Tim22p complex. YOR297c encodes a 21.9-kDa protein that is processed to an ~18-kDa mature form after its import into mitochondria (see below). We have named the gene TIM18 and the protein Tim18p, consistent with the nomenclature for mitochondrial import components (Pfanner et al., 1996).

To determine if the suppression activity of TIM18 is limited to tim54-1, we examined the consequence of multiple copies of TIM18 on the tim23-1 mutant. The tim23-1 mutant was transformed with 2\(\mu\) plasmids containing TIM18, TIM23, TIM17, or an empty vector, and we compared the results with those of tim54-1 cells transformed with 2\(\mu\) plasmids carrying TIM18, TIM54, TIM22, or an empty vector (Figure 1A). In the tim54-1 mutant, all transformants grew at 24°C, but only cells that contained TIM54, TIM22, or TIM18 grew at 34°C. Multiple copies of either TIM18 or TIM22 suppressed the growth defect of the tim54-1 mutant. In contrast, TIM18 did not allow tim23-1 cells to grow at 34°C. Only TIM17- or TIM23-containing plasmids rescued the growth defect of tim23-1. Therefore, we found that there is a specific genetic interaction between TIM18 and tim54-1 similar to that seen with TIM22. Our results raise the possibility that Tim18p is part of the Tim54p-Tim22p pathway.

Figure 1 (cont).
but grew slower than wild-type cells. Our results indicate that TIM18 is not an essential gene. We found that tim18::HIS3 mutants are cold sensitive for growth. The tim18::HIS3 disruption in diploid strain 605 was sporulated, and meiotic progeny were allowed to grow on YEP medium containing 2% glucose for 5 d at 34, 30, or 24°C. Lanes 1–9 show different tetrads, and individual spores are labeled A–D. (B) Multiple copies of TIM22 suppress the growth defect of tim18::HIS3. tim18::HIS3 strain 992 (tim18Δ) was transformed with the following high-copy plasmids: 2µ-TIM22 plasmid pH1202 (Kerscher et al., 1997), 2µ-TIM54 plasmid pOK31 (Kerscher et al., 1997), 2µ-TIM18 plasmid pOK94, or the empty vector pRS426. Wild-type TIM18 ura3 strain 993 was transformed with the empty vector pRS426. Cells were grown at 34°C in YEPD, diluted in 10-fold steps, spotted onto YEPD plates at 24°C, and incubated for 5 d.

in their growth after about eight divisions. The microcolonies formed from these spores did not undergo additional growth even after prolonged incubation. We found that the cold-sensitive growth defect was limited to glucose-containing medium. As shown in Figure 2B, tim18::HIS3 cells pregrown at 34°C and then spotted onto glucose-containing medium displayed a clear growth defect at 24°C compared with wild-type cells. At 30°C, the growth defect of tim18::HIS3 was detectable, but it was less than at 24°C. By comparison, tim18::HIS3 cells grown on medium containing raffinose or glycerol/ethanol medium grew at rates indistinguishable from those of wild-type cells at all temperatures tested.

tim18::HIS3 disruptions in a second strain background displayed a more extreme growth defect than those in the 605 background (not shown). Haploid tim18::HIS3 cells derived from strain 1082 were inviable on glucose medium at
18, 24, 30, and 34°C. \textit{tim18::HIS3} cells were able to grow on glycerol or raffinose medium, although they grew at reduced rates compared with wild-type cells. The reason that \textit{tim18::HIS3} disruptions show a strain-specific phenotype is not known. However, disruptions of other yeast genes, such as \textit{CHC1} (Munn et al., 1991) and \textit{HRD2} (Yokota et al., 1996), often yield distinct results in different strain backgrounds. We found that \textit{tim18::HIS3} cells cannot tolerate the loss of mitochondrial DNA (Figure 2C). \textit{tim18::HIS3} and \textit{TIM18} cells were grown for 3 days at 30°C on medium containing 25 μg/ml ethidium bromide (YEPD+EtBr) to induce loss of mitochondrial DNA (Goldring et al., 1970) and then tested for growth on different carbon sources. \textit{TIM18} cells were able to grow on glucose medium (YEPD) after ethidium bromide treatment but were unable to grow on nonfermentable medium (YEPgly/eth) as a result of loss of mitochondrial DNA. In contrast, \textit{tim18::HIS3} cells pregrown on ethidium bromide failed to grow on either YEPD or YEPgly/eth medium. We conclude that \textit{tim18::HIS3} cells that have lost mitochondrial DNA are inviable.

\textit{tim18::HIS3} Is Lethal in Combination with \textit{tim54-1}

Because multiple copies of \textit{TIM18} suppress the temperature-sensitive growth defect of the \textit{tim54-1} mutant, \textit{Tim18p}, like the \textit{Tim54} protein, may play a role in import. Supporting this idea, we found that \textit{tim18::HIS3 tim54-1} double mutants were inviable, exhibiting a synthetic-lethal phenotype. \textit{tim18::HIS3/tim54-1} diploid strain 1009 was sporulated, and the meiotic progeny were allowed to grow at 24°C on medium containing glycerol and ethanol (Figure 3A). In 20 tetrads (9 are shown in Figure 3A), we failed to recover any progeny carrying both \textit{tim54-1} and \textit{tim18::HIS3}. Spores inferred to be double mutants germinated but arrested their growth after a few divisions. The result that the combination of \textit{tim18::HIS3} and \textit{tim54-1} is lethal provides additional genetic evidence for an interplay between \textit{Tim18p} and \textit{Tim54p}. \textit{tim18::HIS3} did not exhibit synthetic lethality with \textit{tim23-1} (Kerscher, unpublished observations), indicating that the interaction of \textit{Tim18p} and \textit{Tim54p} is specific.

\textbf{Multiple Copies of TIM22 Suppress \textit{tim18::HIS3}}

We previously showed that the \textit{tim54-1} mutant was suppressed by high levels of \textit{TIM22} expression (Kerscher et al., 1997). We found a similar interaction between \textit{tim18::HIS3} and \textit{TIM22}. We transformed \textit{tim18::HIS3} cells with 2μg/ml \textit{TIM18} plasmid pOK66, \textit{TIM22} plasmid pOK1030, or the empty vector pRS423. Transformants were streaked onto YEP medium containing 2% glycerol and ethanol and incubated at 24°C for 5 d or at 34°C for 3 d.
the function of Tim18p can be bypassed by increased levels of Tim22p and suggest that Tim18p, like Tim22p, is part of the protein import pathway.

**Tim18p Is Homologous to Two Other Yeast Proteins**

The predicted protein from the TIM18 sequence (yeast ORF YOR297c) is a protein of 21.9 kDa (Figure 4A). Hydropathy analysis suggested that Tim18p is a membrane protein with three potential membrane-spanning segments (Figure 4A, underlined amino acids). In addition, the amino terminus of Tim18p has many of the characteristics of a mitochondrial presequence (Roise and Schatz, 1988; Claros and Vincens, 1996). The first 44 amino acids of Tim18p contain five basic residues, no acidic amino acids, numerous polar residues, and no long stretches of hydrophobic amino acids. When plotted on a helical wheel (Claros and Vincens, 1996), the basic residues of the amino terminus cluster to one side of the helix. Tim18p is predicted to contain a matrix processing protease site at amino acid position 44/45 (Claros and Vincens, 1996).

The Tim18 protein is homologous to two other proteins in the yeast genome. One of these proteins, Sdh4p, is 39.5% identical to Tim18p and has been reported to encode a membrane anchor for the mitochondrial IM succinate dehydrogenase complex (Bullis and Lemire, 1994). The other protein is 36.4% identical to Tim18p and is an uncharacterized ORF (YLR164w). Both SDH4p and Ylr164p are 52.6% identical to each other. The two Tim18p homologues, however, do not suppress the tim54-1 mutant (Figure 4B).

**Figure 5.** TIM18 encodes a mitochondrial inner membrane protein with its carboxyl terminus facing the mitochondrial intermembrane space. (A) Tim18p is a mitochondrial protein. tim18::HIS3 strain 992 containing plasmid pOK1032, which expresses the Tim18p-HA fusion protein, was grown in YEP medium containing 2% glycerol and ethanol. Cells were homogenized (H) and separated into a mitochondrial pellet fraction (M) and crude cytosol (C) by centrifugation. Aliquots of homogenate, mitochondria, and cytosol representing cell equivalents were subjected to SDS-PAGE. Immune blots were decorated with antibodies to the HA epitope (Tim18p-HA), antiserum to the β-subunit of the F1-ATPase (F1β), or antiserum to hexokinase (Hex) and detected by chemiluminescence. (B) Tim18p is imported into mitochondria and processed to a mature form. Mitochondria were isolated from wild-type strain D273-10b and incubated for 30 min at 30°C with the 35S-labeled Tim18p protein. After all manipulations, mitochondria were reisolated by centrifugation and analyzed by SDS-PAGE and phosphorimaging. Lane 1, 20% of the Tim18p precursor added to each import reaction. Lane 2, translation product of Tim18p(44–192), a truncated version of Tim18p lacking its first 43 amino acids. Lane 3, import in the presence of 25 μM CCCP and 0.5 μM valinomycin (∆Ψ). Lane 4, import. Lane 5, import followed by digestion with 100 μg/ml trypsin for 30 min. The precursor (p) and mature (m) forms of Tim18p are indicated. (C) Tim18p-HA is an integral membrane protein. Tim18p-HA mitochondria were treated with either 0.1 M sodium carbonate or 1.5 M sodium chloride or left untreated (BB) and then separated into supernatant (S) and pellet (P) fractions by centrifugation. After SDS-PAGE, proteins were analyzed by immune blotting with antibodies to the HA epitope (Tim18p-HA), F1β, a peripheral membrane protein, and PiC, an integral membrane protein. (D) Tim18p-HA is located in the mitochondrial IM. Tim18p-HA mitochondria were sonicated, and membrane vesicles were loaded on sucrose step gradients. After centrifugation, fractions were collected and analyzed by immune blotting with antibodies to the HA epitope (Tim18p-HA), the IM protein F1β, or the OM protein OM45. Fracton 1 represents the top of the gradient. (E) The carboxyl terminus of Tim18p-HA faces the IMS. Tim18p-HA mitochondria were digested with 50 μg/ml proteinase K (PK) for 30 min on ice and analyzed by immune blotting with antibodies to the HA epitope (Tim18p-HA), F1β, Tim23p, or the OM protein Tom70p. To expose proteins located in the IMS, the mitochondrial OM was ruptured by osmotic shock (OS) and then treated with protease.
transformed into the tim54-1 mutant, and tested for their ability to rescue the growth defect of tim54-1 at 34°C. Multiple copies of SDH4 or YLR164w did not allow the tim54-1 strain to grow at the nonpermissive temperature. Only cells that expressed high levels of Tim18p, or a Tim18p-HA fusion protein (see below), grew at 34°C.

Tim18p Is Located in the Mitochondrial IM with Its Carboxyl Terminus Facing the Intermembrane Space

To identify the function of Tim18p, we determined the location of Tim18p in the cell. First, we inserted a HA epitope
tag at the carboxyl terminus of Tim18p, forming Tim18p-HA. We found that Tim18p-HA is functional, because the growth defect of the tim18::HIS3 disruption is rescued by Tim18p-HA (Kerscher, unpublished results) and multiple copies of TIM18-HA suppress the growth defect of the tim54-1 mutant (Figure 4B). Cells expressing Tim18p-HA were homogenized and separated into a mitochondrial fraction and a postmitochondrial supernatant (Figure 5A). We found by immune blotting that Tim18p-HA cofractionated with the β-subunit of the F1–ATPase (F1β), a mitochondrial protein. No Tim18p-HA was found in the supernatant along with the cytosolic hexokinase protein.

Supporting our localization data, we found that Tim18p was imported into isolated mitochondria. Tim18p synthesized in vitro yielded an ~22-kDa 35S-labeled protein after SDS-PAGE (Figure 5B, lane 1). When incubated with energized mitochondria, Tim18p was processed to an ~18-kDa protein (Figure 5B, lane 4) that was protected from externally added protease (Figure 5B, lane 5). When the potential across the mitochondrial IM was dissipated with CCCP, import and processing of Tim18p were inhibited (Figure 5B, lane 3). Tim18p is predicted to contain a mitochondrial processing protease site at amino acid position 44/45 (Claros and Vincens, 1996). To test this possibility, we constructed a truncated version of Tim18p, Tim18p(44–192), lacking its first 43 amino acids. In vitro synthesized Tim18p(44–192) comigrates with the imported and processed form of Tim18p (Figure 5B, compare lanes 2 and 5). Our results thus indicate that Tim18p is a mitochondrial protein with an amino-terminal cleavable presequence.

Tim18p is located in the mitochondrial IM. First, mitochondria were isolated from cells expressing Tim18p-HA and treated with either sodium carbonate or sodium chloride (Figure 5C). Tim18p-HA and the IM-localized PiC protein (Phelps et al., 1991) remained in the membrane pellet after carbonate or salt treatment. In contrast, F1β, a peripheral membrane protein (Chen and Douglas, 1987), was found in the supernatant after carbonate treatment. To determine in which of the two mitochondrial membranes Tim18p resides, we prepared membrane vesicles from Tim18p-HA mitochondria and separated them into OM and IM fractions on sucrose gradients. As shown in Figure 5D, Tim18p-HA cofractionates with the IM vesicle fraction, along with the F1β protein. OM45p, a marker for the OM (Yaffe et al., 1989), migrates near the top of the gradient in fractions separate from Tim18p-HA and F1β.

The carboxyl terminus of Tim18p faces the intermembrane space (IMS). Tim18p-HA mitochondria were treated with protease either in the presence or in the absence of an intact OM (Figure 5E). Immune blots showed that the IM proteins Tim18p-HA, F1β, and Tim23p were all resistant to digestion in intact mitochondria. In contrast, Tom70p, an OM protein that faces the cytosol is removed by protease treatment. When the mitochondrial OM was disrupted by osmotic shock, Tom70p, Tim23p, and Tim18p-HA were all digested, and only F1β, which faces the matrix, was protected from the protease. Our antibodies to Tim23p recognize a domain that faces the IMS, which is accessible to digestion in mitochondria (Ryan et al., 1998). Therefore, we conclude that the carboxyl-terminal HA tag on Tim18p-HA is located in the IMS.

**Tim18p Is Part of the Tim54p-Tim22p Complex in the IM**

The genetic experiments described above suggest that Tim18p physically interacts with Tim54p and Tim22p. To test this idea directly, we asked if Tim54p or Tim22p could be immune precipitated along with Tim18p. Tim18p-HA mitochondria were solubilized with digitonin-containing buffer, and Tim18p-HA was precipitated with antibodies to the HA epitope (Figure 6A). Using antibodies to the HA epitope to decorate immune blots, we found that the majority of Tim18p-HA was located in the pellet. Only a small amount of the Tim18p-HA was not precipitated by the HA antibodies and remained in the supernatant. When we examined our fractions with antibodies to Tim22p, we found that virtually all of the Tim22 protein coprecipitated with Tim18p-HA, indicating that Tim22p physically associates with the Tim18p protein in mitochondria. Using antibodies to Tim54p, we found that part, but not all, of Tim54p interacts with Tim18p. Only about half of Tim54p appeared to interact tightly with Tim18p. This result was similar to the results of previous experiments, in which we found that half of total Tim54p associated with Tim22p (Kerscher et al., 1997). We also found that the Tim23p or Tim17p proteins did not coprecipitate with Tim18p-HA, indicating that Tim18p’s interaction with Tim22p and Tim54p is specific (Figure 6A). In addition, an abundant IM protein not involved in import, PIC, did not associate with Tim18p-HA.

Supporting our observations that Tim18p is a member of the Tim54p-Tim22p complex, we found that all three proteins were present in an ~300-kDa complex after blue native electrophoresis (Figure 6B). Mitochondria isolated from cells expressing Tim18p-HA, Tim54p-HA, or Tim22p-HA were solubilized with digitonin-containing buffer, and the membrane protein complexes were separated on blue native polyacrylamide gradient gels. The mobility of the different proteins was monitored by immune blotting with antibodies to the HA epitope. Tim18p-HA comigrated with Tim54p-HA and Tim22p-HA, both of which were previously shown to be part of a 300-kDa complex (Koehler et al., 1996b; Kurz et al., 1999). Tim18p was not found in an ~90-kDa complex containing Tim23p and Tim17p (Dekker et al., 1997). Tim54p, Tim22p, and Tim18p thus appear to be part of the same translocon.

Also supporting our conclusion that Tim18p associates with Tim54p and Tim22p, we found that the mobility of the Tim54p-Tim22p complex was altered in mitochondria lacking Tim18p (Figure 6C). Mitochondria isolated from wild-type (WT) and tim18::HIS3 (Δ) cells were subjected to blue native electrophoresis. In tim18::HIS3 mitochondria, we found that Tim54p and a Tim54p-HA fusion protein migrated in a complex of ~200 kDa, in contrast to the ~300-kDa complex seen with wild-type mitochondria. Other IM complexes, such as the Tim23p-Tim17p translocon or the PIC dimer, were not affected by the absence of Tim18p. To examine the distribution of Tim22p, mitochondria from wild-type or tim18::HIS3 cells were first subjected to blue native electrophoresis, and the lane was then cut out and analyzed by SDS-PAGE. Immune blots showed that Tim22p was located in an ~300-kDa complex in wild-type mitochondria and in an ~200-kDa complex in mitochondria lacking Tim18p. In contrast, the OM protein, Tom40p, was located in
an ~400-kDa complex (Dekker et al., 1997) in both wild-type and tim18::HIS3 mitochondria.

Several proteins of the intermembrane space, including Tim12p, Tim10p, and Tim9p, interact with the Tim54p-Tim22p complex (Koehler et al., 1998a,b; Sirrenberg et al., 1998; Adam et al., 1999). All of Tim12p is part of the TIM complex, whereas only a fraction of Tim10p and Tim9p associate with Tim54p and Tim22p. We found that the interaction of Tim12p with the TIM complex does not require Tim18p. As shown in Figure 6D, Tim12p is located in an ~300-kDa complex containing Tim54p and Tim22p in wild-type mitochondria and in an ~200-kDa complex in tim18::HIS3 mitochondria.

tim18::HIS3 Cells Are Cold Sensitive for the Import or Stability of Several IM Proteins

Because Tim18p is part of the Tim54p-Tim22p complex, it is possible that Tim18p also plays a role in the import of proteins into mitochondria. Tim54p and Tim22p are required for the insertion of a number of polytopic proteins into the IM, including PiC and the Tim22 protein (Sirrenberg et al., 1996; Kerscher et al., 1997). However, we found that mitochondria isolated from tim18::HIS3 cells were not defective in the import of several proteins, including PiC and Cox4p (Kerscher, unpublished observations). To determine if Tim18p plays a more subtle role in import, we took advantage of the cold-sensitive growth defect of tim18::HIS3 cells grown on glucose-containing medium. We grew wild-type and tim18::HIS3 strains in YEPD medium at 34°C, at which tim18::HIS3 cells grow at wild-type rates, and then shifted the cells to 24°C, at which tim18::HIS3 cells grow very slowly. For comparison, we also grew TIM18 and tim18::HIS3 cells at 30 and 34°C. Total protein was then isolated from these cells, and the level of different mitochondrial proteins was determined by immune blotting. As shown in Figure 7, tim18::HIS3 cells grown at 34°C contained similar amounts of all mitochondrial IM proteins examined, including PiC, Tim22, subunit IV of cytochrome oxidase (Cox4p), Tim54p, Tim23p, and the α-subunit of the F1-ATPase (Flα). In contrast, tim18::HIS3 cells grown at 24°C contained greatly reduced levels of PiC, Tim22p, and Cox4p compared with wild-type cells. At 30°C, the levels of PiC, Tim22, and Cox4p in tim18::HIS3 cells were reduced, but the amounts were higher than in tim18::HIS3 cells grown at 24°C. Cells lacking Tim18p appear to be cold sensitive for the import or stability of at least several IM proteins. Whether this defect is a direct consequence of the lack of Tim18p or an indirect effect (e.g., caused by the reduced amount of Tim22p) requires further study. Our results also indicate that Tim18p may not be required for import of all IM proteins, because we found wild-type levels of Tim54p, Tim23p, and Flα in tim18::HIS3 cells grown at 24, 30, or 34°C.

DISCUSSION

We have identified a new mitochondrial IM protein named Tim18p. Several observations suggest that Tim18p, like Tim54p and Tim22p, plays a role in the import of proteins into mitochondria. For example, we identified TIM18 as a high-copy suppressor of the temperature-sensitive tim54-1 mutant. We previously showed that TIM22, when present in multiple copies, likewise rescues the growth defect of tim54-1 cells (Kerscher et al., 1997). Tim18p and Tim22p, therefore, both show a similar genetic interplay with Tim54p. We also found that the tim18::HIS3 disruption is lethal in combination with the tim54-1 mutation. Moreover, we showed that the cold-sensitive growth defect of the tim18::HIS3 disruption is suppressed by multiple copies of TIM22. Our results thus provide genetic evidence that Tim18p, Tim22p, and Tim54p interact and suggest that these three proteins function at a common step in the import pathway.

We also found evidence for physical interaction between Tim18p, Tim54p, and Tim22p. These proteins form a complex together, because all three proteins coimmunoprecipitate from mitochondrial extracts, and they comigrated as an ~300-kDa complex during blue native electrophoresis. The 300-kDa complex is separate from an ~90-kDa complex containing Tim23p and Tim17p and from the ~400-kDa TOM complex in the OM (Dekker et al., 1997). Interestingly, the integrity of the 300-kDa complex requires the Tim18 protein. Blue native electrophoresis of mitochondria isolated from tim18::HIS3 cells showed that Tim54p and Tim22p migrate together as a smaller complex of ~200 kDa, whereas other IM complexes, such as the Tim23p-Tim17p translocase, were not affected. Whether the 100-kDa decrease in size of the translocase represents the loss of multiple Tim18p subunits or the loss of other proteins dependent on Tim18p for assembly awaits further study. Regardless, our results indicate that Tim18p is a new member of the Tim54p-Tim22p translocase.

Tim18p is homologous to two proteins in yeast, Sdh4p (39.5% identical) and the uncharacterized ORF YLR164w.
(36.4% identical). Although both Sdh4p (Bullis and Lemire, 1994) and the YLR164w protein (Kerscher, unpublished data) are mitochondrial IM proteins, neither homologue appears to be part of the Tim54p-Tim22p-Tim18p complex. Multiple copies of SDH4 or YLR164w did not suppress the \textit{tim54-1} mutant. Furthermore, in preliminary experiments, we observed that the YLR164w and Sdh4p proteins do not immune precipitate along with Tim18p (Kerscher, unpublished data). Although the role of YLR164w is unknown, Sdh4p is proposed to function as the membrane anchor for other members of the succinate dehydrogenase complex (Bullis and Lemire, 1994). It is possible, therefore, that Tim18p plays an anchor function in the TIM complex. Arguing against this possibility, we found that Tim18p is not required for the interaction of Tim12p with Tim54p and Tim22p. Whether Tim10p, Tim9p, or other import proteins use Tim18p for their association with Tim54p-Tim22p awaits further studies.

\textit{TIM18} encodes a 21.9-kDa protein with a cleavable presequence. After its import into mitochondria, Tim18p was processed to an \textsim 18-kDa mature form. Tim18p is predicted to carry a cleavage site for the mitochondrial processing protease, MPP, between residues 44 and 45 (Claros and Vencins, 1996). Hydrophy analysis suggests that Tim18p contains three transmembrane segments, and Tim18p was located in the IM after mitochondrial fractionation. A HA tag inserted at the carboxyl terminus of Tim18p was digested when mitoplasts were treated with protease. We conclude that the mature form of Tim18p is inserted in the mitochondrial IM with three transmembrane segments, with its amino terminus facing the matrix and its carboxyl terminus exposed to the intermembrane space.

The genetic interactions between \textit{TIM18}, \textit{TIM22}, and \textit{TIM54} and our evidence for physical interaction suggest that Tim18p, like Tim22p and Tim54p, functions in the insertion of polytopic proteins into the IM. Consistent with such a role, we found that \textit{tim18} mutants contain lower steady-state amounts of several IM proteins. Nevertheless, Tim18p is not an essential protein and thus differs from Tim54p and Tim22p. Disruptions of \textit{TIM54} and \textit{TIM22} are inviable under all growth conditions (Sirrenberg et al., 1996; Kerscher et al., 1997), whereas \textit{tim18::HIS3} cells are inviable under special assay conditions (Sirrenberg et al., 1996; Kerscher et al., 1997). Cox4p, however, is a subunit of a complex in the IM, and its assembly requires chaperone activity, including that of the AAA complexes (Arlt et al., 1998). Mutants defective in chaperone activity of Yta10p and Yta12p, subunits of the m-AAA complex, have reduced levels of Cox4p similar to that seen in \textit{tim18::HIS3} cells. In addition, disruptions of \textit{YME1} (Thorsness et al., 1993; Weber et al., 1996), a subunit of the i-AAA complex, gives rise to a cold-sensitive growth defect on glucose-containing medium similar to \textit{tim18::HIS3}. Furthermore, both \textit{tim18::HIS3} and \textit{yme1} mutants cannot tolerate the loss of mitochondrial DNA (Thorsness et al., 1993). We found in preliminary studies that the import, stability, or assembly of AAA complex subunits is not defective in \textit{tim18::HIS3} mutants (Kerscher, unpublished data). The interesting possibility that Tim18p itself is a chaperone or mediates an interaction with chaperones, such as the i-AAA or m-AAA complex, awaits future studies.

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REFERENCES


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Lithgow, T., Junne, T., Wachter, C., and Schatz, G. (1994). Yeast mitochondria lacking the two import receptors Mas20p and Mas70p
can efficiently and specifically import precursor proteins. J. Biol. Chem. 269, 15325–15330.


