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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 coimmune precipitated 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 translocases 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-subunit processing protease called MPP (McAda and Douglas, 1982; Yaffe *et al.*, 1985; Jensen and Yaffe, 1988; Pollock *et al.*, 1988; Witte *et al.*, 1988; Yang *et al.*, 1988). Some proteins destined for the mitochondrial inner membrane (IM) carry a cleavable presequence followed by one or more hydrophobic membrane-spanning segments (Stuart and Neupert, 1996). The transmembrane segments are proposed to either function as stop-transfer sequences in the IM (Miller and Cumsky, 1991, 1993) or facilitate the insertion of the polypeptide into the IM after its complete import into the matrix (Mahlke *et al.*, 1990; Herrmann *et al.*, 1997).

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Mitochondrial precursor proteins bind to one of several receptors on the mitochondrial surface (Hase *et al.*, 1983; Hines *et al.*, 1990; Steger *et al.*, 1990; Moczko *et al.*, 1993; Ramage *et al.*, 1993; Gratzer *et al.*, 1995; Hönlinger *et al.*, 1995; Lithgow *et al.*, 1995; Moczko *et al.*, 1997). Precursors are then translocated across the outer membrane (OM) via the TOM complex, which includes Tom40p, Tom22p, Tom7p, Tom6p, and Tom5p (Alconada *et al.*, 1995; Honlinger *et al.*, 1996; Dietmeier *et al.*, 1997; Hill *et al.*, 1998). The mitochondrial IM appears to have at least two separate import machines. One multiple subunit complex, the Tim23p-Tim17p translocon, is required to translocate proteins across the IM into the matrix (Emtage and Jensen, 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 translocase 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-

berg *et al.*, 1996; Kerscher *et al.*, 1997; Davis *et al.*, 1998; Koehler *et al.*, 1998a; Endres *et al.*, 1999). Tim54p and Tim22p are integral membrane proteins that associate with several proteins in the intermembrane space, including Tim12p, Tim10p, and Tim9p (Koehler *et al.*, 1998b; Sirrenberg *et al.*, 1998; Adam *et al.*, 1999). Tim12p, Tim10p, and Tim9p are homologous proteins and are thought to play a role in shuttling imported proteins from the TOM complex in the OM to the Tim54p-Tim22p complex in the IM (Koehler *et al.*, 1998a; Sirrenberg *et al.*, 1998; Adam *et al.*, 1999). Recently, two new members of the Tim12p-Tim10p-Tim9p family have been identified, Tim13p and Tim8p, but their role in import is not clear (Koehler *et al.*, 1999).

To identify additional members of the Tim54p-Tim22p insertion complex, we isolated genes that, when present in multiple copies, suppress the temperature-sensitive growth defect of the *tim54-1* mutant. We identified a new gene, called *TIM18*, which encodes an integral protein residing in the mitochondrial IM. Both genetic and biochemical experiments suggest that Tim18p is a member of the Tim54p-Tim22p complex.

MATERIALS AND METHODS

Strains and Relevant Genotypes

MATα tim54::URA3 trp1Δ63 leu2Δ1 ura3-52 strain 735 contains *tim54-1* on *TRP1-CEN6* plasmid pOK24 (Kerscher *et al.*, 1997). *MATa tim23-1 ura3 trp1 his3 leu2* strain 574 (Ryan *et al.*, 1998) and *MATα tim54-1 trp1Δ63 leu2Δ1 ura3-52 his3-Δ200* strain 809 (Kerscher *et al.*, 1997) have been described. *MATa/MATα trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 ura3-52/ura3-52* strain 605 was constructed by crossing strain FY833 to strain FY834 (Winston *et al.*, 1995). *MATa/MATα trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 ura3Δ0/ura3Δ0* strain 1082 was constructed by crossing strain BY4733 to strain BY4734 (Brachmann *et al.*, 1998). *tim54-1/tim18::HIS3* diploid strain 1009 was constructed by crossing *tim54-1* strain 809 to *tim18::HIS3* strain 994 (see below). Standard yeast genetic techniques and media were used (Rose *et al.*, 1988).

Isolation of TIM18

Yeast strain 735 contains the *tim54::URA3* disruption and pOK24, which carries *tim54-1* on a *TRP1-CEN6*–containing plasmid (Kerscher *et al.*, 1997). Strain 735 was transformed with a library containing genomic DNA fragments in the *2μ-LEU2* plasmid YEp13 (Nasmyth and Tatchell, 1980). Five thousand Leu$^+$ transformants were selected at 24°C and then replica plated to YEP medium containing 2% glycerol and 2% ethanol as the sole carbon source. Thirty-one transformants that grew at 34°C were isolated, and the *LEU2*-containing plasmids were isolated. Seven of the 31 plasmids were able to rescue the *tim54-1* defect upon retransformation. PCR analysis showed that four of the plasmids contained either *TIM22* (three isolates) or *TIM54* (one isolate). The remaining three plasmids were partially sequenced, and two plasmids contained overlapping inserts of DNA from chromosome XV. Plasmid pOK94 carries the *tim54-1*–suppressing activity on an ~6-kilobase pair (kbp) genomic DNA fragment from chromosome XV. Subcloning experiments localized the *tim54-1*–suppressing activity to an ~1.26-kbp *Eco*RI fragment containing a single ORF, YOR297c. Plasmid pOK66, which carries the YOR297 *Eco*RI fragment inserted into plasmid pRS423 (Sikorski and Hieter, 1989), suppressed the *tim54-1* temperature-sensitive phenotype. One plasmid contained sequences from chromosome VII and has not been characterized.

Plasmid Constructions

2μ-HIS3 plasmid pOK59 carries *TIM54* on a 3-kbp *Cla*I fragment inserted in pRS423 (Sikorski and Hieter, 1989) and was constructed by homologous recombination in yeast (Oldenburg *et al.*, 1997) between a *Pvu*II fragment derived from pOK31 (Kerscher *et al.*, 1997) and *Xho*I-*Eco*RI–digested pRS423. Plasmid pJH203 (Holder, unpublished data) was constructed by insertion of an *Eco*RI 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′-GGAATTCCCTGATTCGCACCTTC-3′) and 235 (5′-GGAATTCGTCACCACTAACCAAAC-3′) and PCR (Saiki *et al.*, 1985). After *Eco*RI digestion, the PCR product was inserted into pRS426 (Sikorski and Hieter, 1989), forming pOK90. *2μ-SDH4* plasmid pOK91 was constructed by isolating a *Pvu*I 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 *Xho*I-*Bam*HI–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 (TCTTAGAAATGCAAAAAAAAAGAAAAAGTATGGGTGAGTCAGATTGTACTGAGAGTGCAC) and 222 (ATGCGAGGTGCAACAACTGAGTAATTTAATACCTTTGGTACTGTGCGGTATTTCACACCG) and plasmid pRS303 (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/his3* diploid strains 605 and 1082. His$^+$ diploid transformants were isolated and shown by PCR analysis to have one of two copies of *TIM18* disrupted (*tim18::HIS3*). *MATα tim18::HIS3* strain 992, *MATa tim18::HIS3* strain 994, *MATα TIM18* strain 993, and *MATa TIM18* strain 995 are the meiotic products from *tim18::HIS3/TIM18* diploid strain 987 (strain 605 background).

tim54::KAN strain 1078, which lacks the *TIM54* ORF, was constructed by PCR-mediated gene replacement into diploid strain 605 with the use of oligonucleotides 382 (GCTTTAAAGTCCATTGTTCTCAAAAGAAGCTCAATAGACCAGATTGTACTGAGTGCAC) and 383 (CGTCGATCGTGCATGATGATAAAACATAATATATATCCAACTGTGCGGTATTTCACACCG) and *kanMX4* plasmid pRS400 (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 *Not*I 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 (GGAATTCGTGTTAATG) and 227 (ATAGTTTAGCGGCCGCCGTTTCTTCCAAATATATAC), digested with *Eco*RI and *Not*I, and inserted into Bluescript SK II$^+$ (Stratagene, La Jolla, CA). A 114-bp *Not*I 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 *Aat*II and *Sac*I, and an *Aat*II-*Sac*I fragment from pOK48 (Kerscher *et al.*, 1997) containing the 3′-untranslated region of *TIM23* was inserted to yield pOK74. *TIM18-HA* was isolated from pOK74 as a 2.2-kbp *Pvu*II fragment and inserted into the *Hin*dIII-*Xho*I–digested *LEU2-CEN6* plasmid pRS315 (Sikorski and Hieter, 1989) by homologous recombination in yeast (Oldenburg *et al.*, 1997), forming pOK1032. pOK1032 was transformed into *MATα tim18::HIS3* strain 992 (making strain 1032). *2μ-TIM18-HA* plasmid pOK1030 was constructed as described

for pOK1032 except that a 2.2-kbp *Pvu*II fragment containing *TIM18-HA* from pOK74 was inserted into the *LEU2-2μ* plasmid pRS425 (Sikorski and Hieter, 1989).

Subcellular and Submitochondrial Fractionation

Yeast cells were grown to OD_{600} 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 Na_2CO_3, pH 11, or 1 M NaCl and centrifuged for 30 min at ~30 pounds per square inch in a Beckmann (Fullerton, CA) Airfuge. For analysis, proteins were separated on SDS-polyacrylamide gels (Laemmli, 1970; Haid and Suissa, 1983) and transferred (Haid and Suissa, 1983) to Immobilon P membranes (Millipore, Bedford, MA). Membranes were decorated with antibodies (Haid and Suissa, 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 (GAATTCCATGGGGAATCTGACTC) and 220 (CCGCTCGAGAGGTGCAACAACTGAG). After digestion with *Xho*I and *Eco*RI, the *TIM18* PCR fragment was inserted into *Sal*I-*Eco*RI–digested pSP64 (Promega, Madison, WI), forming plasmid pOK67. pOK68, which expresses a truncated version of *TIM18* lacking the first 43 amino acids, *TIM18*(44-192), was constructed as described above except that oligonucleotide 223 (CCGCTCGAGATGGCTGAAAATAAAATAAAC) was substituted for primer 220. Labeled Tim18p and Tim18p(44–192) proteins were synthesized with the use of 1.5 mCi/ml [^{35}S]methionine (Amersham, Arlington Heights, IL) in the SP6-Quick system (Promega) according to the manufacturer's instructions. Mitochondria for in vitro imports were isolated from strain D273-10b (Sherman, 1964) as described (Daum *et al.*, 1982) except that SEH buffer (250 mM sucrose, 1 mM EDTA, 20 mM HEPES-KOH, pH 7.4) was sometimes used in place of breaking buffer. Fifteen microliters of reticulocyte lysate containing precursor proteins was added to 80 μg of mitochondria (1 mg/ml) in import buffer (Scherer *et al.*, 1992) in each import reaction. Imports were stopped by incubation on ice and by the addition of valinomycin (Sigma Chemical) to 0.5 μM and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma Chemical) to 25 μM. To remove nonimported proteins, mitochondria were digested with 100 μg/ml trypsin (Sigma Chemical) for 30 min on ice, followed by the addition of a fivefold molar excess of soybean trypsin inhibitor (Sigma Chemical). After SDS-PAGE, labeled proteins were visualized by autoradiography (Bonner and Laskey, 1974) or exposed to a Molecular Dynamics (Sunnyvale, CA) Phosphor screen overnight, scanned with a Molecular Dynamics Storm 860 PhosphorImager, and analyzed with ImageQuant Software (Molecular Dynamics).

Immune Precipitations and Blue Native Electrophoresis

For immune precipitations, mitochondria were isolated from *tim18::HIS3* strain 992 that expresses Tim18p-HA from plasmid 1032. Mitochondria were solubilized in 0.5% digitonin, 50 mM NaCl, 30 mM HEPES-KOH, pH 7.4, 1 mM PMSF, 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride (Calbiochem-Novabiochem, La Jolla, CA)], 1 μg/ml leupeptin (Calbiochem-Novabiochem), and 1 μg/ml aprotinin (Sigma Chemical) at a protein concentration of 1 mg/ml. Unsolubilized material was removed from detergent lysates by centrifugation at 12,500 × *g* for 10 min. To 500 μl of lysate, 200 μl of a 1:1 slurry of agarose beads coupled to antibodies directed to the HA epitope was added. After rocking for 4 h at 4°C, immune complexes were recovered by centrifugation at 1300 × *g* for 1 min and washed. Equal amounts of pellets and supernatants were separated by SDS-PAGE and analyzed by immune blotting. HA antibodies were coupled to agarose beads with the use of the Immuno-Pure IgG orientation kit (Pierce) according to the manufacturer's instructions.

For blue native gel electrophoresis, mitochondria were isolated from wild-type strain 993, *tim18::HIS3* strain 992, strain 1032 that expresses Tim18p-HA, strain 494 that expresses Tim54p-HA (Kerscher *et al.*, 1997), and strain 800 that expresses Tim22p-HA (Kerscher *et al.*, 1997). Fifty micrograms of mitochondria from each strain was solubilized in buffer containing 1% digitonin and subjected to blue native electrophoresis as described (Schagger and von Jagow, 1991; Dekker *et al.*, 1996, 1997).

Antiserum and Antibodies

Antiserum 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). Antisera 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::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*–suppressing activity was localized to an ~1.26-kbp *Eco*RI fragment containing a single

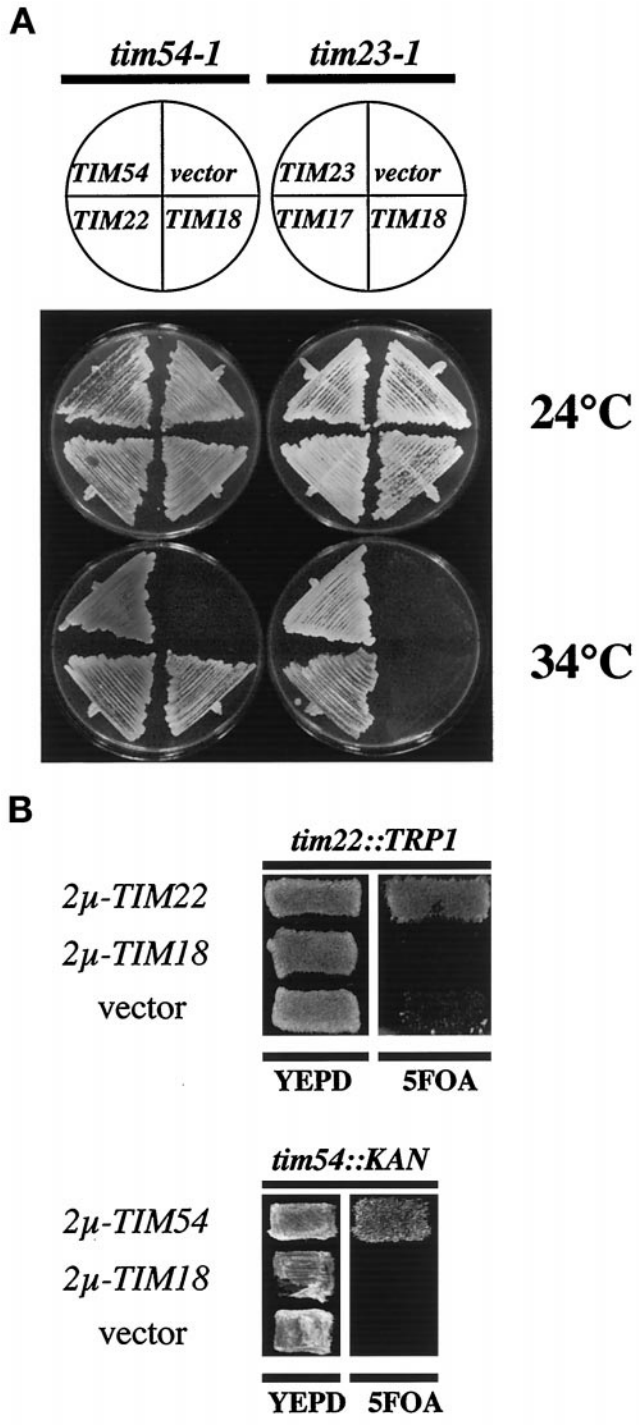

Figure 1. (A) Multiple copies of *TIM18* suppress the growth defect of *tim54-1* mutants but not *tim23-1* mutants. *tim54-1 his3* strain 735 was transformed with one of the following high-copy plasmids: 2μ-*TIM18* plasmid pOK66, 2μ-*TIM54* plasmid pOK59, 2μ-*TIM22* plasmid pJH203, or the empty vector pRS423 (Sikorski and Hieter, 1989). *tim23-1 leu2 ura3* strain 574 was transformed with 2μ-*TIM18* plasmid pOK66, 2μ-*TIM23* plasmid pKR21 (Ryan *et al.*, 1998), 2μ-*TIM17* plasmid pKR7 (Ryan *et al.*, 1994), or the empty vector pRS426 (Sikorski and Hieter, 1989). Transformants were streaked onto YEP medium containing 2% glycerol and ethanol and incubated at 24 or 34°C for 5 d. (B) Multiple copies of *TIM18* do not suppress the *tim54::KAN* or *tim22::TRP1* disruptions. *tim22::TRP1* strain 935, which carries the *TIM22-URA3* plasmid pJH202 (Kerscher et al., 1997), was transformed with 2μ-*TIM18* plasmid pOK66, 2μ-*TIM22* plasmid pJH203, or the empty vector pRS423 (Sikorski and Hieter, 1989). *tim54::KAN* strain 1078, which carries the *TIM54-URA3* plasmid pOK30 (Kerscher *et al.*, 1997), was transformed with 2μ-*TIM18* plasmid pOK66, *CEN-TIM54* plasmid pOK22 (Kerscher *et al.*, 1997), or the empty vector pRS424 (Sikorski and Hieter, 1989). Transformants were patched onto YEPD medium and then replica plated onto 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 2μ plasmids containing *TIM18*, *TIM23*, *TIM17*, or an empty vector, and we compared the results with those of *tim54-1* cells transformed with 2μ 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.

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 2μ-*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.

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 haploid progeny were allowed to grow at 24, 30, or 34°C on rich medium containing glucose as the carbon source (Figure 2A). 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^+ and were assumed to carry the *tim18::HIS3* disruption, whereas the other two colonies were His^- and thus carried the wild-type *TIM18* gene. At 30°C, *tim18::HIS3* cells formed colonies

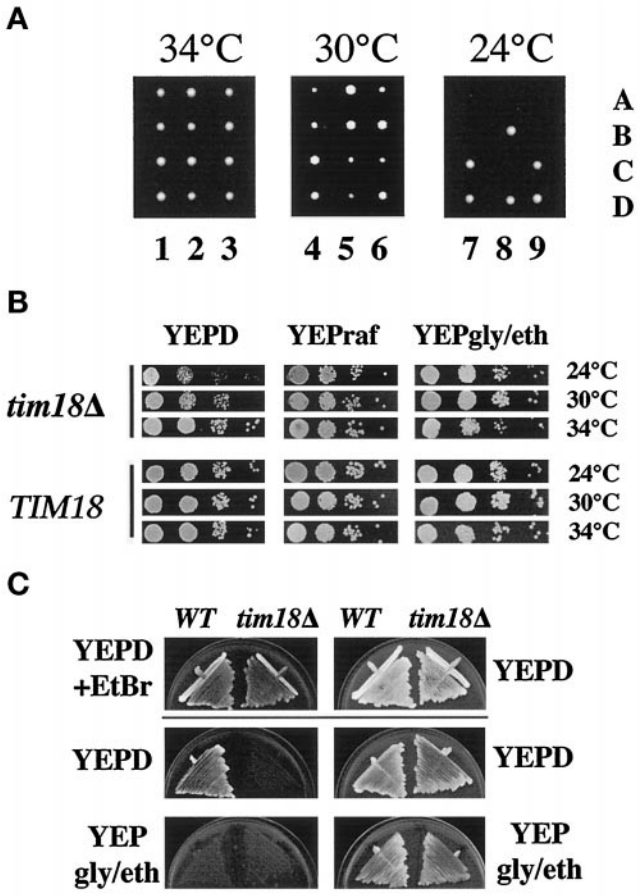

Figure 2. *TIM18* is required for normal cell growth. (A) *tim18::HIS3* disruptions are cold sensitive for growth. The *tim18::HIS3* disruption in diploid strain 605 was sporulated, and meiotic progeny were allowed to grow on YEP medium containing 2% glucose for 5 d at 34, 30, or 24°C. Lanes 1–9 show different tetrads, and individual spores are labeled A–D. (B) The cold-sensitive growth defect of *tim18::HIS3* is limited to glucose medium. *tim18::HIS3* strain 994 and *TIM18* strain 993 were pregrown at 34°C on YEP medium containing 2% glucose (YEPD), YEP medium containing 2% raffinose (YEPraf), or YEP medium containing 2% glycerol and ethanol (YEPgly/eth). Two OD_{600} units of cells were diluted in 10-fold increments, and 10 μl of each dilution (starting with the 1:100 dilution) was spotted onto YEPD, YEPraf, or YEPgly/eth medium and incubated at 24, 30, or 34°C for 3–8 d. (C) *tim18::HIS3* cells cannot tolerate loss of mitochondrial DNA. *TIM18* and *tim18::HIS3* cells were grown at 30°C on YEPD medium in the presence (YEPD+EtBr) or absence (YEPD) of 25 μg/ml ethidium bromide, streaked onto YEPD or YEPgly/eth medium lacking ethidium bromide, and incubated at 30°C for 5 d. WT, wild type.

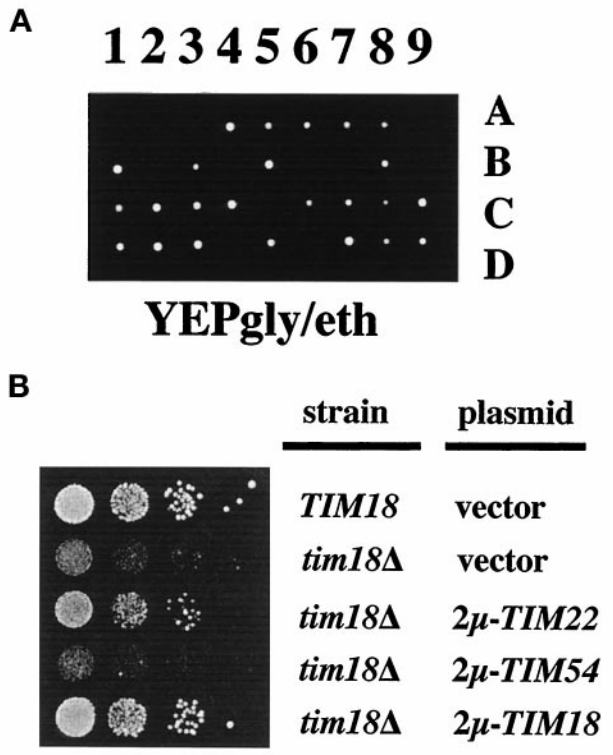

Figure 3. *TIM18* genetically interacts with both *TIM54* and *TIM22*. (A) *tim18::HIS3* is lethal in combination with *tim54-1*. *tim54-1/tim18::HIS3* diploid strain 1009 was sporulated, and meiotic progeny were allowed to grow for 8 d at 24°C on YEP medium containing 2% glycerol and ethanol (YEPgly/eth). 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 (*tim18*Δ) was transformed with the following high-copy plasmids: 2μ-*TIM22* plasmid pJH202 (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.

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 on glucose-containing medium. When tetrads from *tim18::HIS3/TIM18* diploids were grown at 24°C, only two of the four spores grew into distinct colonies (Figure 2A). These colonies were shown to be His^- and therefore contained the wild-type *TIM18* gene. His^+ spores containing *tim18::HIS3* germinated, grew into very small colonies, and then arrested 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

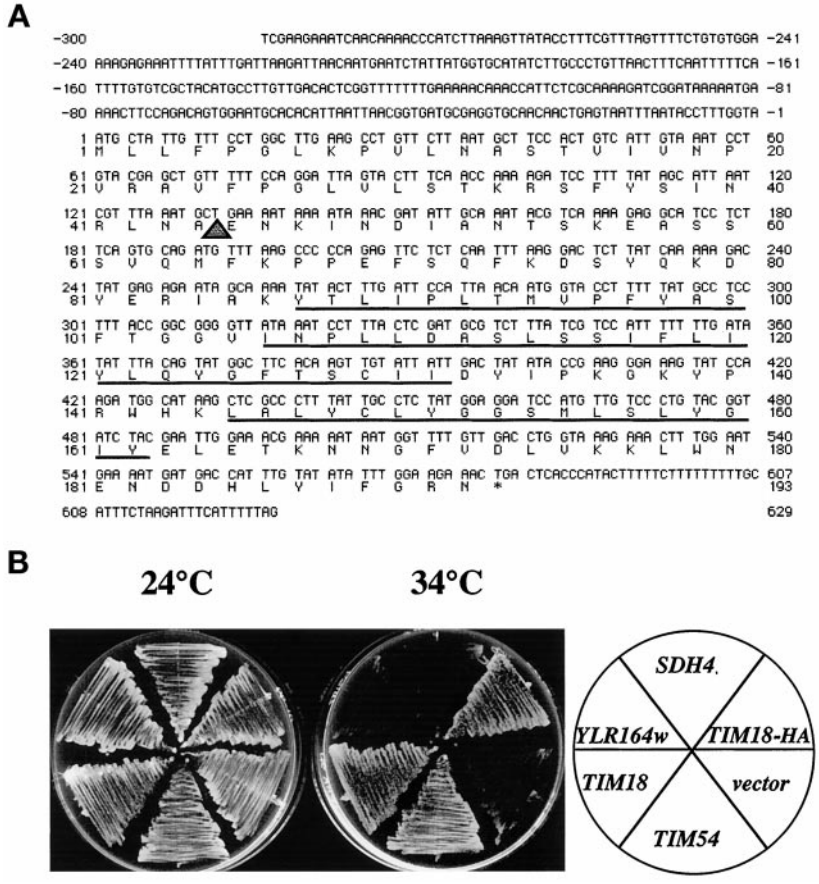

Figure 4. The Tim18 protein is homologous to two other yeast proteins. (A) DNA sequence of *TIM18* and its predicted protein product. The underlined amino acids represent potential membrane-spanning domains predicted from hydropathy analysis. The triangle indicates a potential mitochondrial processing protease cleavage site. (B) Increased levels of Sdh4p and YLR164w, proteins homologous to Tim18p, do not suppress the *tim54-1* growth defect. *tim54-1* strain 809 was transformed with one of the following high-copy plasmids: 2μ-*SDH4* plasmid pOK91, 2μ-*YLR164w* plasmid pOK90, 2μ-*TIM18* plasmid pOK66, 2μ-*TIM18-HA* 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.

18, 24, 30, and 34°C. *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 *tim18::HIS3* disruptions show a strain-specific phenotype is not known. However, disruptions of other yeast genes, such as *CHC1* (Munn *et al.*, 1991) and *HRD2* (Yokota *et al.*, 1996), often yield distinct results in different strain backgrounds.

We found that *tim18::HIS3* cells cannot tolerate the loss of mitochondrial DNA (Figure 2C). *tim18::HIS3* and *TIM18* cells were grown for 3 d 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. *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, *tim18::HIS3* cells pregrown on ethidium bromide failed to grow on either YEPD or YEPgly/eth medium. We conclude that *tim18::HIS3* cells that have lost mitochondrial DNA are inviable.

tim18::HIS3 Is Lethal in Combination with tim54-1

Because multiple copies of *TIM18* suppress the temperature-sensitive growth defect of the *tim54-1* mutant, Tim18p, like the Tim54 protein, may play a role in import. Supporting this idea, we found that *tim18::HIS3 tim54-1* double mutants were inviable, exhibiting a synthetic-lethal phenotype. *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 *tim54-1* and *tim18::HIS3*. Spores inferred to be double mutants germinated but arrested their growth after a few divisions. The result that the combination of *tim18::HIS3* and *tim54-1* is lethal provides additional genetic evidence for an interplay between Tim18p and Tim54p. *tim18::HIS3* did not exhibit synthetic lethality with *tim23-1* (Kerscher, unpublished observations), indicating that the interaction of Tim18p and Tim54p is specific.

Multiple Copies of TIM22 Suppress tim18::HIS3

We previously showed that the *tim54-1* mutant was suppressed by high levels of *TIM22* expression (Kerscher *et al.*, 1997). We found a similar interaction between *tim18::HIS3* and *TIM22*. We transformed *tim18::HIS3* cells with 2μ-containing plasmids carrying either *TIM18*, *TIM22*, *TIM54*, or an empty vector and tested the cells for growth at 24°C on glucose-containing medium. As shown in Figure 3B, *tim18::HIS3* cells containing either the *TIM18* or *TIM22* gene grew similar to wild-type cells on YEPD at 24°C. High levels of *TIM54*, on the other hand, did not suppress the cold-sensitive growth defect of *tim18::HIS3*. Our results show that

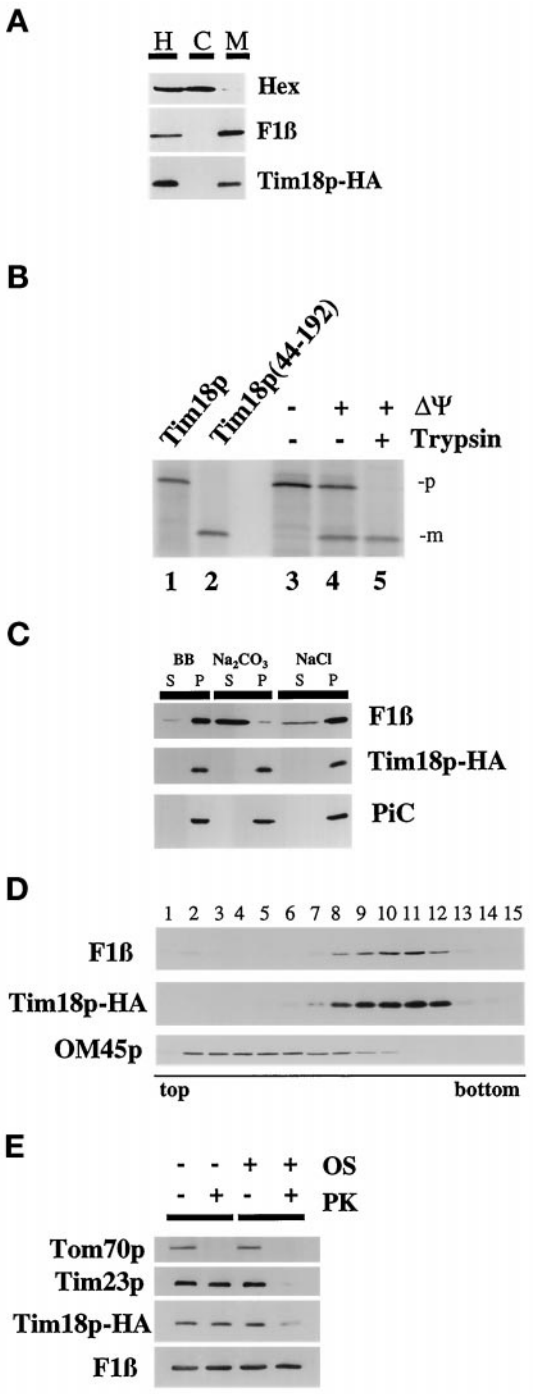

Figure 5. *TIM18* encodes a mitochondrial inner membrane protein with its carboxyl terminus facing the mitochondrial intermembrane space. (A) Tim18p is a mitochondrial protein. *tim18::HIS3* strain 992 containing plasmid pOK1032, which expresses the Tim18p-HA fusion protein, was grown in YEP medium containing 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 F_1-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 ^{35}S-labeled Tim18 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 (−ΔΨ). 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. Fraction 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.

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). *SDH4* and *YLR164w* were cloned into 2μ-containing plasmids,

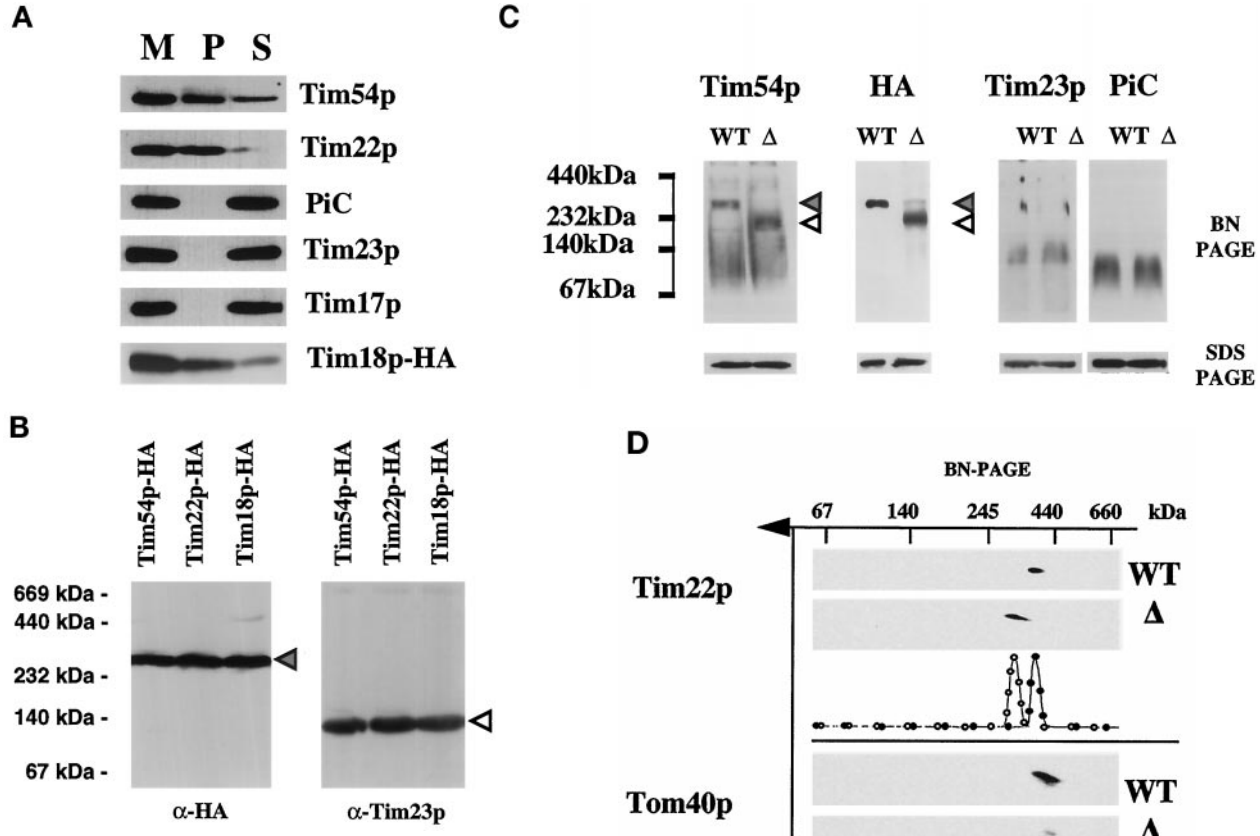

Figure 6. The Tim18 protein is part of the Tim22p-Tim54p complex. (A) Tim54p and Tim22p coprecipitate with Tim18p-HA. Tim18p-HA mitochondria were solubilized in 0.5% digitonin buffer. Mitochondrial extracts (M) were immune precipitated with antibodies against the HA epitope. Immunoprecipitates (P) and supernatants (S) were analyzed by SDS-PAGE, and duplicate samples were immune blotted with antibodies to the HA epitope, Tim22p, Tim54p, Tim23p, Tim17p, and PiC and then detected by chemiluminescence. (B) Tim18p-HA, Tim54p-HA, and Tim22p-HA are part of an ~300-kDa complex. Mitochondria were isolated from cells expressing Tim18p-HA, Tim54p-HA, or Tim22p-HA, solubilized in 1% digitonin buffer, and separated by blue native electrophoresis (6–16.5% polyacrylamide). Immune blots were decorated with antibodies to HA or with antiserum to Tim23p. The protein complexes at ~300 kDa (dark arrowhead) and ~90 kDa (white arrowhead) are indicated. The molecular masses of standards run in adjacent lanes are indicated. (C) Tim18p is required for formation of the ~300-kDa complex containing Tim54p. Strain 494, which expresses Tim54p-HA, *TIM18* (wild-type) strain 993, *tim18::HIS3* strain 992, and strain 992 carrying *TIM54-HA* plasmid pJH301 (Kerscher et al., 1997) were grown at 30°C on either galactose- or glycerol/ethanol-containing medium. Isolated mitochondria were solubilized in 1% digitonin buffer and separated by blue native electrophoresis (BN-PAGE). Mitochondria were also subjected to SDS-PAGE. Immune blots were decorated with antibodies to Tim54p, the HA epitope of Tim54p (HA), Tim23p, or PiC. The dark arrowheads indicate an ~300-kDa complex in wild-type (WT) mitochondria, and the white arrowheads mark an ~200-kDa complex in *tim18::HIS3* (Δ) mitochondria. (D) *tim18::HIS3* mitochondria contain an ~200-kDa complex containing Tim22p and Tim12p. Mitochondria from TIM18 (WT) strain 993 and *tim18::HIS3* (Δ) strain 992 were first subjected to blue native electrophoresis (BN-PAGE) and then to SDS-PAGE. Immune blots were decorated with antibodies to Tim22p, Tim12p, or Tom40p. Films were quantitated by scanning densitometry, and the results are shown below each pair of immune blots. (●) WT; (○) Δ.

Figure 6 (cont).

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 F_1-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 ^{35}S-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 mitoplasts (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 Tim18-HA, indicating that Tim22p physically associates with the Tim18 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., 1998b; 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 F_1-ATPase (F1α). 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 F1α in *tim18::HIS3* cells grown at 24, 30, or 34°C.

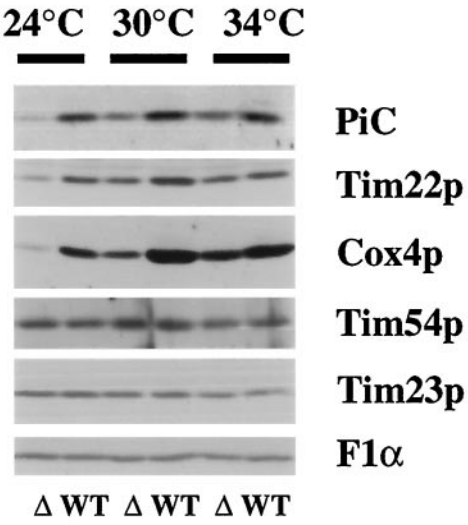

Figure 7. *tim18::HIS3* cells contain reduced steady-state levels of several IM proteins. *TIM18* (WT) strain 995 and *tim18::HIS3* (Δ) strain 994 were grown at 34°C in YEPD medium, diluted, and then grown at 24, 30, or 34°C to an OD_{600} of ~1.5. Total protein was isolated and separated by SDS-PAGE. Immune blots (75 μg of protein) were decorated with antibodies to PiC, Tim22p, Cox4p, Tim54p, Tim23p, or the α-subunit of the F_1-ATPase (F1α) and detected by chemiluminescence.

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 coimmune precipitated 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 translocon, were not affected. Whether the 100-kDa decrease in size of the translocon 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 translocon.

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 *tim54-1* mutant. Furthermore, in preliminary experiments, we observed that the YLR164w and Sdh4 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.

TIM18 encodes a 21.9-kDa protein with a cleavable presequence. After its import into mitochondria, Tim18p was processed to an ~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 Vincens, 1996). Hydropathy 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 *TIM18*, *TIM22*, and *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 *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 *TIM54* and *TIM22* are inviable under all growth conditions (Sirrenberg *et al.*, 1996; Kerscher *et al.*, 1997), whereas *tim18::HIS3* cells are cold sensitive for growth on glucose-containing medium. Several possibilities may explain why Tim18p is not essential under most growth conditions. For example, the function of Tim18p may be redundant, overlapping with the activity of another mitochondrial protein. In this case, we speculate that the second Tim18p-like activity is either absent or reduced when cells are grown on glucose, thus explaining why *tim18::HIS3* mutants are defective only on glucose medium. An alternative possibility is that Tim18p is required for efficient, but not basal, levels of import. For example, the Tom6 and Tom7 proteins are nonessential subunits of the TOM complex and modulate the assembly and disassembly of the TOM complex in the mitochondrial OM, but their role in import can be detected only under special assay conditions (Alconada *et al.*, 1995; Honlinger *et al.*, 1996). A third possibility why the Tim18 protein is not essential is that Tim18p may be required for the import of some, but not all, proteins. For example, each import receptor in the mitochondrial OM mediates the import of a subset of proteins (Lithgow *et al.*, 1995). Similarly, the TRAM protein of the endoplasmic reticulum translocon is required to translocate only some substrates (High *et al.*, 1993). We are currently testing these possibilities by examining the import of many different proteins into *tim18::HIS3* mitochondria.

Finally, our results also raise the possibility that Tim18p could play a role in the assembly of IM protein complexes. In particular, we found that *tim18::HIS3* cells grown on glucose medium at 24°C contain reduced levels of Cox4p, in addition to low amounts of Tim22p and PiC. Although Tim22p and PiC are inserted into the IM by Tim54p-Tim22p (Sirrenberg *et al.*, 1996; Kerscher *et al.*, 1997), the Cox4p precursor carries an amino-terminal cleavable presequence and is imported into the mitochondrial matrix via the Tim23p-Tim17p complex (Emtage and Jensen, 1993). 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 *tim18::HIS3* cells. In addition, disruptions of *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 *tim18::HIS3*. Furthermore, both *tim18::HIS3* and *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 *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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