The TIM23 complex mediates import into mitochondria of nuclear encoded preproteins with a matrix-targeting signal. It is composed of the integral membrane proteins Tim17 and Tim23 and the peripheral membrane protein Tim44, which recruits mitochondrial Hsp70 to the sites of protein import. We have analyzed the functions of these constituents using a combined genetic and biochemical approach. Depletion of either Tim17 or Tim23 led to loss of import competence of mitochondria and to a reduction in the number of preprotein-conducting channels. Upon depletion of Tim44, mitochondria also lost their ability to import proteins but maintained normal numbers of import channels. In the absence of Tim44 precursor protein was specifically recognized. The presequence was translocated in a Δψ-dependent manner across the inner membrane and cleaved by matrix-processing peptidase. However, the preprotein did not move further into the matrix but rather underwent retrograde sliding out of the TIM23 complex. Thus, the TIM23 complex is composed of functionally independently modules. Tim17 and Tim23 are necessary for initiating translocation, whereas Tim44 and mitochondrial Hsp70 are indispensable for complete transport of preproteins and for unfolding of folded domains of preproteins.

Mitochondrial precursor proteins are imported into mitochondrial subcompartments by preprotein translocases located in the outer and inner membranes of the organelle. The outer membrane contains a general translocase, the TOM (translocase of the outer membrane) complex, which is probably used by all nuclear encoded preproteins (1, 2). The mitochondria contain two distinct translocases of the inner membrane, the TIM22 and TIM23 complexes, that mediate import and membrane insertion of different classes of precursor proteins. The TIM22 complex specifically imports members of the mitochondrial carrier family and the precursors of Tim23, Tim22, and Tim17 (3–8). The TIM23 complex mediates import into the space matrix and membrane insertion of precursors that contain a positively charged matrix-targeting signal (2). The energy required for import of precursors into the matrix is provided by the inner membrane potential, Δψ, and by ATP in the matrix (2, 9, 10). Translocation of the presequence across the inner membrane import channel is triggered by Δψ (11, 12). ATP is used by mitochondrial Hsp70 (mt-Hsp70)1 to drive further translocation of the polypeptide chain across the inner membrane (13–18).

We show that the integral inner membrane proteins Tim17 and Tim23 and the peripheral inner membrane protein Tim44 are required to form a fully functional translocase. One TIM23 complex has the capacity to import one precursor protein/min in vitro. Depletion of either of the essential Tim components does not significantly affect the expression levels of the remaining proteins but severely reduces the overall kinetics of precursor import. Depletion of Tim17 or depletion of Tim23 reduces the number of functional TIM23 complexes. In contrast, when Tim44 is depleted, Tim17 and Tim23 form a functional protein-conducting import channel. This channel interacts in a Δψ-dependent manner with a preprotein and promotes translocation of the matrix-targeting signal across the inner membrane. However, the Tim44-depleted translocase cannot recruit mt-Hsp70 to the import site to drive complete translocation of folded or unfolded preproteins into the matrix.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotide Primers**—Fragments containing the LEU2 gene and the Gal10 promoter were amplified by polymerase chain reaction using Yep51-Tim23 (19) as template. For integration into the yeast genome the primer pairs contained extensions homologous to the promoter regions of Tim17 and Tim44, respectively: T17UTR-LEU2, 5′-ACG GCC AGG CCA GCA GTC TG CG TGG ATG AGC TGG TGG CCC GTT GTT CAG GAA CTC CAG TGG GAT AAT ACT CAG GAG-3′; T17-10.5′-ACC ACC GAA ATC ATT TAG TAT GAC TTG AAT TCT TAC GGA TAT GTA CTT TTT CCC-3′; and T44-GAL10, 5′-CCG TGT GGG AAT ACT CAG G-3′. Depletion of either of the essential Tim components does not significantly affect the expression levels of the remaining proteins but severely reduces the overall kinetics of precursor import. Depletion of Tim17 or depletion of Tim23 reduces the number of functional TIM23 complexes. In contrast, when Tim44 is depleted, Tim17 and Tim23 form a functional protein-conducting import channel. This channel interacts in a Δψ-dependent manner with a preprotein and promotes translocation of the matrix-targeting signal across the inner membrane. However, the Tim44-depleted translocase cannot recruit mt-Hsp70 to the import site to drive complete translocation of folded or unfolded preproteins into the matrix.

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1. The abbreviations used are: mt-Hsp70, mitochondrial Hsp70; PAGE, polyacrylamide gel electrophoresis; DHFR, dihydrofolate reductase; MTX, methotrexate; WT, wild type; PK, proteinase K.

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insert was then reintroduced into the BamHI-SalI sites of the pYep51-Tim23 vector to obtain pYep51–12hisTim23.

Replacement of the Endogenous Tim23 Promoter—A fragment comprising about 250 base pairs of a 5’-untranslated region of Tim23 amplified with primers TIM23UU and TIM23UL was cloned into the HindIII-HindIII sites of pYep51–12hisTim23, replacing the yeast 2µ origin of replication. The resulting plasmid linearized with HindIII was transformed into a haploid yeast strain 334 (20) and integrated into a chromosome. LEU+ colonies were analyzed by polymerase chain reaction and controlled for galactose-regulated expression of Tim23. The resulting strain was called Tim23(Gal10).

Replacement of Tim17 and Tim44 Promoters—A fragment containing yeast Gal10 promoter and Leu2 marker was amplified from pYep51-Tim23 (19) with primer pairs Tim17UTR-LEU2, T17-GAL10 and T44UTR-LEU2, T44-GAL10 for subsequent integration into the chromosome in front of Tim17 and Tim44 genes, respectively. The haploid yeast strain 334 was transformed with the amplified fragments, and Leu+ colonies were selected. The strains with galactose-regulated expression of Tim17 and Tim44 are designated Tim17(Gal10) and Tim44(Gal10), respectively.

Gel Filtration Analysis—Mitochondria (1 mg/ml) were lysed with 1% digitonin, 150 mM sodium acetate, 2 mM EDTA, 4 mM EGTA, and 20 mM Tris/HCl, pH 7.4. The supernatant of a clarifying spin (46,000 × g, 20 min) was subjected to gel filtration on Superose 12 and Superose 6 columns (Amersham Pharmacia Biotech). The samples were analyzed by SDS-PAGE and Western blotting.

In Vitro Synthesis of Precursor Proteins and Import into Isolated Mitochondria—The mitochondria were isolated as described previously (19, 21). The precursor proteins were synthesized by in vitro transcription-translation in rabbit reticulocyte lysate (22). Translocation of precursors into mitochondria was performed in import buffer containing 3% bovine serum albumin (fatty acid-free), 500 mM sorbitol, 50 mM KCl, 10 mM magnesium acetate, 2 mM KH2PO4, 2.5 mM EDTA, 2.5 mM MnCl2, and 50 mM HEPES-KOH, pH 7.2. A standard assay also contained 2.5 mM malate, 2.5 mM succinate, 5 mM NADH, 2.5 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 1 mg/ml mitochondria, and 1–3% reticulocyte lysate with the radiolabeled precursor protein. Protein import and translocation of mitochondria was carried out on ice for 20 min and stopped by the addition of 1 mM phenylmethylsulfonyl fluoride.

The mitochondria were washed in HS buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.2) and resuspended in a sample buffer. Translocation arrest of DHFR fusion proteins with methotrexate (MTX) was performed as described by Ungermann et al. (23, 24).

Purification and Unfolding of Recombinant pSu9(1–69)DHFR—pSu9(1–69)DHFR containing a C-terminal Hisa tag was cloned in pQE60. The plasmid was transformed into Escherichia coli strain BL21 carrying pREP4 (Promega). pSu9(1–69)DHFR was expressed as a soluble protein and purified on nickel-nitrilotriacetic acid-agarose. The protein was eluted in 100 mM NaCl, 500 mM imidazole, 50 mM Tris/HCl, pH 8, and then it was desalted on a PD10 column (Amersham Pharmacia Biotech) equilibrated with import buffer without bovine serum albumin. To unfold the preprotein, pSu9(69)DHFR was precipitated with ammonium sulfate, and the pellet was resuspended in 8 mM urea, 20 mM Tris/HCl, pH 7.5.

RESULTS

Analysis of Strains with Reduced Expression of Tim17, Tim23, and Tim44—All constituents of the TIM23 complex are essential for the viability of yeast. To study the role of individual components, the promoters of Tim17, Tim23, and Tim44 were replaced in the chromosome by homologous recombination by the inducible Gal10 promoter (3). Tim23 was expressed with a Hisa tag at the N terminus (see “Experimental Procedures”). The resulting strains grew like the parental wild type strain when galactose was present in the growth medium. When the cells were shifted to galactose-free medium, they stopped growing after approximately four divisions (Fig. 1A). The mitochondrial proteins were prepared from cells grown for 24 h in galactose-free medium.

The expression levels of the components of the TIM23 complex were analyzed by Western blotting (Fig. 1B). The mitochondria were essentially depleted of the respective Tim component controlled by the Gal10 promoter. Interestingly, the depletion of an individual Tim component did not severely affect the expression levels of the other components of the TIM23 complex. Furthermore, the mitochondria contained Tom40, Tom22, and Mge1p, proteins examined as markers of the outer membrane, inner membrane, and the matrix, respectively (Fig. 1B). This suggests that the integrity of the mitochondria was not affected by the depletion protocol.

Tim17, Tim23, and Tim44 are organized in a high molecular mass assembly, the TIM23 complex, in the inner membrane (19). When mitochondrial membranes are solubilized with digitonin, the integral membrane proteins Tim23 and Tim17 remain in a stable subcomplex, whereas the association of the peripheral membrane protein Tim44 with this subcomplex is labile and sensitive to salt concentration (19, 25). To investigate how the depletion of an individual component affects the formation of the TIM23 complex, mitochondria were solubilized with digitonin in the presence of 150 mM potassium acetate and analyzed by gel filtration (Fig. 2). Tim23 (upper panels) and Tim17 (lower panels) eluted in a high molecular mass subcomplex when WT mitochondria were analyzed. When Tim17 was depleted Tim23 eluted in a lower molecular mass form, and Tim17 eluted in a lower molecular mass form when Tim23 was depleted. Because Tim44 is not associated with Tim23 and Tim17 in the presence of 150 mM potassium acetate, the elution profiles of Tim23 and Tim17 were not significantly affected by

FIG. 1. Characterization of yeast strains and mitochondria with regulated expression of Tim44, Tim23, and Tim17. A, depletion of TIM components affects growth of yeast cells; growth curves of yeast strains with galactose-regulated expression of Tim44, Tim23, and Tim17. Haploid yeast strain 334 was grown in the presence of 2% glucose and 1% galactose (+ gal) or in the presence of the glucose only (--gal). The plots present the relative number of cells at indicated times. B, depletion of individual TIM components does not significantly affect expression levels of mitochondrial markers. The mitochondria were isolated from the indicated strains after incubation without galactose for 24 h to deplete the indicated Tim protein (+ ). Mitochondrial proteins were analyzed by SDS-PAGE, Western blotting, and immunoblotting with antibodies against components of the TIM23 complex and mitochondrial marker proteins for the outer membrane (Tom40), the inner membrane (Tom22), and the matrix (Mge1).
The depletion of Tim44, and the elution of Tim44 was not affected by depletion of Tim23 or Tim17 (not shown). Depletion of Tim17 or Tim23 did not affect the association of Tim44 with the inner membrane (not shown). In summary, the data indicate that in the absence of one component of the TIM23 complex, the remaining partners are expressed and accumulate to significant levels. In the absence of Tim44, Tim17 and Tim23 assemble into a stable subcomplex.

**Tim17 and Tim23 Are Part of a Protein-conducting Import Channel**—To analyze the role of Tim17 and Tim23 in protein import, we expressed the chimeric precursor pSu9(1–69)DHFR in E. coli and purified chemical amounts of the recombinant preprotein. To challenge the mitochondrial import machinery, 2 μg of recombinant pSu9(1–69)DHFR were incubated with mitochondria (50 μg) from WT cells and with mitochondria from cells depleted of Tim17 (Tim17−/−) or Tim23 (Tim23−/−). The imported protein was detected with antibodies against DHFR and quantified by densitometry (Fig. 3, A and B). pSu9(1–69)DHFR was rapidly and efficiently imported into WT mitochondria. The import was linear for 10 min, and the observed import kinetics corresponded to an initial rate of import of ~9 pmol (250 ng) of pSu9(1–69)DHFR/min/mg of mitochondrial protein. The mitochondria contain about 8.5 pmol of TIM23 complex/mg of protein (26). Accordingly, about 1 precursor was imported per min and import site. The kinetics of import of chemical amounts of pSu9(1–69)DHFR into mitochondria from cells depleted of Tim17 and from cells depleted of Tim23 were about 40-fold reduced (Fig. 3B, lower panels). Likewise, when radiolabeled pSu9(1–69)DHFR (fmol) was used, the amounts of preprotein imported in a 3-min period were reduced in these mitochondria. This shows that mitochondria depleted of Tim17 or Tim23 contained significantly fewer functional import channels than WT mitochondria.

**Tim44 Is Required for Import of Folded and Unfolded Preproteins**—The mitochondria from cells depleted of Tim44 contained approximately normal levels of Tim17 and Tim23 (Fig. 1) and also normal levels of mt-Hsp70 (not shown). To characterize the role of Tim44 in protein import, recombinant pSu9(1–69)DHFR (2 μg) was incubated with energized mitochondria from Tim44−/− cells (Fig. 4, left panel). The kinetics was more than 10-fold slower than the kinetics of import into WT mitochondria, which imported ~1 precursor/min/import site. This indicates that Tim44 is required for the import of pSu9(1–69)DHFR. To distinguish whether Tim44 is primarily required for unfolding of the DHFR domain or also for translocation of the unfolded polypeptide chain, pSu9(1–69)DHFR (2 μg) was denatured with urea and then added to the energized mitochondria (Fig. 4, right panel). Urea-denatured pSu9(1–69)DHFR was rapidly imported into WT mitochondria, whereas kinetics of import into Tim44−/− mitochondria was drastically reduced. The addition of methotrexate, which stabilizes the folded DHFR domain, inhibited import of native precursor but not of urea-denatured pSu9(1–69)DHFR (not shown), indicating that refolding of the DHFR moiety was negligible under the conditions used. Thus, Tim44 is required for efficient translocation of an unfolded polypeptide chain across the inner membrane.

Yeast cells harboring the ssc1-l-2 mutation encode a temperature-sensitive mt-Hsp70 that has an increased affinity for precursor proteins (27). On the other hand, the Ssc1-1 protein also has a significantly reduced, although measurable affinity for protein import (28, 29).
for Tim44 (15). Under nonpermissive conditions mitochondria from ssc1-1 cells do not import folded preproteins (28). In contrast unfolded preproteins were imported into ssc1-1 mitochondria with considerable efficiency (27, 28). These experiments were performed with radiochemical quantities of preproteins, i.e. under conditions where import sites (pmol) were in vast excess over the amount of radiolabeled preproteins (fmol). Accordinglly, only one round of translocation could be observed. To determine import activity on a quantitative level, chemical amounts of urea-unfolded pSu9(1–69)DHFR (100 pmol) were incubated with energized mitochondria (50 μg corresponding to ~0.4 pmol import sites) from WT and ssc1-1 cells, and import was measured (Fig. 5, upper panel). Import of the unfolded precursor into ssc1-1 mitochondria was significantly reduced when compared with import into WT mitochondria. We then measured import of urea-denatured radiochemical amounts pSu9(1–69)DHFR. Import of the radiochemical amounts of unfolded precursor (fmol) into ssc1-1 mitochondria (0.4 pmol import sites) was almost as efficient as import into WT mitochondria (Fig. 5, lower panel). The results suggest that the reduced affinity of Ssc1-1p for Tim44 was sufficient to promote translocation of a single unfolded preprotein by a TIM23 complex before the import reaction was analyzed. However, despite its high affinity for unfolded proteins, Ssc1-1p did not support several rounds of translocation/import site as measured with chemical amounts of preprotein. Corresponding results were obtained when chemical and radiochemical amounts of unfolded preprotein were imported into mitochondria depleted of Tim44 (Fig. 5). Import of chemical amounts of unfolded precursors into Tim44↓ mitochondria was not detectable, indicating the strict requirement of Tim44 for translocation of an unfolded polypeptide chain across the inner membrane. By contrast, radiochemical amounts of unfolded precursors were imported. The efficiency of import was, however, significantly reduced when compared with import into WT mitochondria. The residual import of radiochemical precursors may therefore be attributed to trace amounts of Tim44 still present in the depleted mitochondria.

Together, these observations indicate that the interaction of mt-Hsp70 with Tim44 is required for import of unfolded preproteins. When this interaction is compromised, either by deletion of Tim44 or by a mutation in mt-Hsp70 that lowers its affinity for Tim44, translocation of an unfolded polypeptide chain across the inner membrane is severely reduced. The requirement of mt-Hsp70 and Tim44 for import of unfolded proteins becomes masked under conditions when only a single round of translocation is observed.

**Fig. 4.** Tim44 is required for import of folded and unfolded preprotein. The mitochondria (50 μg) from WT cells or from cells depleted of Tim44 were incubated with 2 μg of recombinant pSu9(1–69)DHFR which was either added in native form (folded) or denatured by treatment with 8 μM urea and then added to the import reaction (unfolded). The import assays were terminated after the indicated time periods, and the samples were analyzed and quantified as described in the legend to Fig. 3.

**Fig. 5.** Interaction of mt-Hsp70 and Tim44 is required for import of unfolded precursors. Upper panel, urea-denatured recombinant pSu9(1–69)DHFR (2 μg) was incubated for 3 min at 25 °C with energized mitochondria (50 μg) from the indicated yeast strains. The ssc1-1 phenotype was induced by preincubation of the mitochondria for 10 min at 37 °C. The mitochondria from WT cells were also preincubated at 37 °C. Samples were analyzed by SDS-PAGE and immunoblotting with antibodies against DHFR and quantified by densitometry. a.u., arbitrary units. Lower panel, radiolabeled pSu9(1–69)DHFR was synthesized in reticulocyte lysate, denatured with urea, and added to the import assay containing mitochondria from WT cells, ssc1-1 cells, which were preincubated at 37 °C, and from Tim44↓ cells. The samples were incubated for 3 min at 25 °C and then analyzed by SDS-PAGE and quantified with a PhosphorImager. Import into WT mitochondria was set to 100%.
The TIM23 complex is composed of the integral inner membrane proteins Tim17 and Tim23 and the peripheral inner membrane protein Tim44 (19). In the inner membrane the TIM23 complex is organized as a dimer, stabilized on the outside of the inner membrane by interactions of the intermediate domain of Tim23 and on the inner face of the inner membrane by dimerization of Tim44 (2, 12, 25). The components of the TIM23 complex are essential for the viability of yeast under all conditions (2, 29, 30). In this report we describe a study on the functional organization of the TIM23 complex. Although a number of efforts by various groups have been performed to address the role of the individual known Tim components, little is known about their detailed function in the complex. Regulated gene expression under the control of a repressible promoter offers an opportunity to deplete mitochondria of single Tim components and study the structural and functional properties of the compromised translocation machinery. We show here that depletion of a single Tim component does not significantly affect the expression levels of the other Tim components. Each component was imported and accumulated independently in a stable manner in the inner membrane. These observations are consistent with results from experiments in which overexpressed single Tim components were found to be stable in the mitochondria (Refs. 15 and 19 and data not shown).

To address the activity of the TIM23 complex we established an in vitro import assay that uses chemical amounts of precursor proteins. This is essential because import of radiochemical amounts often does not allow us to draw quantitative conclusions. Using energized mitochondria from WT cells — 9 pmol of preprotein/mg of mitochondrial protein was imported at 25 °C. Mitochondria contain about 8.5 pmol of TIM23 complex/mg of protein (26). The observed rate of import therefore indicates that an individual TIM23 complex imports approximately one precursor/min. Thus, 1 min is required to move a precursor protein across the outer and inner membranes, which corresponds to a distance of less than 200 Å. In an aqueous environment the protein would diffuse over the same distance in about 10⁻⁵ s (31). Compared with random diffusion, translocation of a preprotein through a protein-conducting channel is a relatively slow process. Brownian motion could therefore easily drive translocation of preproteins across the import channel at the observed rates even when diffusion of segments of the polypeptide chain in the protein conducting import channel would be severely constrained.

How does depletion of single components affect the function of the translocase? Depletion of any one of the Tim components resulted in the loss of import competence, demonstrating that each component is essential for protein translocation. Remarkably, the translocase depleted of Tim44 remained partially functional. In the absence of Tim44, Tim17 and Tim23 assemblaged into preprotein-conducting channels. These channels were apparently sealed because depletion of Tim44 did not result in a loss of Δψ. In the presence of a preprotein the channels opened and promoted translocation of the presequence across the inner membrane in a Δψ-dependent manner. Thus, the capacity of the translocase to form channels and interact in a Δψ-dependent manner with a preprotein does not require Tim44. This is in agreement with the observation of Dekker et al. (32) that Tim44 did not co-purify with the 600 K complex, consisting of a preprotein in transit, components of the TOM complex, Tim17 and Tim23. Accordingly, Tim44 may not be a subunit of the translocation channel of the inner membrane. However, as shown here the presence of the Tim44 is absolutely crucial for the import of preproteins. When challenged

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**DISCUSSION**

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with chemical quantities of preprotein, mitochondria depleted of Tim44 imported neither folded nor unfolded pSu9(1–69)/DHFR. One role of Tim44 is to recruit mt-Hsp70 to the import sites (2, 13–15). This recruitment allows rapid binding of the chaperone to the incoming precursor to trap segments of the preprotein as they emerge from the import channel (2, 16, 23–25). The loss of mitochondrial import competence upon depletion of Tim44 indicates that mt-Hsp70 without assistance by its co-chaperone cannot efficiently bind to an incoming preprotein. Moreover, even Ssc1-1p, which displays an increased affinity for polypeptide substrates but a reduced affinity for unfolded polypeptides, could not drive import of unfolded preprotein. Thus, an increase in substrate affinity of mt-Hsp70 cannot compensate for its co-chaperone cannot efficiently bind to an incoming preprotein and translocates the presequences in a manner across the inner membrane. Complete translocation of Tim44 indicates that mt-Hsp70 without assistance by Tim44 imported neither folded nor unfolded pSu9(1–69)/DHFR. One role of Tim44 is to recruit mt-Hsp70 to the translocon to support the targeting function of Tim44. The requirement for co-chaperones that recruit Hsp70s to mediate recognition of their respective substrate proteins appears to be a general feature of chaperones that recruit Hsp70s to mediate recognition of their substrates is supported by the results of studies using chemical amounts of preprotein where import sites (pmol) are present in huge excess of precursors (fmol). Therefore they do not measure import rates of the compromised translocases. In contrast, we determined import rates using chemical amounts of pSu9 DHFR. Thereby it became obvious that depletion of Tim44 as well as inactivation of Ssc1-1p severely affects import of folded and unfolded precursors. This shows that Tim44 is an essential component of the translocation machinery.

In summary, our data demonstrate that the translocase is composed of functionally distinct modules that are associated with individual subunits. Tim17 and Tim23 are part of a protein-conducting channel that specifically recognizes precursor proteins and translocates the presequences in a Δψ-dependent manner across the inner membrane. Complete translocation across the inner membrane of folded and unfolded preproteins strictly requires the peripheral membrane protein Tim44 and a mt-Hsp70 protein that can interact with Tim44.

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Modular Structure of the TIM23 Preprotein Translocase of Mitochondria
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