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Sequential Processing of a Mitochondrial Tandem Protein: Insights into Protein Import in *Schizosaccharomyces pombe*

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The sequencing of the genome of *Schizosaccharomyces pombe* revealed the presence of a number of genes encoding tandem proteins, some of which are mitochondrial components. One of these proteins (pre-Rsm22-Cox11) consists of a fusion of Rsm22, a component of the mitochondrial ribosome, and Cox11, a factor required for copper insertion into cytochrome oxidase. Since in *Saccharomyces cerevisiae*, Cox11 is physically attached to the mitochondrial ribosome, it was suggested that the tandem organization of Rsm22-Cox11 is used to covalently tie the mitochondrial ribosome to Cox11 in *S. pombe*. We report here that pre-Rsm22-Cox11 is matured in two subsequent processing events. First, the mitochondrial presequence is removed. At a later stage of the import process, the Rsm22 and Cox11 domains are separated by cleavage of the mitochondrial processing peptidase at an internal processing site. In vivo data obtained using a tagged version of pre-Rsm22-Cox11 confirmed the proteolytic separation of Cox11 from the Rsm22 domain. Hence, the tandem organization of pre-Rsm22-Cox11 does not give rise to a persistent fusion protein but rather might be used to increase the import efficiency of Cox11 and/or to coordinate expression levels of Rsm22 and Cox11 in *S. pombe*.

About 10 to 15% of the nuclear genes of eukaryotic organisms encode mitochondrial proteins (30, 49). These proteins are typically synthesized with N-terminal presequences in the form of amphiphilic helices with one positively charged face and one hydrophobic face (54). These presequences function as targeting signals which mediate their selective translocation across the TOM complex of the outer membrane of mitochondria and across the TIM23 translocase of the inner membrane. Following their membrane potential-dependent transfer into the matrix, the presequences are bound by the mitochondrial Hsp70 chaperone (mtHsp70), which, together with other components of the import motor, energetically drives the import of the entire protein into the matrix. Finally, the presequence is proteolytically removed by the mitochondrial processing peptidase (MPP) (for reviews, see references 12, 24, 26–28, 32, 40, 44, 50, and 51).

Over the last two decades, the import of proteins into mitochondria was extensively studied, mainly by using the fungi *Saccharomyces cerevisiae* and *Neurospora crassa* as model systems, and our current picture of the mitochondrial protein import is deduced almost entirely from experiments with these two fungal species. The studies which were performed to characterize the import apparatuses of mitochondria in animals, plants, and protists suggested that the basic principles and components of this transport process are widely conserved among eukaryotes. Each system thereby showed specific features such as the absence of certain components of the import apparatus, the presence of additional factors, or variations in the properties of the presequences (3, 17, 22, 34). The fungus *Schizosaccharomyces pombe* has been widely used as a model system for many cell biological processes. Nevertheless, hardly any studies focused on the import of proteins into mitochondria of *S. pombe*. Interestingly, the sequencing of the genome of *S. pombe* revealed the presence of a number of genes encoding fusion proteins (55, 56), several of which are predicted to be mitochondrial components (Fig 1). These gene products contain classical mitochondrial targeting signals at their N termini, followed by a sequence which represents the homologues of two mitochondrial proteins arranged in tandem. It is unclear whether these tandem proteins are proteolytically processed or remain as fusion proteins in mitochondria of *S. pombe*.

In order to follow the biogenesis of mitochondrial proteins in *S. pombe* experimentally, we established an in vitro assay in which the import of preproteins into isolated mitochondria of *S. pombe* can be monitored. We used these conditions to characterize the biogenesis of one of the tandem proteins of *S. pombe*, SPAC1420.04c. This protein is depicted in Fig. 1A. Its N-terminal 39 amino acid residues show the hallmarks of a classical mitochondrial presequence; residues 136 to 467 are homologous to Rsm22 of *S. cerevisiae*, a protein of the small subunit of the mitochondrial ribosome (47); and finally, residues 568 to 739 show sequence similarity to Cox11, a factor required for the insertion of the copper cofactor into cytochrome oxidase (5, 52). In *S. cerevisiae*, the mature Cox11 protein is anchored in the inner membrane by an N-terminal transmembrane domain and exposes the large copper-binding domain into the intermembrane space. Interestingly, Cox11 was recently found in physical association with the mitochondrial ribosome, which may facilitate the insertion of copper into subunit 1 of the cytochrome oxidase right after its synthesis in the organelle (25). On the basis of the ribosomal associ-

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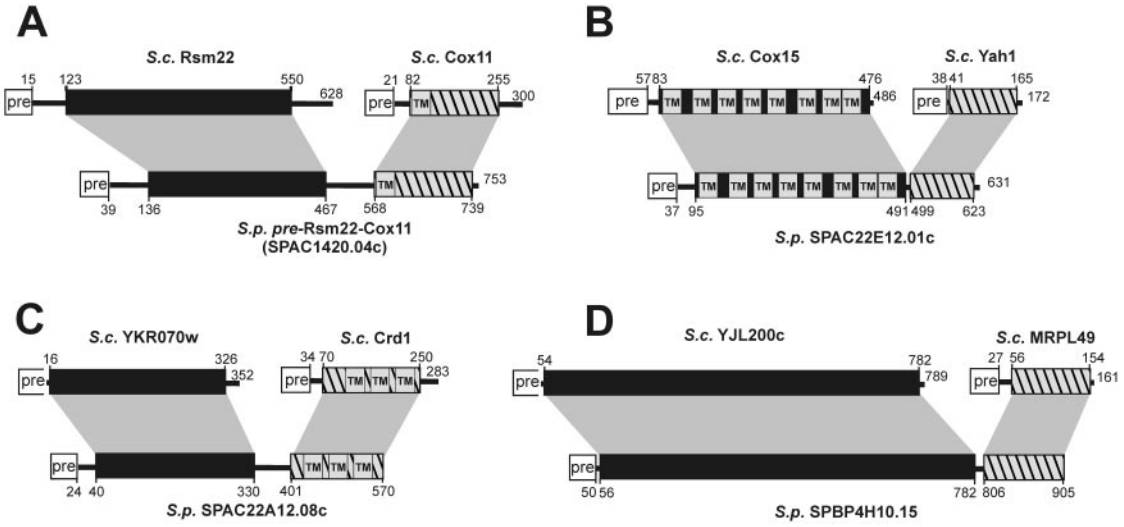

FIG. 1. Schematic representation of mitochondrial fusion proteins of *S. pombe* and of their homologues in *S. cerevisiae*. The proteins depicted are (A) SPAC1420.04c, (B) SPAC22E12.01c. (C) SPAC22A12.08c, and (D) SPBP4H10.15 (55, 56). Numbers indicate amino acid positions in the proteins. Black boxes depict conserved regions of the proteins and are labeled according to the *S. cerevisiae* nomenclature. Mitochondrial targeting sequences (pre) were predicted using the TargetP or Mitoprot algorithm (9, 11); positions of the predicted processing sites are indicated. Transmembrane domains (TM) of the various proteins are indicated. *S.c.*, *S. cerevisiae*; *S.p.*, *S. pombe*.

ation of Cox11 in *S. cerevisiae*, it was speculated that the expression of Cox11 as a tandem protein with a ribosomal subunit in *S. pombe* might covalently tie Cox11 to the mitochondrial ribosome (6). In favor of this idea, Carr et al. (5) used an unprocessed artificial fusion of the Rsm22 and Cox11 proteins of *S. cerevisiae* that mimicked the tandem protein of *S. pombe* and obtained full complementation of *RSM22* and *COX11* deletion mutants. Due to the tandem organization of the Cox11 protein in *S. pombe*, we refer to this mitochondrial preprotein as pre-Rsm22-Cox11 in this study.

Using radiolabeled pre-Rsm22-Cox11 precursor, we could show that this fusion protein is efficiently imported and cleaved in two sequential processing steps to give rise to three polypeptides: the N-terminal presequence, a mature fragment corresponding to the Rsm22 segment, and a C-terminal, membrane-embedded Cox11 protein. The cleavage between both mature proteins is catalyzed by MPP and occurs in a region which shows the characteristics of a classical mitochondrial presequence. Thus, the tandem organization of this protein is not maintained in the endogenous protein, but Rsm22 and Cox11 are present in *S. pombe* as distinct polypeptides like in other species. We suggest that the tandem organization of mitochondrial proteins in *S. pombe* might be used to improve the efficiency by which these proteins are imported into mitochondria and/or to coordinate expression levels of the fused proteins.

MATERIALS AND METHODS

Strains and growth media. The *S. pombe* strains used in this study were L972 (h^{-s}) (31) and the isogenic strain HE620 (h^{+s} *leu1-32 ura4-D18*) (strain collection of the Institut für Genetik, TU Dresden). *S. pombe* cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic minimal medium lacking leucine and supplemented with uracil with 3% glucose as a carbon source (19). For experiments with *S. cerevisiae*, the wild-type strain YPH499 (*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3*), a temperature-sensitive *ssc1-3* mutant (PK83) (14), and a mutant harboring the *TIM23* gene under control of the *GAL10* promoter (Tim23 ↓) (36) were used. *S. cerevisiae* cells were grown in lactate medium (21) supplemented with 0.1% galactose or glucose. For cloning, *Escherichia coli* strain DH5α (BRL) was used. Media were as described previously (46).

Constructs and plasmids. Genomic DNA isolated from strain L972 served as a template to obtain the *pre-RSM22-COX11* open reading frame with primers 1 (5′-TAT TTA GGA TCC ATG CCC ATT CTA ACA TGC AG-3′) and 2 (5′-TAT TTA GAA TTC TCA GTT GAG TTT AGT TAA AAG ATT G-3′) or primers 3 (5′-TAT TTA GAA TTC ATG CCC ATT CTA ACA TGC AGA TAT AAA ATT-3′) and 4 (5′-TAT TTA GGA TCC TCA GTT GAG TTT AGT TAA AAG ATT G-3′). The PCR fragments were cut with BamHI and EcoRI at the underlined restriction sites and cloned into the expression vector pGEM3 or pGEM4 (Promega). For expression of the pre-Rsm22-Cox11ΔC protein lacking the C-terminal 54 amino acid residues, the pGEM4 plasmid harboring the pre-Rsm22-Cox11 open reading frame was digested with SpeI and religated. For generation of a strain carrying a triple hemagglutinin (HA) epitope tag at the C terminus of the *pre-RSM22-COX11* gene, three copies of the HA tag were introduced by means of overlap extension PCR (42) using primers 5 (5′-GGC AAT CTT TTA ACT AAA CTC AAC CTG GTT CCG CGT GGA-3′) and 6 (5′-TCC ACG CGG AAC CAG GTT GAG TTT AGT TTA AAG ATT GCC-3′) and flanking primers 7 (5′-TAT TTA CTC GAG ATG CCC ATT CTA ACA TGC AGA TAT AAA ATT CTG-3′) and 8 (5′-ATT ATT CCA TGG CTA TTA GCG GCC GCA CTG AGC AGC-3′). The resulting 2,429-bp fragment was digested with XhoI and NcoI and cloned into pJR1-3XL (38), yielding pJR1-3XLSp*cox11*HA. All sequences of the cloned fragments were verified by DNA sequencing.

Isolation of import-competent mitochondria from *S. pombe*. Import-competent mitochondria were isolated from *S. pombe* essentially as described by Moore et al. (37) with slight modifications. Briefly, cells were grown in YPD medium to late exponential phase and collected at 2,000 × *g* for 10 min at room temperature. The cell pellet was washed with distilled water and incubated with 2 ml/g of 0.1 M Tris-HCl (pH 9.3) and 0.3% β-mercaptoethanol for 10 min at 30°C. The cells were centrifuged at 2,000 × *g* for 10 min (room temperature), washed with 0.5 M KCl and 10 mM Tris-HCl (pH 7.0), and resuspended in 3 ml/g of 1.2 M sorbitol and 20 mM phosphate buffer (pH 7.4). Zymolyase (1 mg/ml) was added, the suspension was incubated for 15 min at 30°C, and then 2 mg/ml *Trychoderma harzianum* lysing enzymes (Sigma) was added and the cells were further incubated for 15 min at 30°C. All of the following steps were carried out at 4°C. The obtained spheroplasts were centrifuged at 400 × *g* for 10 min and washed with 1.2 M sorbitol, 10 mM MOPS (morpholinepropanesulfonic acid) (pH 6.8), and 0.1% bovine serum albumin. The spheroplasts were resuspended in 6.7 ml/g lysis buffer (0.65 M mannitol, 10 mM MOPS [pH 6.8], 2 mM EDTA, 0.5% bovine serum albumin) with 1 mM phenylmethylsulfonyl fluoride and gently broken in a glass homogenizer. Intact cells and cell debris were removed by centrifugation at 1,000 × *g* for 10 min. The supernatant was centrifuged at 17,000 × *g* for 10

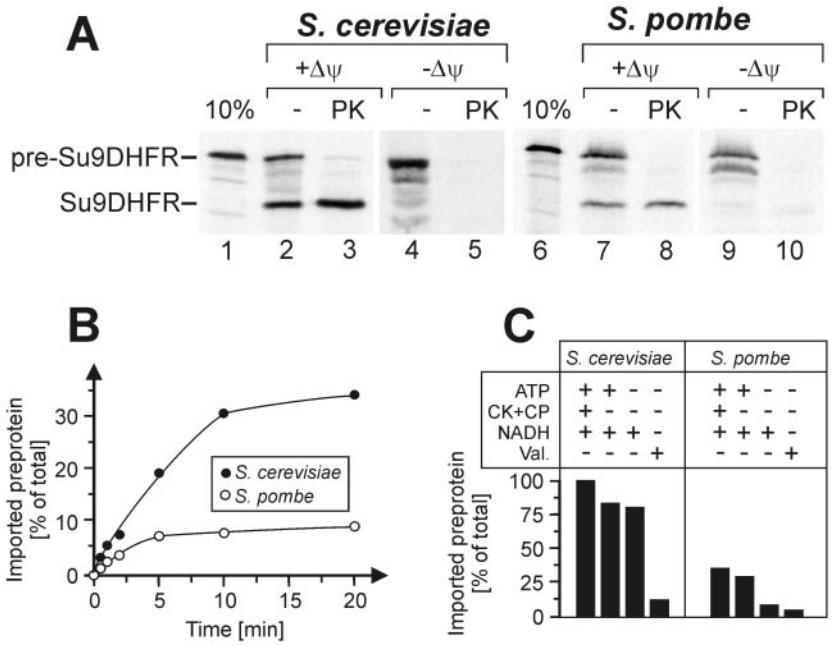

FIG. 2. Pre-Su9DHFR can be imported into isolated mitochondria of *S. cerevisiae* and of *S. pombe*. (A) Pre-Su9DHFR was synthesized in the presence of [^{35}S]methionine in reticulocyte lysate and incubated with isolated mitochondria of *S. cerevisiae* and *S. pombe* in the presence of NADH and ATP (+$\Delta\psi$) or the presence of valinomycin to deplete the membrane potential (−$\Delta\psi$). After the import reaction, the samples were incubated in the absence or presence of proteinase K (PK). Mitochondria were reisolated, washed, and dissolved in sample buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. (B) Mitochondria of *S. cerevisiae* and *S. pombe* were incubated for different time periods with radiolabeled pre-Su9DHFR as described for panel A. The amount of matured and protease-protected Su9DHFR was quantified and is expressed in relation to the total amount of added preprotein after correction for the respective methionine contents of the preprotein and the mature species. (C) Pre-Su9DHFR was incubated with isolated mitochondria for 10 min at 20°C in the absence or presence of ATP, malate, succinate, creatine phosphate and creatine kinase (CK+CP), NADH, or valinomycin (Val.). The amount of imported protein was quantified and expressed in relation to that of the total precursor protein.

min, and the pellet obtained was resuspended in 0.7 M sorbitol, 1 mM EDTA, and 20 mM HEPES (pH 7.4). Residual cell debris was removed by centrifugation at 1,000 × *g* for 10 min, and the mitochondria were collected by centrifugation of the supernatant at 12,000 × *g* for 10 min. The mitochondria were gently resuspended in 0.5% bovine serum albumin, 0.7 M sorbitol, 1 mM EDTA, and 20 mM HEPES (pH 7.4) to a protein concentration of 10 mg/ml and shock frozen in liquid nitrogen.

Protein import into isolated mitochondria. Precursor proteins were synthesized in reticulocyte lysate (Promega) in the presence of [^{35}S]methionine and imported into isolated mitochondria essentially as described previously (43); 2 mM NADH, 2 mM ATP, and an ATP-regenerating system containing 2.5 mM malate, 2.5 mM succinate, 1 mM creatine phosphate, and 0.1 mg/ml creatine kinase were added during the import reaction to obtain a highly energized state of the mitochondria. To dissipate the membrane potential, 2 μM valinomycin was added during the import reaction. Nonimported precursor protein was removed by incubation with proteinase K (100 μg/ml) for 30 min on ice. The protease was inactivated by addition of 2 mM phenylmethylsulfonyl fluoride.

Mitochondrial subfractionation. For hypotonic swelling to selectively rupture the outer membrane, mitochondria were incubated in the presence of 60 mM sorbitol and 20 mM HEPES (pH 7.4) for 30 min on ice.

RESULTS

Proteins can be imported into isolated mitochondria of *S. pombe*. The import of radiolabeled precursor proteins into purified mitochondria is a widely used experimental approach. Import protocols were optimized before for several fungal (*S. cerevisiae* and *N. crassa*) and mammalian mitochondria. While it was shown before that preproteins can be imported into isolated *S. pombe* mitochondria (1, 8, 37, 39), detailed import studies with mitochondria of *S. pombe* are still lacking. In order to test the import competence of mitochondria from *S. pombe*, we used a fusion protein of subunit 9 of the F_1F_o ATPase of *N. crassa* and mouse dihydrofolate reductase (pre-Su9DHFR) as a model substrate. This protein can be efficiently imported in vitro into mitochondria of various organisms and represents one of the best characterized precursor proteins. When mitochondria were purified from *S. pombe* cells according to published procedures (23, 37, 39), they turned out to be not competent to import pre-Su9DHFR (data not shown). We then isolated mitochondria from *S. pombe* cells by using a protocol which was adapted from the procedure by which import-competent *S. cerevisiae* mitochondria are isolated (see Materials and Methods for details). As shown in Fig. 2A, these mitochondria were able to import pre-Su9DHFR. Pre-Su9DHFR was synthesized in reticulocyte lysate in the presence of [^{35}S]methionine, resulting in a radiolabeled precursor form (Fig. 2A, lane 1). Upon incubation of this protein with mitochondria isolated from *S. cerevisiae* or *S. pombe*, pre-Su9DHFR was converted to a faster-migrating mature form. This mature species remained inaccessible to added protease, indicating its complete import into the mitochondria (Fig. 2A, lanes 3 and 8). Dissipation of the membrane potential prevented import of pre-Su9DHFR into mitochondria of both *S. cerevisiae* and *S. pombe*. Noteworthy is that the efficiency of the import into *S. pombe* mitochondria was lower than of import that into mitochondria of *S. cerevisiae*. The overall kinetics of the import reaction, however, were similar (Fig. 2B). The re-

duced import efficiency might be a consequence of the generally lower energetic state of mitochondria of *S. pombe*. This lower energetic state might also explain why the import of Su9-DHFR into *S. pombe* mitochondria required the addition of external ATP whereas addition of ATP was dispensable for protein import into isolated mitochondria of *S. cerevisiae* (Fig. 2C). From this we conclude that mitochondria of *S. pombe* can be used for in vitro import studies with radiolabeled precursor proteins. However, at least in our in vitro assay, protein import into *S. pombe* mitochondria occurs with significantly lower efficiency than import into mitochondria of *S. cerevisiae*.

Pre-Rsm22-Cox11 can be imported into isolated *S. pombe* mitochondria. In order to characterize the biogenesis of pre-Rsm22-Cox11, we synthesized the pre-Rsm22-Cox11 precursor protein in reticulocyte lysate. This resulted in a band with an apparent molecular mass of 80 kDa (Fig. 3A, lane1, pre), roughly matching the 87 kDa which was calculated for pre-Rsm22-Cox11. In addition, two smaller translation products were generated, most likely by reinitiation at internal ATG codons. The smaller of these two by-products (indicated by an asterisk) was of ca. 55 kDa and was almost as prominent as the full-length protein. Upon incubation of the reticulocyte lysate with mitochondria, three smaller fragments which were at least partially resistant to added protease appeared (Fig. 3A, lane 3, a to c). These fragments were specific for the import reaction, as their appearance was dependent on the membrane potential (Fig. 3A, lanes 4 and 5). The two larger fragments (a and b) were completely resistant to added proteinase K, whereas the smaller one was partially degraded. It appears likely that upon import, pre-Rsm22-Cox11 is converted into two fragments of about 50 (band a) and 23 (band c) kDa, which roughly correspond to the sizes of the Rsm22 and Cox11 domains, respectively, of the precursor proteins. Fragment b (30 kDa) presumably originated from a comparable cleavage of the smaller translation product. This suggests that the Rsm22 and Cox11 domains of the pre-Rsm22-Cox11 protein are separated by proteolysis during or following their import into mitochondria.

To determine whether the pre-Rsm22-Cox11 protein is indeed internally processed in vivo, we generated a plasmid (pJR1-3XLSp*cox11*HA) which allowed the expression of pre-Rsm22-Cox11 with three C-terminal HA tags in *S. pombe* cells. In extracts of this mutant, HA-specific antibodies recognized a protein of 25 kDa which was absent in wild-type cells (Fig. 3B, lanes 1 and 2). This matches the masses observed for the processed Cox11 domain (23 kDa) plus the triple HA tag. A signal corresponding to an unprocessed precursor form was not observed, indicating that the Cox11 part of pre-Rsm22-Cox11 does not remain fused to Rsm22 in vivo. Upon addition of protease, the signal of the Cox11 domain was strongly diminished but no smaller fragments were generated (Fig. 3B, lane 3). A similar signal reduction was observed for cytochrome c_1, an inner membrane protein which exposes a large domain into the intermembrane space, suggesting that the outer membrane of the purified *S. pombe* mitochondria was partially ruptured. The inner membrane was intact, since no reduction was observed for the matrix protein aconitase. Upon lysis of the mitochondria with detergent, Cox11 was accessible to protease and degraded (Fig. 3B, lane 5). This suggests that Cox11, like in *S. cerevisiae*, exposes the C terminus into the intermembrane space.

Pre-Rsm22-Cox11 is matured in two subsequent processing steps. To assess the kinetics of the processing of pre-Rsm22-Cox11 precursor, we incubated the preprotein for various time periods with isolated *S. pombe* mitochondria. The samples were divided into three aliquots. One aliquot was directly applied to the sodium dodecyl sulfate (SDS) gel (Fig. 4A, lanes 2 through 7), the second was incubated with proteinase K (lanes 8 through 13), and the third was first 10-fold diluted in order to rupture the outer membranes and then treated with protease (lanes 14 through 19). The import of pre-Rsm22-Cox11 precursor again led to the generation of fragments a to c. Upon hypotonic swelling and treatment with protease, the Cox11 domain (fragment c) was completely degraded, indicating that this species is at least largely accessible from the intermembrane space.

At early time points during the import reaction, a processing intermediate appeared which migrated slightly faster than pre-Rsm22-Cox11 and which presumably represented a protein species lacking the mitochondrial targeting sequence (Rsm22-Cox11) (Fig. 4A, lanes 2 through 7 and enlargement in the inset). After 5 min of incubation, this species declined and fragments a to c appeared (Fig. 4A and B). Dissipation of the mitochondrial membrane potential by addition of valinomycin prevented the generation of this Rsm22-Cox11 intermediate (Fig. 4C).

To verify the nature of fragments a to c, a C-terminally truncated variant of pre-Rsm22-Cox11 was generated and used for import experiments (Fig. 4D). This truncated preprotein gave also rise to three fragments: two larger ones which were identical to fragments a and b of pre-Rsm22-Cox11 and a third fragment (c′) which was about 7 kDa smaller than fragment c. This confirmed that fragment c represents the C-terminal Cox11 domain whereas fragments a and b were generated from an N-terminal region of pre-Rsm22-Cox11. From this we conclude that the pre-Rsm22-Cox11 protein is matured in two subsequent processing steps: first the N-terminal presequence is proteolytically removed at early stages of the import reaction, and in a second step, Rsm22-Cox11 is further proteolytically processed, giving rise to distinct proteins that correspond to the Rsm22 and Cox11 domains, respectively (Fig. 4E).

Pre-Rsm22-Cox11 is properly imported and processed in mitochondria of *S. cerevisiae*. While in *S. pombe*, several mitochondrial proteins are synthesized as tandem proteins, no tandem proteins had been identified so far in *S. cerevisiae*. We therefore asked whether the import and processing of tandem proteins is a specific ability of *S. pombe* mitochondria or whether mitochondria isolated from *S. cerevisiae* are likewise able to import and process pre-Rsm22-Cox11. As shown in Fig. 5A, incubation of mitochondria isolated from *S. cerevisiae* gave rise to three fragments which closely resembled the fragments observed in *S. pombe* mitochondria. Upon hypotonic swelling and incubation with protease, fragments a and b remained unaffected whereas fragment c was degraded (Fig. 5A, lane 4). This indicates that fragment c, like in *S. pombe*, represents the C-terminal Cox11 part of the tandem protein. Moreover, after short incubation times, the Rsm22-Cox11 import intermediate appeared, showing that the presequence is removed initially during the import of the protein (Fig. 5B). Thus, the machinery for import and processing for proteins in

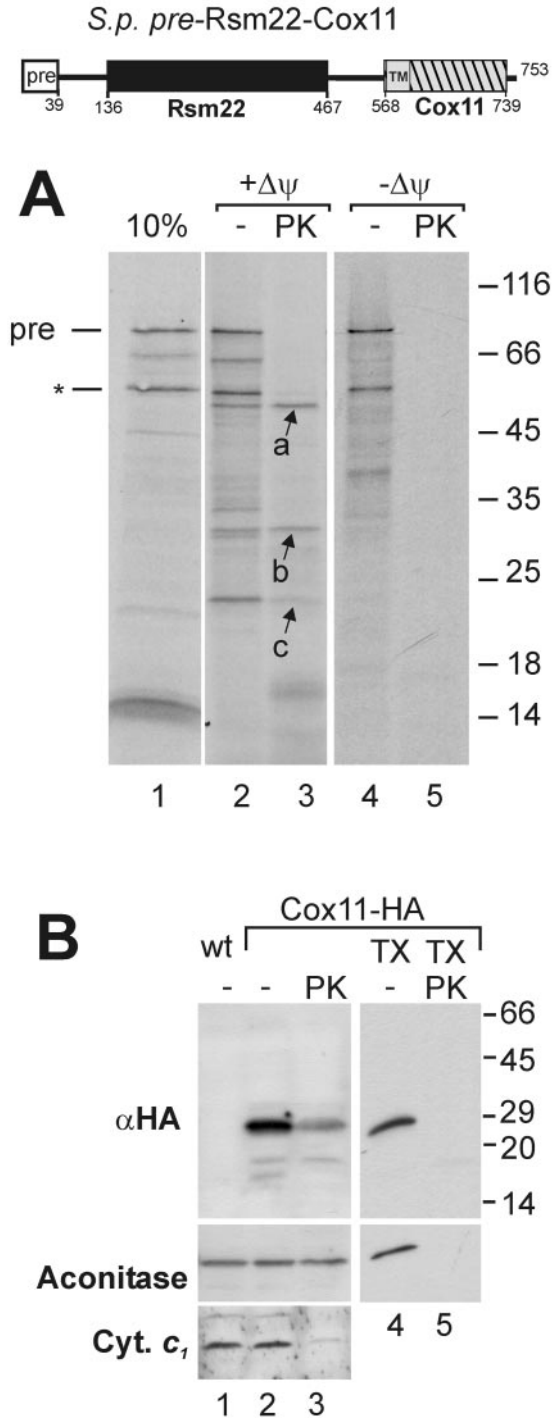

FIG. 3. Pre-Rsm22-Cox11 can be imported into isolated mitochondria. (A) Radiolabeled pre-Rsm22-Cox11 was incubated with isolated *S. pombe* mitochondria for 20 min at 30°C in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of valinomycin. The samples were divided, and proteinase K (PK) was added to half of the samples. After 30 min on ice, mitochondria were reisolated, washed, and dissolved in sample buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Protease-resistant fragments of the imported precursor protein are indicated by arrows. Lane 1 shows 10% of the precursor protein used for each of the import reactions shown in lanes 2 to 5. Positions of molecular weight standards are indicated. (B) *S. pombe* wild-type (wt) mitochondria or mitochondria of a strain harboring a plasmid for expression of a C-terminally HA-tagged version of pre-Rsm22-Cox11 were incubated in the absence or presence of proteinase K. In the samples shown in lanes 4 and 5, the mitochondria were lysed by incubation with 1% Triton X-100 (TX) prior to the protease treatment. Proteins were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected by Western blotting using HA-specific antibodies. Western blot signals of the matrix protein aconitase and the inner membrane protein cytochrome c_1 (Cyt. c_1) are shown as controls.

mitochondria of *S. cerevisiae* is able to correctly transport and process the pre-Rsm22-Cox11 tandem protein.

The *S. cerevisiae* system allowed assessment of the relevance of specific components of the import machinery for the biogenesis of pre-Rsm22-Cox11. We therefore analyzed the kinetics of import of the tandem protein into mitochondria lacking functional mtHsp70 (*ssc1-3*) or Tim23 (Tim23 ↓) or into wild-type mitochondria as a control. As shown in Fig. 5C, the import of pre-Rsm22-Cox11 into the mtHsp70 mutant was significantly reduced, and in the absence of Tim23, no pre-Rsm22-Cox11 was translocated into the mitochondria. This suggests that mtHsp70 drives the translocation of pre-Rsm22-Cox11 through the protein-conducting channel of the TIM23 complex.

The internal cleavage site of Rsm22-Cox11 shows the characteristic features of a mitochondrial presequence. In order to identify the region at which the Rsm22-Cox11 protein is internally processed, we generated C-terminally truncated versions of pre-Rsm22-Cox11 by digest of the expression plasmid at unique restriction sites (Fig. 6A). Following digestion with ScaI, PstI, and KpnI, radiolabeled preproteins which were terminated at amino acid residues 470, 504, and 549, respectively, were synthesized. These variants were imported into *S. pombe* mitochondria. Nonimported material was removed by protease treatment, and the sizes of the resulting fragments were compared to that of the Rsm22 part of the wild-type pre-Rsm22-Cox11 protein (Fig. 6A). Cleavage of the DNA with ScaI and PstI resulted in shortened versions of the Rsm22 fragment, whereas cleavage with KpnI had no or almost no effect on the size of the Rsm22 fragment. This indicates that the processing of the Rsm22-Cox11 protein occurs at around position 549 of the precursor protein. Interestingly, this region of the protein shows the typical features of a mitochondrial targeting sequence: it is rich in hydroxylated and positively charged amino acid residues and poor in negatively charged residues (Fig. 6B). The presence of this internal presequence-like region around the internal processing site might explain the unexpected import of N-terminally shortened variants of pre-Rsm22-Cox11 (see above), as it might serve as an internal targeting sequence which can direct the protein into mitochondria.

The presence of a presequence-like internal region between the Rsm22 and Cox11 domains is compatible with an internal cleavage of the tandem protein by MPP, which then would cleave the precursor twice, initially after the presequence and then between the Rsm22 and Cox11 moieties. MPP is a conserved heterodimeric enzyme which recognizes and cleaves substrate proteins reliably in vitro (13). In order to test whether pre-Rsm22-Cox11 is processed by MPP, we employed an in vitro digestion assay with MPP purified from *S. cerevisiae* mitochondria (33). This yielded an active enzyme which was able to cleave a radiolabeled mitochondrial preprotein (pre-

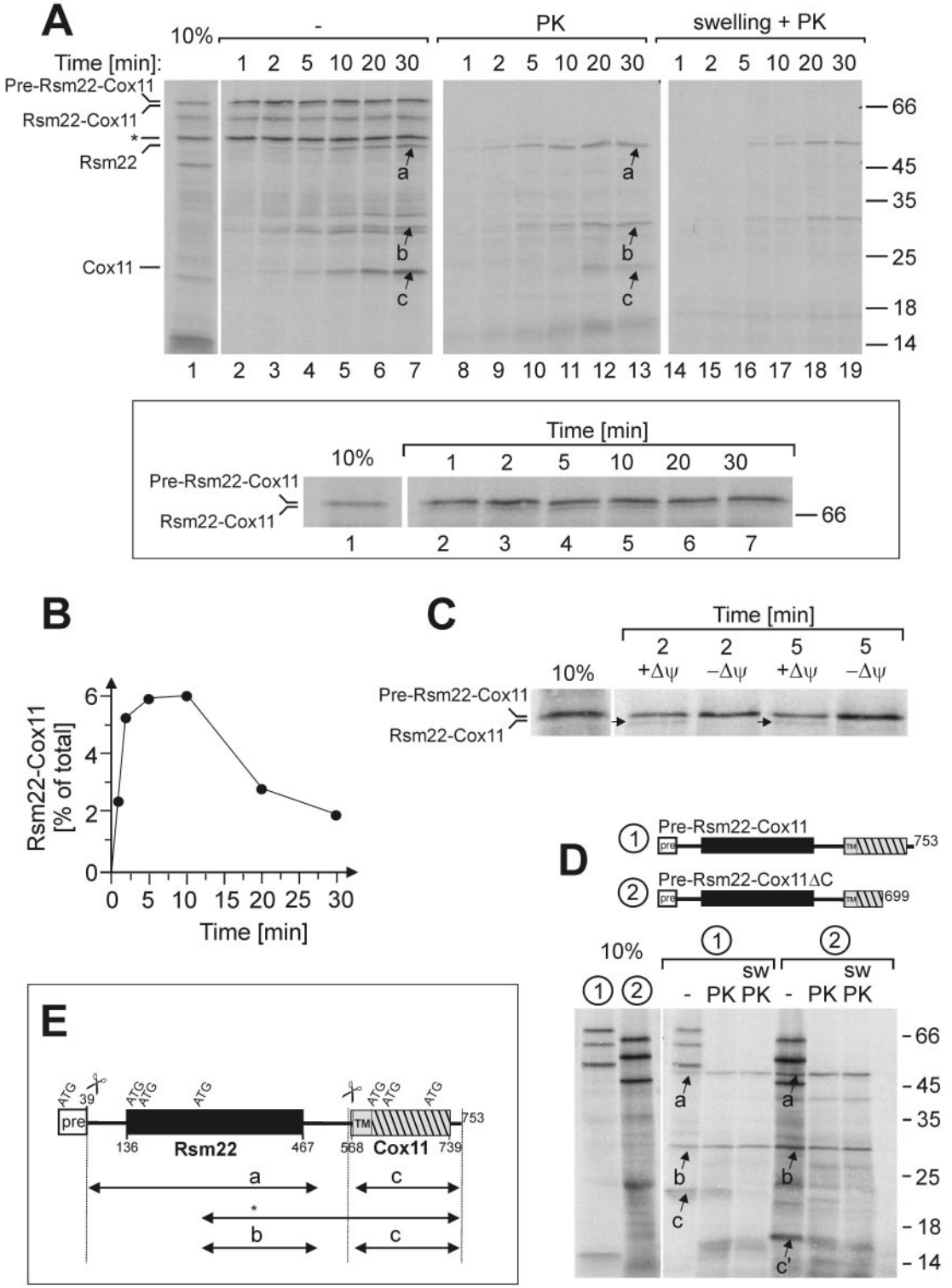

FIG. 4. Pre-Rsm22-Cox11 is processed in two steps. (A) Pre-Rsm22-Cox11 was imported at 30°C for the time periods indicated. The mitochondria were reisolated and divided into three aliquots. One was mock treated (lanes 2 to 7), one was incubated with proteinase K (PK) (lanes 8 to 13), and one was diluted 10-fold in 20 mM HEPES (pH 7.4) and treated with protease (lanes 14 to 19). The mitochondria were further treated as described for Fig. 3. The inset at the bottom shows an enlargement of a section of the upper panel. (B) The radioactive signal of the matured Rsm22-Cox11 species for each time point was quantified by densitometry. The values are depicted as percentages of the signals of the total precursor protein used for the import experiments following correction for the specific methionine contents of the precursor and the Rsm22-Cox11 protein. Thus, after 5 to 10 min of import, about 6% of the imported protein was present as N-terminally processed Rsm22-Cox11 intermediate. At later stages of the import process, this species was decreased and instead the fully matured Rsm22 and Cox11 species accumulated. (C) The removal of the presequence of pre-Rsm22-Cox11 depends on the membrane potential. Pre-Rsm22-Cox11 was imported in

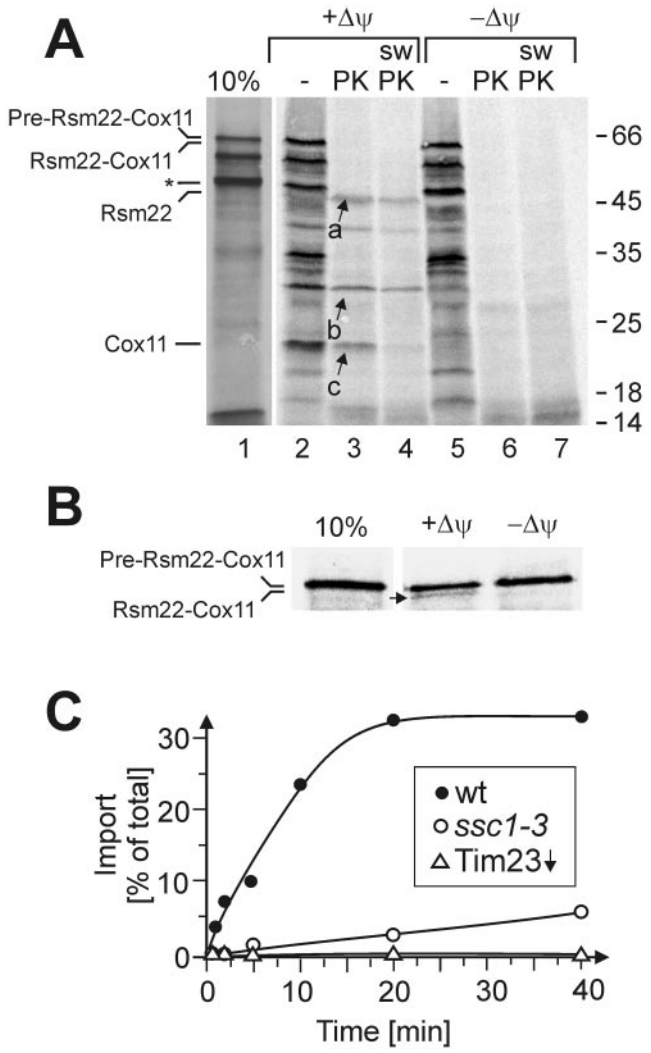

FIG. 5. Pre-Rsm22-Cox11 can be imported and correctly processed in *S. cerevisiae* mitochondria. (A) The import of radiolabeled pre-Rsm22-Cox11 into mitochondria of *S. cerevisiae* was assessed as described for Fig. 4D. PK, proteinase K; sw, rupture of outer membrane of mitochondria by hypotonic swelling. (B) The presequence of pre-Rsm22-Cox11 is removed upon import into isolated *S. cerevisiae* mitochondria. Pre-Rsm22-Cox11 was incubated for 5 min with *S. cerevisiae* mitochondria, and the generation of the Rsm22-Cox11 intermediate was analyzed as described for Fig. 4. (C) Mitochondria were purified from wild-type cells (wt), a Tim23-depleted strain (Tim23 ↓), or an *ssc1-3* mutant. The mitochondria were resuspended in import buffer and incubated for 10 min at 37°C to induce the temperature-sensitive phenotype of the *ssc1-3* mutant. To assess the kinetics of the import reaction, radiolabeled pre-Rsm22-Cox11 was added and left for various time periods. Nonimported material was removed by protease treatment, and the amount of imported Rsm22 fragment was quantified. Following correction of the specific methionine content of the Rsm22 fragment and the pre-Rsm22-Cox11 precursor, the percentage of imported protein was calculated for each time point.

Oxa1) into its mature part and its presequence (Fig. 6C, left panel). The in vitro translation of pre-Rsm22-Cox11 and pre-Rsm22-Cox11ΔC led to the synthesis of the respective full-length proteins and two shorter variants (Fig. 6C, right panel, bands 1 to 3), presumably resulting from initiation at downstream ATG codons as described above. Upon incubation with purified MPP, these proteins were converted to three fragments of identical size (f_1 to f_3) and two fragments with pre-protein-specific mobility (f_4 and f_5). Fragments f_1 and f_3 thereby match fragments a and b of the import reactions. Since their size is not altered in the C-terminally truncated protein, these fragments represent N-terminal parts of the proteins. Presumably, fragments 1 to 3 therefore are products of radiolabeled proteins 1 to 3, respectively. The smaller fragments 3 and 4, on the other hand, represent C-terminal parts of the tandem protein. However, they are larger than fragments c and c′ of the import reaction. This would be compatible with an internal cleavage of the tandem protein by MPP which is followed by a subsequent processing by an unknown peptidase (Fig. 6D).

DISCUSSION

The sequencing of the genome of *S. pombe* revealed the presence of a number of genes encoding fusion proteins, several of which are predicted to be mitochondrial components. These gene products contain classical mitochondrial targeting signals at their N termini followed by sequences which show similarity to two mitochondrial proteins arranged in tandem. It is unclear whether these tandem proteins are proteolytically processed or remain as fusion proteins in mitochondria of *S. pombe*. Employing an in vitro import assay with *S. pombe* mitochondria, we showed that one of these fusion proteins, pre-Rsm22-Cox11, is efficiently imported and cleaved in two sequential processing steps, thereby giving rise to three polypeptides: the N-terminal presequence, a mature fragment corresponding to the matrix-located soluble Rsm22 segment, and a C-terminal Cox11 protein that is at least partially exposed to the intermembrane space. Evidence for an internal processing of the tandem protein was also obtained in vivo by use of an *S. pombe* strain expressing the fusion protein with a C-terminal HA tag.

The data presented here suggest that pre-Rsm22-Cox11 is imported by the TIM23 translocase (Fig. 7). The observed import defect in *ssc1-3* mitochondria indicates that the matrix chaperone mtHsp70 is vital for the import of pre-Rsm22-Cox11. This chaperone typically is critical for the translocation of extended matrix regions across the inner membrane. In contrast, mtHsp70 is dispensable for inner membrane proteins in which, like in yeast Cox11, the transmembrane domain directly follows the presequence (15). Thus, the fusion of Cox11

the absence or presence of a membrane potential for 2 or 5 min as indicated. The generation of the Rsm22-Cox11 intermediate was analyzed as for panel A. (D) Pre-Rsm22-Cox11 and pre-Rsm22-Cox11ΔC were synthesized in vitro and incubated with *S. pombe* mitochondria. Each import reaction mixture was divided into three aliquots. One was mock treated, and one was incubated with proteinase K. In the third sample, the outer membrane of the mitochondria was ruptured by hypotonic swelling (sw) and the sample was treated with protease. The generated fragments were analyzed as for panel A. (E) Schematic representation of the protein fragments which are produced from pre-Rsm22-Cox11 following import into mitochondria. ATG codons allowing internal starts of the translation products are depicted. The fragments observed in the in vitro import experiments are indicated and labeled as in panel A. See text for details.

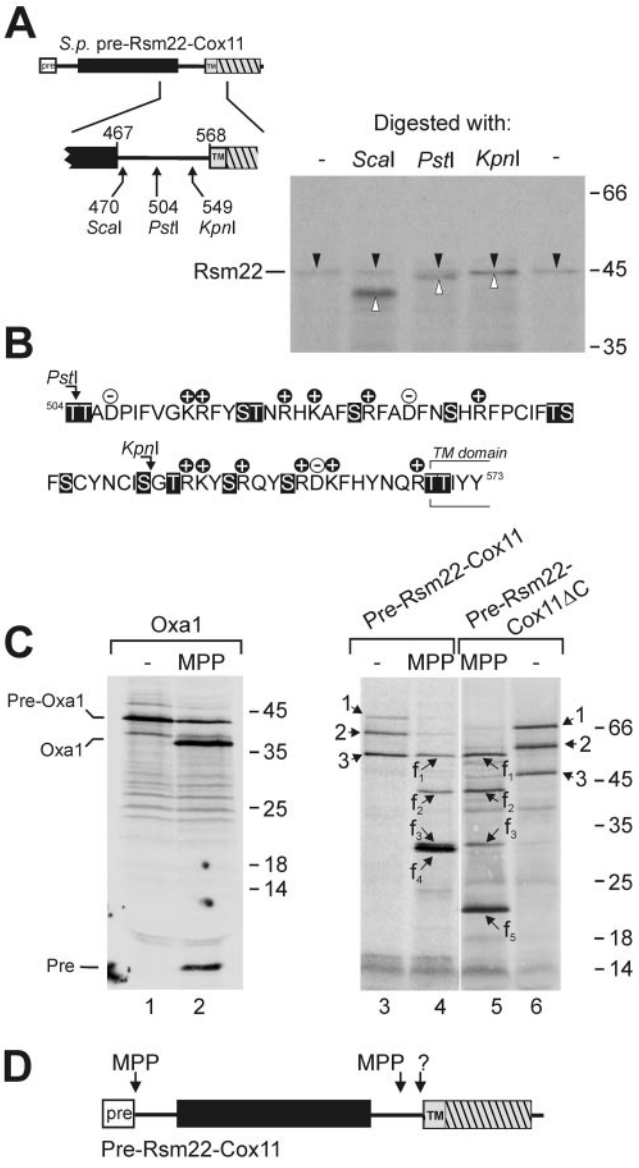

FIG. 6. MPP can proteolytically separate the Rsm22 and Cox11 domains of pre-Rsm22-Cox11. (A) The plasmid for in vitro synthesis of pre-Rsm22-Cox11 was digested at unique restriction sites in order to produce truncated versions of the radiolabeled preprotein. The upper panel shows the positions of the restriction sites in the corresponding protein sequence of pre-Rsm22-Cox11. The truncated variants of the preprotein were imported into *S. pombe* mitochondria. Nonimported protein was removed by protease treatment, and the size of the processed Rsm22 fragment was assessed by SDS-polyacrylamide gel electrophoresis and autoradiography. Black arrowheads depict the Rsm22 fragment resulting from maturation of the undigested plasmid. White arrowheads show the positions of the fragments which resulted from the truncated preproteins. (B) Amino acid sequence of the region around the internal processing of pre-Rsm22-Cox11. Hydroxylated residues are highlighted by black boxes, and charged residues are depicted. The hydrophobic transmembrane (TM) domain of the Cox11 part is indicated. The arrows point to the C termini of the truncated versions of the precursor protein. (C) Radiolabeled pre-Oxa1, pre-Rsm22-Cox11, and pre-Rsm22-Cox11ΔC were incubated in 10 mM NaCl and 20 mM Tris (pH 7.4) in the absence or presence of 5 μg purified MPP (33) for 3 h at 30°C. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The positions of the mature Oxa1 (Oxa1) and the Oxa1 presequence (pre) are indicated. Signals 1, 2, and 3 depict the three translation products obtained by in vitro synthesis with the plasmids for expression of the tandem proteins. The resulting fragments are indicated. See text for details. (D) Schematic representation of pre-Rsm22-Cox11 and its processing sites.

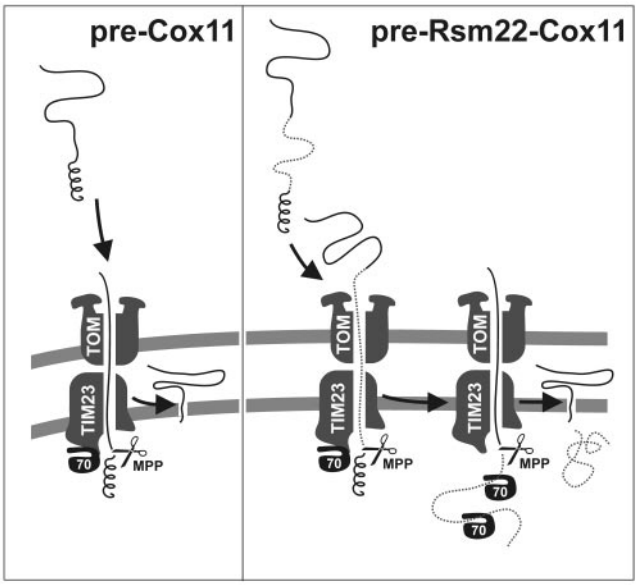

FIG. 7. Model for the topogenesis of Cox11 in *S. cerevisiae* (left) and *S. pombe* (right). In *S. cerevisiae*, as in most organisms, Cox11 is expressed in the cytosol as precursor protein with a mitochondrial presequence (pre-Cox11). Proteins of this type typically follow a stop-transfer pathway via the TOM and TIM23 translocases. During or following membrane insertion, the presequences are removed by MPP. The pre-Rsm22-Cox11 tandem protein of *S. pombe* is matured by at least two subsequent processing events. First, the presequence is removed by MPP early during the import process. Then, the Rsm22-Cox11 intermediate is further imported in a reaction that is driven by mtHsp70 until the internal processing site reaches the matrix. There, the Rsm22 and Cox11 domains are proteolytically separated by MPP. Presumably this second cleavage by MPP is followed by a subsequent processing step, as the endogenous Cox11 protein is smaller than the in vitro processing product of MPP.

to Rsm22 might have converted it into a preprotein that is imported by the assistance of the import motor, thereby potentially rendering the import process more efficient. Interestingly, some of the other mitochondrial tandem proteins found in the *S. pombe* genome also consist of one hydrophobic and one hydrophilic partner (Fig. 1). It seems conceivable that the tethering to a hydrophilic polypeptide improves the efficiency of the import reaction both because it increases the solubility of the protein in the cytosol and because the hydrophilic region can serve as a handle for mtHsp70 and the mitochondrial import motor to drive the translocation across the inner membrane. This might be especially beneficial in *S. pombe*, where the mitochondrial import process apparently is less robust than that in *S. cerevisiae*.

Three examples of natural mitochondrial tandem proteins which, like pre-Rsm22-Cox11, are internally processed were studied before. These are (i) the pre-Cox15-Yah1 tandem protein of *S. pombe*, where Yah1 transfers electrons to Cox15, an

TABLE 1. Similarities of components of the mitochondrial import machinery

Component	*S. pombe* orthologue	Identity (%) with human orthologue[a]		
		S. pombe	*S. cerevisiae*	*N. crassa*
Tom70	SPAC6B12.12	25.9	22.8	**29.8**
Tom40	SPBC8D22.22	25.4	23.8	**28.4**
Tom22	SPAC17H9.16	**22.7**	17.5	19.1
Tom20	SPAC6F12.07	**31.5**	24.8	23.2
Tob55	SPAC17C9.06	31.2	24.1	**32.1**
Tim50	SPBC8D2.21c	**30.0**	28.5	27.1
Tim44	SPBC14C8.02	23.4	26.3	**26.4**
Tim23	SPCC16A11.09c	**28.1**	22.3	25.6
Tim22	SPBC25H2.04c	37.2	38.9	**45.8**
Tim21	SPBC1289.09	19.6	19.2	**20.3**
Tim17	SPAC3A12.16c	46.6	47.1	**51.3**
Tim16	SPBC713.10	37.3	39.3	**46.4**
Tim14	SPAC824.06	44.1	38.9	**51.5**
Tim13	SPAC17C9.09c	**44.7**	34.1	36.0
Tim10	SPAC222.03c	**48.1**	37.5	37.5
Tim9	SPAC24B10.05	40.3	**46.2**	32.5
Tim8	SPAC13G6.04	**36.1**	35.3	31.7
Mia40	SPAC57A10.11c	39.7	**49.4**	41.4
Erv1	SPAC3G6.08	38.9	43.2	**55.6**
Mas1	SPBP23A10.15c	**49.5**	44.2	49.1
Mas2	SPBC18E5.12c	29.2	34.9	**36.7**
Oct1	SPAC1F3.10c	**31.1**	29.6	**31.1**
Oxa1	SPAC9G1.04	26.9	**35.6**	20.7
	SPBP4H10.03	27.4		
Hsp70	SPAC13G7.02c	65.2	65.3	**65.9**
Mge1	SPBC3B9.19	38.1	**38.8**	**38.8**
Mdj1	SPCC4G3.14	28.8	30.6	**31.3**
Hsp60	SPAC12G12.04	56.4	56.1	**58.3**
Hsp10	SPCC550.06c	43.4	**44.6**	21.6

[a] The protein sequences of *S. pombe*, *S. cerevisiae*, and *N. crassa* were aligned with their orthologues in human, and the percentages of identical residues were calculated. The pairs with the highest degree of identity are shown in boldface.

enzyme of heme *a* biosynthesis (2, 4, 18); (ii) the *N*-acetylglutamate kinase and *N*-acetyl-γ-glutamyl-phosphate reductase of *N. crassa* (16); and (iii) the succinate dehydrogenase subunit B and S14 subunit of the mitochondrial ribosome in rice and maize (41). Why these fusion proteins appeared and were maintained during evolution remains unclear. It is possible that the genomic rearrangements which resulted in the expression of these fusion proteins were physiologically neutral, since the processing sites in the tandem proteins allowed their proteolytic separation in the matrix. However, at least the first two of these three protein pairs represent molecular interaction partners, suggesting that the tandem organization does not simply reflect the result of random genomic reshuffling processes but rather provides specific advantages. Besides an improved process for import of inner membrane proteins, such beneficial effects of the tandem organization might be that the processing of one fused precursor warrants equal expression levels of the two proteins. In addition, the separation of the tandem protein in the matrix ensures that both proteins emerge at the same entry site in the mitochondrion, which might help the partnering of cooperating proteins. Whatever the molecular basis for the benefit is, it most likely is not generally advantageous, as only few examples of such tandem proteins are present in the genomes sequenced so far. Moreover, the observation that tandem arrangements typically are not conserved among species indicates that these few examples reflect relatively recent gene rearrangements and that their persistence in evolution is short.

In this study we assessed the experimental parameters for in vitro import experiments with *S. pombe* mitochondria. In general, the conditions which permit efficient protein import into these mitochondria are reminiscent to the conditions established for mitochondria of *S. cerevisiae*. The limited growth of *S. pombe* on nonfermentable carbon sources and the resulting lower energetic state of the mitochondria isolated from glucose-grown cells are disadvantages of this organism. However, the use of *S. pombe* as a model system to study cell biological processes has often been very valuable, as in several respects *S. pombe* is more similar to mammalian cells than is *S. cerevisiae*. For example, *S. pombe* has been extremely useful for unraveling the organization of the cytoskeleton (48, 57) and the processes of cell division, meiosis, and the cell cycle (7, 10, 20, 45), of pre-mRNA splicing (29), and of RNA interference (35, 53). Moreover, *S. pombe* served as a powerful model to study the molecular basis of human diseases. About 50 of the 4,824 open reading frames in the *S. pombe* genome show high similarity to human disease genes, some of which are not present in *S. cerevisiae* (56). From the primary sequence, the components of the protein import machinery of *S. pombe* are similarly distant from their human orthologues as those of *S. cerevisiae* or *N. crassa* (Table 1). The profound knowledge of the cell biology and molecular genetics of *S. pombe* make it a highly interesting model to study the processes of mitochondrial biogenesis in the future in order both to identify the organism-specific features and to extract the general principles of mitochondrial protein sorting.

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