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Role of the Intermembrane-Space Domain of the Preprotein Receptor Tom22 in Protein Import into Mitochondria

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Tom22 is an essential component of the protein translocation complex (Tom complex) of the mitochondrial outer membrane. The N-terminal domain of Tom22 functions as a preprotein receptor in cooperation with Tom20. The role of the C-terminal domain of Tom22, which is exposed to the intermembrane space (IMS), in its own assembly into the Tom complex and in the import of other preproteins was investigated. The C-terminal domain of Tom22 is not essential for the targeting and assembly of this protein, as constructs lacking part or all of the IMS domain became imported into mitochondria and assembled into the Tom complex. Mutant strains of *Neurospora* expressing the truncated Tom22 proteins were generated by a novel procedure. These mutants displayed wild-type growth rates, in contrast to cells lacking Tom22, which are not viable. The import of proteins into the outer membrane and the IMS of isolated mutant mitochondria was not affected. Some but not all preproteins destined for the matrix and inner membrane were imported less efficiently. The reduced import was not due to impaired interaction of presequences with their specific binding site on the *trans* side of the outer membrane. Rather, the IMS domain of Tom22 appears to slightly enhance the efficiency of the transfer of these preproteins to the import machinery of the inner membrane.

The biogenesis of mitochondria depends on the accurate targeting of nucleus-encoded preproteins to the organelle and their import into the correct submitochondrial locations (for reviews, see references 9, 17, 33, 35, 42, 43). These processes are achieved through the action of two independent protein translocation machineries, one in each of the mitochondrial outer and inner membranes (3, 13, 15, 41). In *Neurospora crassa* mitochondria, the protein translocation machinery of the outer membrane is organized into a multisubunit complex (Tom complex) consisting of components which are exposed to the cytosol. These include the proteins Tom20 (formerly termed MOM19; see reference 31), Tom22 (MOM22), and Tom70 (MOM72). Other components such as Tom40 (MOM38), Tom7 (MOM7), and Tom5 (MOM8) are almost completely embedded in the outer membrane (for a review, see reference 22). A complex of similar composition exists in *Saccharomyces cerevisiae* mitochondria.

Although many members of the outer membrane import machinery have been identified and their roles have been postulated (11, 12, 18, 44, 47), precise functions have been ascribed only to proteins which are exposed to the mitochondrial surface. The cytosolic domains of Tom20 and Tom22 cooperate to form a binding site specific for mitochondrial presequences at the organelle's surface (termed the *cis* site) (25, 26). Ionic interactions between the positively charged presequences and negative patches of the cytoplasmic portions of Tom20 and Tom22 seem to underlie this recognition process. Both genetic and biochemical studies suggest that Tom20 and Tom22 are closely associated within the Tom complex and act as the main entry point for preproteins into mitochondria (10, 25, 28). Tom70, on the other hand, increases the import efficiency of only a subset of precursor proteins, including the ADP/ATP carrier (AAC), the phosphate carrier, and cytochrome c_1 (12, 37, 47). Tom37, a surface component interacting with Tom70, has been found only in *S. cerevisiae* (7). The two proteins form a subcomplex which may serve as the binding site for preproteins targeted to mitochondria by MSF, the cytosolic chaperone mitochondrial import stimulating factor (8).

Little is known about the function of the membrane-embedded constituents of the protein import complex and of those parts of the translocation machinery that are exposed to the intermembrane space (IMS). Recently, studies using highly purified outer membranes vesicles have suggested an important role of the *trans* side of the outer membrane in the initiation of protein import into mitochondria. After interacting with the *cis* site, the presequence becomes translocated across the membrane and associates with a presequence-specific binding site (termed the *trans* site) (26). Interaction with the *trans* site drives translocation of the presequence and is accompanied by unfolding of those parts of the precursor protein immediately adjacent to the presequence (26).

According to the membrane arrangement of the known components of the protein import complex, Tom22 is a possible candidate for participating in presequence binding to the *trans* site. It spans the outer membrane once, exposing its N-terminal domain to the cytosol and its C-terminal region to the IMS. The IMS region carries a net negative charge (18) and therefore might be able to interact with positively charged presequences. Other potential functions for the IMS domain of Tom22 include facilitation of the release of preproteins into the lipid bilayer of the outer membrane or into the IMS and the docking of the Tom complex to the inner membrane import machinery.

In this study, we have addressed two central questions con-

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cerning the function of the IMS domain of Tom22. First, we examined its role in targeting and assembly of Tom22 into the outer membrane. Second, the potential requirement of this domain for protein transport of other mitochondrial precursors was analyzed. On the basis of our previous achievements of inactivating genes in *N. crassa* by the method of sheltered disruption (28), we now have developed a genetic method allowing the functional analysis of specific mutant alleles, even if they are deleterious. Using this system, we have generated two mutant Tom22 proteins, lacking either two-thirds or all of the IMS-exposed portion of Tom22. We find that the IMS domain is not essential for the assembly of Tom22 into the Tom complex and does not play a crucial role in the import of other preproteins into mitochondria. Nonetheless, this part of Tom22 may enhance the efficiency of transferring preproteins from the *trans* site of the outer membrane to the translocation machinery of the inner membrane.

MATERIALS AND METHODS

Growth and transformation of *Neurospora* strains. The control strain used in this study is the heterokaryon HP-1, which has the wild-type mitochondrial import apparatus (28). Altered *tom-22* coding sequences were introduced into the HP-1 derivative strain ND-113-1, a heterokaryon in which one nuclear type contains a *tom-22* allele disrupted by a hygromycin resistance gene and the other contains the wild-type *tom-22* allele (28). Spheroplasts of ND-113-1 were transformed as described previously (1, 40) with plasmids pBB22-36 or pDC-3 (see below), to generate strains DC-47A and FD-7-1, respectively. After transformation, spheroplasts were grown in the presence of *p*-fluorophenylalanine and histidine, to select for the *tom-22*-disrupted nuclei, and bleomycin, to select for positive transformants in the nuclei. In previous experiments we had demonstrated that both an intact *tom-22* gene and a bleomycin resistance gene were essential for obtaining transformants under these conditions (28). The colonies were purified by streaking on the same medium, and their homokaryotic state was confirmed through nutritional requirement tests (28).

Neurospora cultures were maintained as described previously (5, 10). All media used to propagate strains DC-47A and FD-7-1 contained 0.2 mg of histidine per ml (28). For mitochondrial isolation, strains HP-1, DC-47A, and FD-7-1 were grown in liquid Vogel's medium (5) for 14 h at 25°C. The linear growth rate of the different *Neurospora* strains was measured along on agar surface in race tubes (5). These provide a convenient and accurate measure of the mycelial elongation rate of *Neurospora* strains (see, e.g., references 10 and 28).

Plasmids. pTom22Δ*Sph*I was generated from the cloned *tom-22* cDNA in pVOLL (18, 28) by digestion of the plasmid with *Sph*I and religation to remove the DNA encoding amino acids 120 to 154 of Tom22. The resulting plasmid was cleaved at the downstream *Hin*dIII site in the pGEM4 (Promega) vector DNA and was treated with Klenow fragment to generate blunt ends. The linker 5′T AGTAGCGGCCGCTACTA was ligated into this DNA to introduce two tandem in-frame stop codons (TAG) and a *Not*I restriction site. This plasmid, pTom22(119), carries the cassette which encodes a truncated protein comprising amino acids 1 to 119 of Tom22 plus two amino acids (QA) derived from vector DNA. pNRC19 was generated from the cDNA sequence in pVOLL by changing the GAC codon for amino acid 106 to the stop codon TAG by site-directed mutagenesis (20).

For expression in *N. crassa*, the appropriate sequences from pTom22(119) and pNRC19 were recloned into plasmids designed for in vivo expression and for selection of transformants with a bleomycin resistance marker. The resulting plasmids are pBB22-36 and pDC-3, respectively (see Fig. 1).

Miscellaneous procedures. The following published procedures were used: standard DNA manipulations (34); raising of antisera (44); blotting of proteins onto nitrocellulose and immunostaining with the chemiluminescence detection system (ECL kit; Amersham) and quantitation of the resulting bands on X-ray film with a Pharmacia Image Master densitometer (24); in vitro transcription and translation reactions using [^{35}S]methionine as a label (ICN Radiochemicals) (45); isolation of mitochondria (10); generation of mitoplasts by digitonin treatment (41); in vitro import of radiolabelled preproteins (28); preparation of outer membrane vesicles (24); and *trans* site binding and unfolding assays (26). For coimmunoprecipitation experiments, radiolabelled Tom22, Tom22(105), or Tom22(119) was imported under standard conditions (16) and the resulting reaction mixtures were diluted with 1 ml of SEM buffer (220 mM sucrose, 1 mM EDTA, 10 mM MOPS [morpholinepropanesulfonic acid] [pH 7.2]). The mitochondria were reisolated by centrifugation, and outer membrane receptor complexes were coimmunoprecipitated as described previously (19).

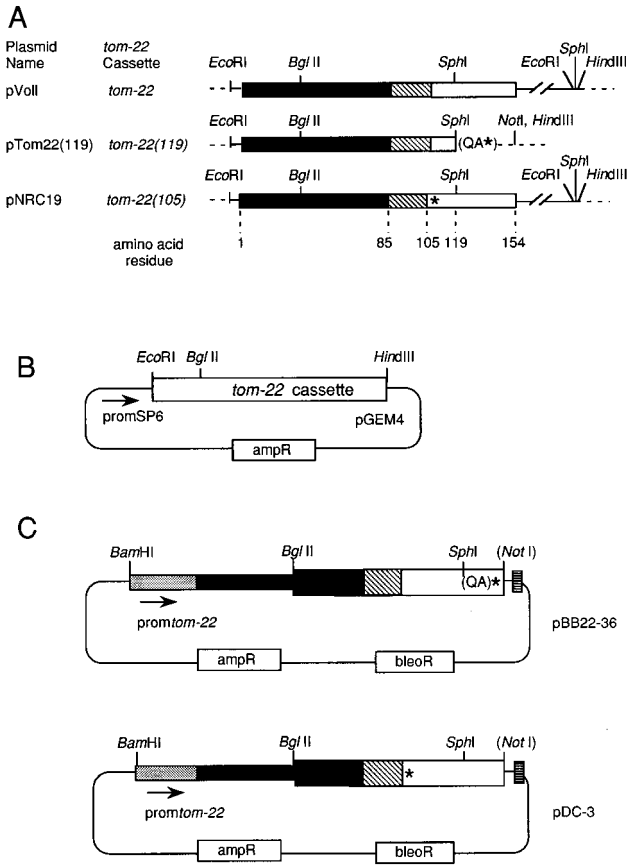
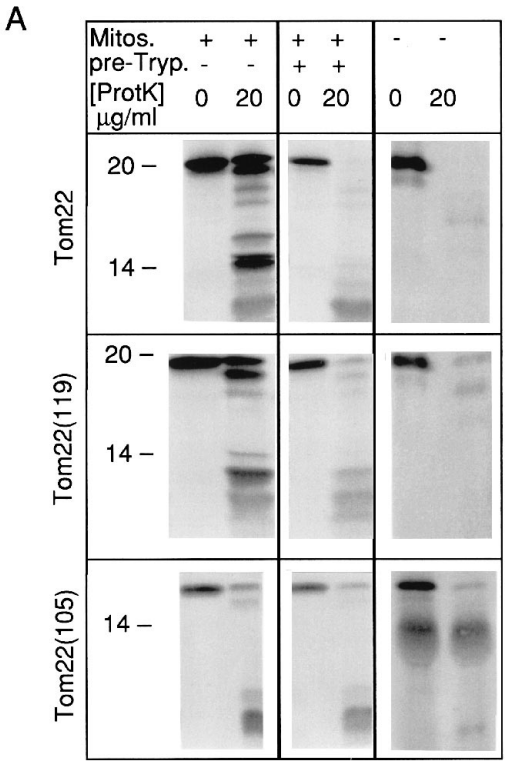
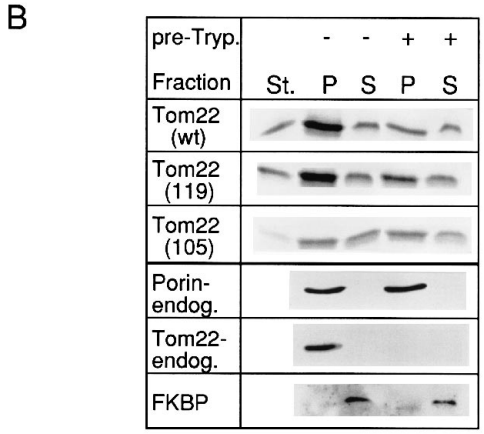


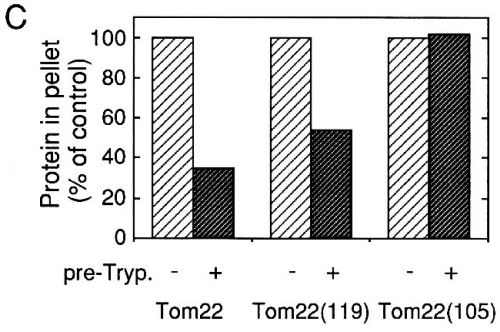
FIG. 1. Deletions in the C terminus of Tom22. (A) Cassettes expressing different forms of Tom22. The cytosolic region, the *trans*-membrane sequence, and the IMS domain of the encoded Tom22 proteins are indicated as solid, hatched, and open boxes, respectively. The thin line represents about 700 bp of noncoding DNA flanking the coding sequence in the original Tom22 cDNA clone, pVOLL (18). Dotted lines indicate vector DNA. The additional 2 amino acids encoded by the vector sequence are shown in parentheses at the end of the truncated *tom-22*(*119*); the asterisk indicates the stop codons in the *tom-22*(*105*) and *tom-22*(*119*) coding sequences. The restriction sites used in constructing the cassettes in panel A and the plasmids in panels B and C are indicated. The *Not*I site that was destroyed during the cloning operations is shown in parentheses. (B) Plasmid used for in vitro transcription and translation of altered Tom22 proteins. The *tom-22* cassettes in pGEM4 (Promega) were positioned downstream of the bacteriophage SP6 promoter (promSP6); ampR indicates the ampicillin resistance gene in pGEM4. (C) The plasmids were constructed in the vector pTZ19R (United States Biochemicals). Plasmid pBB22-36 was created in a multistep process (not shown) using a portion of plasmid pTom22(119) (see panel A), the *Aspergillus nidulans trpC* transcription terminator (small striped box) from pCSN43 (46), a bleomycin resistance gene (bleoR) from plasmid pAB366-2 (2), and a genomic *Bam*HI-*Bgl*II fragment (narrow box) derived from plasmid pR22-B11 (not shown), carrying the amino-terminal portion of the *tom-22* gene as well as about 1.3 kb of upstream sequence (speckled part of narrow box). Plasmid pDC-3 was generated by replacing the 0.3-kb *Bgl*II-*Sph*I fragment of pBB22-36 with the corresponding region of pNRC19 (A). Thus, pBB22-36 and pDC-3 express the *tom-22*(*119*) and *tom-22*(*105*) cassettes, respectively.

RESULTS

Tom22 is imported into mitochondria in the absence of its IMS domain. For the analysis of the Tom22 import requirements, shortened forms of Tom22 lacking all or part of the IMS domain were generated (Fig. 1A). The truncated proteins Tom22(105) and Tom22(119) comprise the first 105 and 119 amino acid residues, respectively, of the Tom22 protein. Tom22(119) carries at its C terminus two additional amino acid residues (QA), which are encoded by vector DNA. These truncated Tom22 coding sequences were cloned in the vector







pGEM4 (Fig. 1B). The corresponding proteins were synthesized by in vitro transcription and translation and imported into mitochondria isolated from wild-type *N. crassa* (Fig. 2). The import of Tom22 can be followed by the generation of characteristic proteolytic fragments which are observed only after integration of Tom22 into the membrane (16, 18). After import, the two Tom22 derivatives were degraded to a discrete set of fragments when mitochondria were treated with proteinase K (Fig. 2A, left panel). As expected from the shorter length of Tom22(105) and Tom22(119), the fragments generated from these proteins migrated slightly faster than the corresponding fragments derived from wild-type Tom22. In the absence of mitochondria these discrete proteolytic products were not formed (Fig. 2A, right panel). When mitochondria were pretreated with trypsin to remove the surface receptors, the amount of the characteristic fragments derived from Tom22 and Tom22(119) was reduced by 96 and 62%, respectively (Fig. 2A, middle panel) (28). This observation reflects the dependence of Tom22 import on the receptor proteins Tom20 and Tom70 (16). In contrast, Tom22(105) was imported in a receptor-independent manner, as the pattern and intensity of the proteolytic fragments remained virtually unaffected by the trypsin pretreatment of the mitochondria (reduction by 14% relative to mock-treated mitochondria).

Membrane insertion of the various Tom22 proteins was assessed by measuring their levels of resistance to extraction with alkaline buffers, a procedure which distinguishes soluble and peripherally membrane-bound proteins from integral proteins such as porin or Tom22 (Fig. 2B). Without the addition of mitochondria, most of the Tom22 precursor was recovered in the supernatant after the extraction procedure (not shown; cf. reference 24). Following the import reaction, a large amount of each of the Tom22 proteins was found in the membrane pellet, indicating that most of the bound material had been inserted into the membrane (Fig. 2B). The fractions of Tom22

FIG. 2. Import of Tom22, Tom22(105), and Tom22(119) into isolated mitochondria. (A) Radiolabelled Tom22, Tom22(105), and Tom22(119) were imported into freshly isolated mitochondria (Mitos.) that were either untreated (−) or pretreated (+) with trypsin (pre-Tryp.) as previously described (23). The import reactions were carried out under standard conditions at 25°C for 7 min (16). The import mixtures were then incubated with or without proteinase K (ProtK) for 15 min at 0°C. After inactivation of the protease with phenylmethylsulfonyl fluoride (final concentration, 1 mM), the samples were diluted with SEM buffer. The mitochondria were reisolated by centrifugation, resuspended in sample buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Radioactively labelled proteins were visualized by fluorography. In parallel to the import reactions, samples containing radiolabelled preproteins were treated in the absence of mitochondria with proteinase K. After the addition of phenylmethylsulfonyl fluoride, the samples were run on a gel without a prior centrifugation step. The thick band in the Tom22(105) samples lacking mitochondria is present after synthesis of this preprotein in rabbit reticulocyte lysate and is normally washed away during mitochondrial reisolation after import reactions. The positions of the 20- and 14-kDa molecular mass markers are indicated to the left of the figure. (B) Alkaline extraction of imported Tom22 proteins. The radiolabelled proteins were imported as described above, and this was followed by reisolation of the mitochondria and washing with alkaline buffer (100 mM sodium carbonate, pH 10.5). The membranes (P) were reisolated by centrifugation for 30 min at 100,000 × *g* at 2°C. Proteins in the supernatant (S) were precipitated with trichloroacetic acid and collected by centrifugation. These samples were analyzed by SDS-PAGE, blotting onto nitrocellulose, and autoradiography. The blot was immunostained for endogenous porin (Porin-endog.), Tom22 (Tom22-endog.), and the soluble FK506-binding protein (FKBP [48]). Note that Tom22 is a trypsin-sensitive surface protein (18). St, 10% input standard. (C) The data from panel B were quantitated by densitometry and, for each Tom22 protein, are given relative to the import into mock-treated mitochondria. The amount of imported material in this case was 18, 28, and 40% of input Tom22, Tom22(119), and Tom22(105) preproteins, respectively. These twofold differences in the import efficiencies are not significant, because different lysate batches of the same preprotein can exhibit a similar degree of variability.

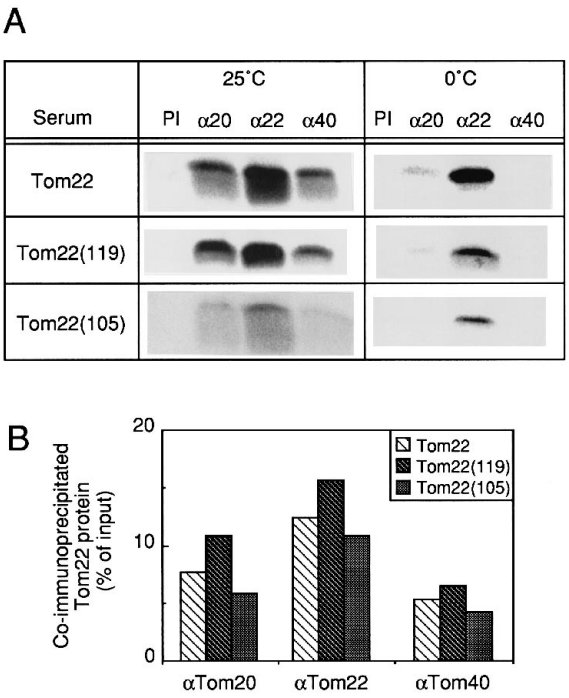


FIG. 3. Assembly of truncated Tom22 into the receptor complex. (A) Import reactions were carried out at 25°C as described above, and the mitochondria were reisolated and dissolved in lysis buffer containing 0.5% digitonin (19). Antisera against Tom20 (α20), Tom22 (α22), Tom40 (α40) were used to immunoprecipitate the Tom complexes, which were then analyzed by SDS-PAGE and autoradiography. As a negative control, the procedure was performed with preimmune serum (PI). To control for nonspecific association of Tom22 with the antisera or the complex after lysis, mitochondria and radiolabelled preproteins were mixed at 0°C and immediately reisolated prior to the precipitation reaction. These conditions allow the association of Tom22 with the mitochondrial surface but result in little import. (B) Quantitative analysis of the coimmunoprecipitated material after import at 25°C. Data shown in panel A were quantitated by densitometry and are given relative to the input precursors. Note that Tom22 and Tom22(119) contain four methionine residues, while in Tom22(105) only two remain.

and Tom22(119) but not that of Tom22(105) in the pellet were largely decreased by using trypsin-pretreated rather than intact mitochondria (Fig. 2C). These results support the conclusion drawn above that removal of the C terminus of Tom22 renders its import independent of surface-exposed receptors. Nonetheless, all three Tom22 proteins became imported with comparable efficiencies into the mitochondrial outer membrane (Fig. 2C). Thus, the C terminus of Tom22 does not harbor essential signals which are important for targeting and membrane insertion.

Tom22 proteins lacking the IMS domain are assembled into the Tom complex. We next examined whether imported Tom22 (105) and Tom22(119) were assembled into the protein import machinery of the outer membrane. The Tom complex can be isolated from detergent-lysed mitochondria by coimmunoprecipitation with antibodies against one component of the complex (19). After import at 25°C, each of the radiolabelled Tom22 proteins could be coprecipitated by specific antibodies against Tom20 and Tom40, demonstrating that the imported Tom22 proteins were firmly associated with the Tom complex (Fig. 3A). The amounts of material coimmunoprecipitated with a particular anti-Tom antibody were similar for all three Tom22 proteins (Fig. 3B), demonstrating that the assembly was unaffected by the truncations. In contrast, after a brief incubation at 0°C (a condition which significantly reduces the import of Tom22) (data not shown), Tom22 proteins could not be coimmunoprecipitated efficiently. Thus, Tom22(105) and Tom22 (119) assemble into the protein import complex in a manner indistinguishable from that of authentic Tom22. In summary, the IMS-exposed segment of Tom22 is dispensable for targeting, membrane insertion, and assembly of the protein. Therefore, the signals harboring this information are localized in the N-terminal segment of the protein.

***Neurospora* strains expressing the truncated Tom22 proteins display normal cell growth.** To initiate the study of the functional role of the IMS domain of Tom22 in mitochondrial protein import, the coding sequences of Tom22(105) and Tom22 (119) were transferred to a *Neurospora* expression vector harboring a selectable bleomycin resistance marker (Fig. 1C). In these plasmids, the *tom-22* genes are downstream of 1.3 kb of genomic DNA, which presumably includes the sequence necessary for the expression of the gene. For transformation, we used the heterokaryotic *Neurospora* strain ND-113-1, which contains two types of nuclei, one with a wild-type *tom-22* gene and another one in which the *tom-22* allele (Δ*tom-22*) is disrupted (28). The latter nuclear type also confers a histidine requirement and resistance to the amino acid analog *p*-fluorophenylalanine. Transformants were plated on medium containing bleomycin, *p*-fluorophenylalanine, and histidine to select for Δ*tom-22* nuclei containing the transformation plasmid. No bleomycin-resistant colonies were obtained when the vector alone was used for transformation (not shown) (cf. reference 28). Single homokaryotic isolates were screened for expression of the mutant Tom22 proteins. Strains DC-47A and FD-7-1, expressing wild-type amounts of Tom22(119) and Tom22(105), respectively, were selected for further investigation (Fig. 4A). The two truncated Tom22 proteins were not recognized by an antiserum raised against the C-terminal 13 amino acid residues of Tom22, thus confirming the deletion of this part of the protein.

The ability of Tom22(105) and Tom22(119) to rescue homokaryons carrying the Δ*tom-22* allele indicated that the truncated proteins are imported and assembled in vivo and that they can complement the defects arising from the lack of wild-type Tom22. The transformation efficiencies with the truncated *tom-22* versions were comparable to that with wild-type *tom-22* (not shown). This argues against the possibility that we have isolated strains harboring suppressors. For further analysis of the strains carrying the Tom22 truncations, we measured their growth along an agar surface contained in long glass tubes, known as race tubes (5). At 25°C, both the DC-47A and FD-7-1 strains grew at rates indistinguishable from those of the wild-type strain, HP-1, and the heterokaryon ND-113-1 (Fig. 4B) (28). The four strains also grew at identical rates at 12 and 37°C, as did several other independent transformants (data not shown). Thus, mitochondria harboring the truncated forms of Tom22 maintain all the functions, including respiratory competence, that are required for normal growth rates.

Cells lacking Tom22 contain reduced amounts of Tom20 in the outer membrane (28). In contrast, cells expressing the truncated forms of Tom22 described in this work contained levels of Tom20 similar to those in the wild-type strain HP-1 (Fig. 4A). Thus, these shortened Tom22 proteins support the maintenance of Tom20 in the membrane. No significant changes in the mitochondrial levels of other members of the Tom complex (Tom40 and Tom70) or in that of porin were found.

Protein import into mitochondria lacking the IMS domain of Tom22. The availability of mutant strains carrying Tom22 with C-terminal truncations facilitated the biochemical analysis of the functional role of the IMS domain of Tom22 in protein import. We first tested the in vitro import of precursor proteins

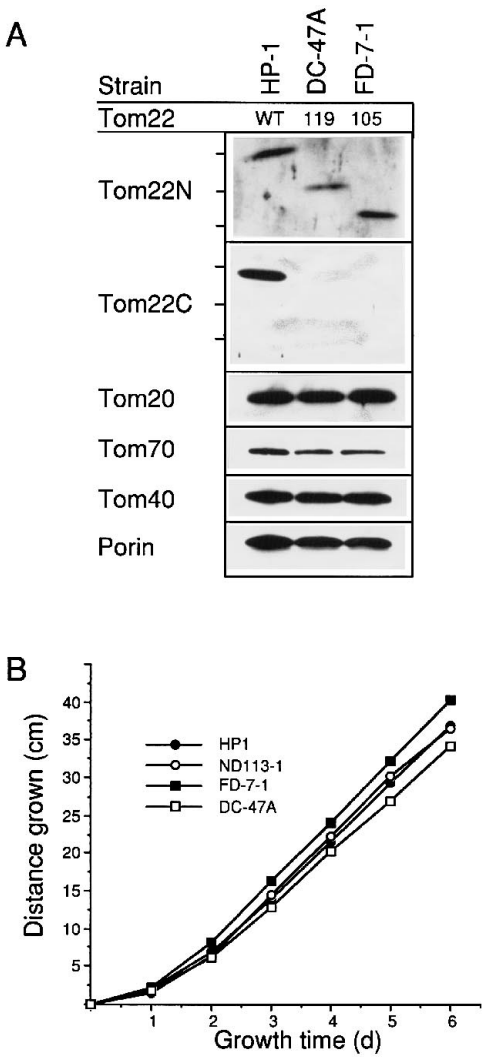


FIG. 4. Expression of Tom22(105) and Tom22(119) in vivo. (A) Protein composition of outer membranes in *Neurospora* strains HP-1, DC-47A, and FD-7-1. Strains were grown in liquid medium, mitochondria were isolated, and 30 μg of protein from each sample was analyzed by SDS-PAGE and immunostaining with the indicated antisera. The positions of the wild-type Tom22, Tom22(119), and Tom22(105) are indicated by dashes to the left of the upper two panels. (B) Growth rates of *Neurospora* strains HP-1, ND-113-1, DC-47A, and FD-7-1 in race tubes. Each strain was inoculated at one end of a race tube, which then was incubated at room temperature. The distance of the hyphal front from the point of inoculation is plotted against time (days). HP-1 and ND-113-1 were grown on minimal medium to maintain them as heterokaryons, while DC-47A and FD-7-1 were grown on histidine-containing minimal medium.

which do not contain cleavable, N-terminal targeting sequences. The import into mutant mitochondria of the outer membrane proteins porin, Tom40, Tom20, and of the IMS protein cytochrome *c* heme lyase (CCHL) was not significantly reduced compared with that into the wild-type organelles (Fig. 5). The import efficiencies of different mitochondrial preparations may vary by ±25%. Therefore, only reductions of more than 50% can be considered significant. Thus, the IMS domain of Tom22 does not seem to play a role in the import of these precursors, none of which crosses the inner membrane nor requires electrochemical potential across this membrane for import. Similar results were observed for the import of the AAC, an inner membrane protein which requires electrochemical potential for its import.

Preproteins destined for the inner membrane and the matrix space were imported into the mutant mitochondria with varying efficiencies (Fig. 5). The precursors of the β-subunit of the F_1-ATPase ($F_1\beta$) and the α-subunit of the matrix-processing peptidase (α-MPP) were imported to similar levels in wild-type and mutant mitochondria. A two to threefold decrease in the import rate was observed for the precursor of the Rieske iron/sulfur protein (Fe/S), and the matrix-targeted fusion protein preSu9-DHFR (a fusion protein comprising amino acids 1 to 69 of subunit 9 of the F_0-ATPase and dihydrofolate reductase) (Fig. 5). The import of the yeast inner membrane protein

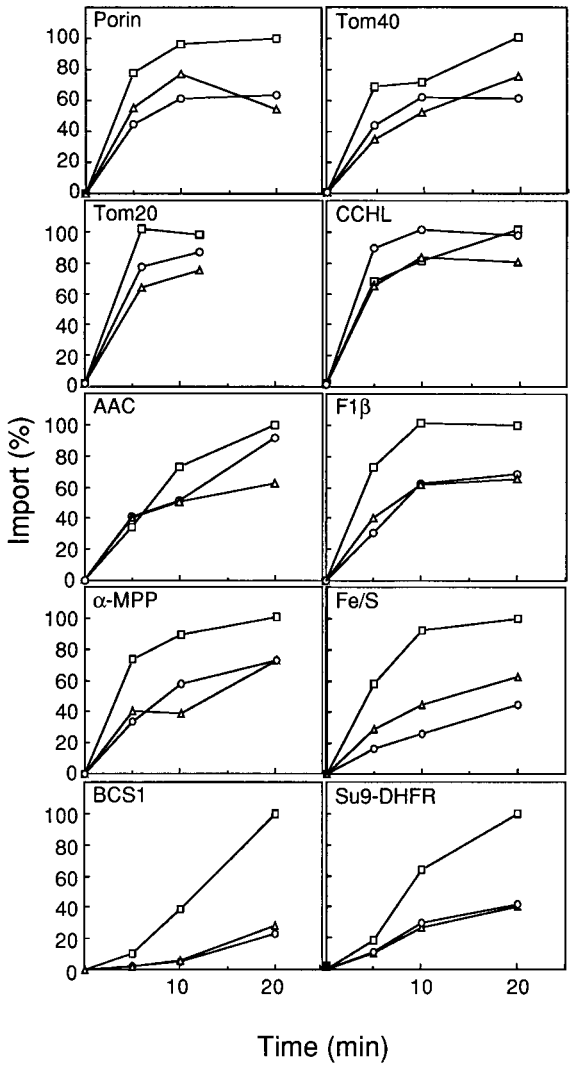


FIG. 5. Import of preproteins into mitochondria lacking the C terminus of Tom22. Radiolabelled precursors were imported into mitochondria isolated from wild-type HP-1 (squares), DC-47A [Tom22(119)] (circles), and FD-7-1 strains [Tom22(105)] (triangles). After the import reaction, the samples were treated with proteinase K to remove nonimported material as previously described (Fig. 2; also see reference 10). Time courses for import were performed at 15°C to ensure protein uptake in the linear range. Precursors of the following proteins were used: porin, Tom40, Tom20, CCHL, AAC, Su9-DHFR (a fusion protein comprising amino acids 1 to 69 of subunit 9 of the F_0-ATPase and dihydrofolate reductase), $F_1\beta$ (β-subunit of the F_1-ATPase), BCS1, (a protein involved in the assembly of the bc_1 complex), Fe/S (Rieske iron/sulfur protein), and α-MPP (α-subunit of the mitochondrial processing peptidase). Import experiments were performed at least three times, and the average data were plotted. The standard error in these experiments was 25%, and for this reason only results differing twofold or more can be considered significant.

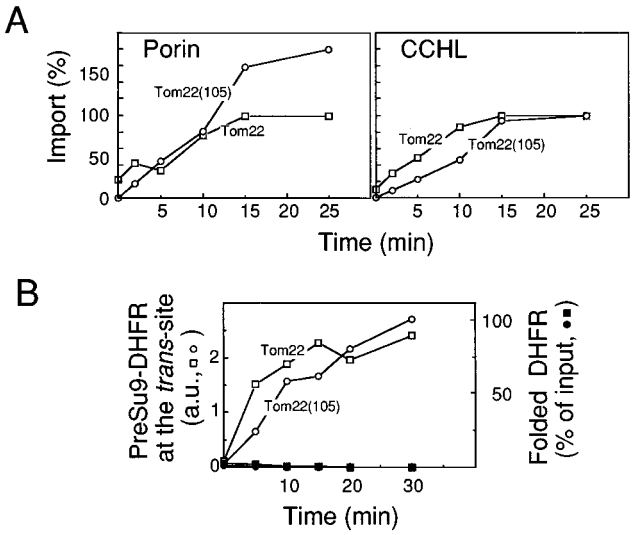


FIG. 6. The C-terminal domain of Tom22 is not involved in presequence binding to the *trans* site. (A) Protein import into outer membrane vesicles isolated from wild-type and FD-7-1 strains. The time courses for import of porin and CCHL into vesicles were performed as described previously (24). The maximum amount of import into the wild-type vesicles was set to 100%. (B) *trans* site binding in outer membrane vesicles. Radiolabelled preSu9-DHFR was incubated with vesicles purified from wild-type or FD-7-1 mitochondria for 20 min at 25°C in 100 μl of import buffer (10 mM MOPS-KOH [pH 7.2], 2.5 mM $MgCl_2$, 20 mM KCl, 0.25 mg of bovine serum albumin per ml) (26). Vesicles were diluted sevenfold with wash buffer (10 mM MOPS-KOH [pH 7.2], 1 mM EDTA, 120 mM KCl) and were reisolated by ultracentrifugation. Pellets were resuspended in import buffer containing 1 mM NADPH and 1 μM methotrexate. One aliquot was precipitated with trichloroacetic acid and analyzed for *trans* site-bound preprotein by SDS-PAGE and densitometry of the fluorographs. Another aliquot was tested for unfolding of the DHFR domain upon *trans* site binding by treatment with proteinase K (100 μg/ml; 15 min; 0°C). After trichloroacetic acid precipitation, samples were further manipulated as described above. a.u., arbitrary units.

BCS1, which contains internal targeting information (6, 30), was reduced about fourfold. In all cases, import into Tom22 (119) and Tom22(105) mitochondria was affected to a similar extent, indicating that the C-terminal 35 amino acid residues of Tom22 are responsible for this effect. In summary, the import rate of some but not all preproteins is reduced in mitochondria harboring the truncated forms of Tom22. However, these effects are much less severe than the >10-fold reduction of import of all these preproteins into mitochondria depleted of either Tom22 (28) or Tom20 (10).

Role of the C terminus of Tom22 in preprotein import. A possible reason for the reduced import of some preproteins into the Tom22(105) and Tom22(119) mitochondria could be the impaired binding of the presequence to the *trans* site, a second presequence-specific binding site on the inner face of the outer membrane (26). Preproteins bound to this site, in contrast to *cis* site-bound preproteins (25), are resistant to high ionic strength (26). As a result of presequence binding to the *trans* site, the immediately following mature part of the preprotein becomes unfolded. To determine the consequences of the deletion of the C terminus of Tom22 for *trans* site binding, we first compared the import efficiency of porin and CCHL into outer membrane vesicles purified from FD-7-1 mutant and wild-type cells (24). No significant differences were observed, showing that the two vesicle preparations were equally efficient in these import reactions (Fig. 6A). Moreover, import of porin and CCHL into vesicles and mitochondria occurred at similar rates (cf. Fig. 6A and Fig. 5).

For *trans* site binding, we utilized the fusion protein preSu9-DHFR (26). The preprotein was incubated with vesicles derived from either wild-type or Tom22(105) mutant mitochondria for 20 min at 25°C. The vesicles were washed with a salt solution to exclusively monitor *trans* site binding (26). Binding of preSu9-DHFR to the *trans* site was identical for both vesicle preparations (Fig. 6B). In both cases, *trans* site binding was accompanied by the quantitative unfolding of the DHFR domain, a further demonstration that the preprotein had entered the translocation machinery. Therefore, the IMS-exposed domain of Tom22 does not form an important part of the *trans* site, nor does it participate in an essential step preceding *trans* site binding.

Finally, we investigated whether the IMS domain of Tom22 plays a role in a step following presequence interaction at the *trans* site. To this end, the efficiency of preprotein import into mitoplasts (i.e., mitochondria with opened outer membranes) was tested. In this case, import occurs directly across the inner membrane (14, 20a). Mitoplasts were generated from wild-type and Tom22(105) mitochondria by digitonin treatment (41). The selective opening of the outer membranes was confirmed by immunostaining for IMS (CCHL), inner membrane (AAC), and matrix (Hsp60) markers (Fig. 7, lower panel). The mitoplasts were then used in protein import assays performed under conditions ensuring the linear uptake of the preprotein (not shown). The efficiencies of import of preSu9-DHFR into

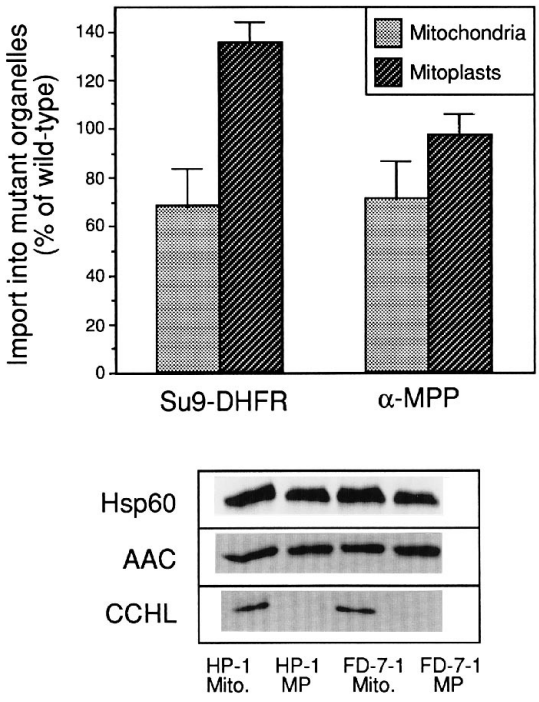


FIG. 7. Protein import is restored to wild-type levels after the opening of the outer membrane. Mitoplasts were generated by incubating mitochondria from HP-1 and FD-7-1 strains for 2 min on ice with 0.17% digitonin (42). Organelles were diluted 10-fold with SEM buffer and reisolated. Import reactions into intact mitochondria (Mito.) or mitoplasts (MP) were performed for 15 min at 15°C as described in the legend to Fig. 5. Preprotein import observed with organelles derived from FD-7-1 is given relative to that of the corresponding wild-type samples. The experiments were repeated four times, and the average data are plotted (upper panel). Bars indicate standard errors. The selective opening of the outer membrane was confirmed by Western blot (immunoblot) analysis of the organelles after protease treatment (lower panel). A representative example is shown. CCHL and the 60-kDa heat shock protein (Hsp60) are protease-sensitive markers for the IMS and matrix, respectively. The inner membrane AAC is not degraded under the conditions used.

wild-type and Tom22(105) mutant mitoplasts were comparable, in contrast to the twofold differences seen with intact mitochondria (Fig. 7, top panel). As a control, we analyzed the import of pre-α-MPP, which did not significantly differ in wild-type and Tom22(105) mitochondria (also Fig. 5). The same finding was made for mitoplasts, showing that the restoration of the import of preSu9-DHFR into Tom22(105) mitoplasts was specific. Thus, the inner membranes of Tom22(105) mitochondria can import preproteins at the same rates as can wild-type organelles. In summary, our data suggest that the C-terminal portion of Tom22 plays an auxiliary function in enhancing the transfer of preproteins from the outer to the inner membrane.

DISCUSSION

In this communication, we present a functional analysis of the IMS-exposed part of Tom22 in the assembly of Tom22 itself and in the import of other preproteins. We demonstrate that the C terminus of Tom22 is dispensable for targeting and assembly of this protein into the outer membrane import complex. Therefore, sorting of Tom22 depends on signals in the N-terminal two-thirds of the protein. Preliminary results suggest that the cytosolic domain of Tom22 may bear at least part of the targeting information, since proteins truncated at their N termini do not acquire a protease-resistant state upon import (4a). In this respect, Tom22 may behave differently from Bcl-2, a tail-anchored protein (21), which spans the mitochondrial outer membrane in the same orientation as Tom22. The C-terminal membrane anchor of Bcl-2 is sufficient for insertion into the outer membrane (29). Tom22 therefore differs from such tail-anchored proteins in that it contains targeting information in a domain other than the C terminus. Our data show that the IMS domain of Tom22 is not passively translocated as cargo but rather requires the function of preprotein receptors. It will be important to determine what causes this receptor dependence and whether it involves direct recognition of the IMS domain of Tom22 by the surface receptors.

The import of the Tom22(105) precursor is reminiscent of that of Tom20, which also occurs efficiently in the absence of surface-exposed components such as Tom20 and Tom22 (10, 28, 39). In contrast to Tom22, Tom20 is anchored in the membrane via an N-terminal hydrophobic sequence and exposes at most two amino acid residues to the IMS (39). One cannot, however, draw the conclusion that all preproteins lacking an IMS domain are inserted into the membrane without the assistance of receptors. For instance, *Neurospora* Tom70 can be imported into the outer membrane in the absence of surface-exposed components (38), even though it has a 38-amino-acid IMS domain. As previously suggested, the receptor dependence may be connected to the folded state of the domain to be translocated across the membrane (43). The IMS domain of the *Neurospora* Tom70 contains a high proportion of proline residues and thus may exist in a flexible conformation that is easily transferred across the membrane.

The development of the sheltered-disruption methodology has facilitated the in vivo analysis of essential genes, such as *tom-22*, in *N. crassa* (28). As demonstrated here, the resulting heterokaryotic strains can be used to express mutant proteins in place of the wild-type version, allowing the dissection of the function of these proteins. The mutants of the essential *tom-22* gene which we have studied here proved to be nondeleterious for cell growth. However, our novel method is also applicable for the study of mutants which exhibit severe growth defects. In these cases, the initial selection would be for a heterokaryotic strain which maintains the wild-type copy of the gene of interest in one type of nucleus and which carries the transformed mutant gene in the disrupted nucleus. As for the analysis of sheltered disruption mutants (28), the transformed heterokaryon would then be cultivated under conditions which select against wild-type nuclei. This results in a cell culture in which only the mutated gene product is expressed.

Applying this novel approach to Tom22, we demonstrate that the import of most mitochondrial preproteins was not affected by the absence of the IMS domain of Tom22. The few preproteins that displayed reduced import efficiencies contain presequences, suggesting that Tom22 could be involved in presequence binding to the *trans* site. This idea was disproven by experiments using outer membrane vesicles. We could demonstrate that the IMS domain of Tom22 is not essential for *trans* site binding or for any step preceding it. This result is corroborated by the inability to detect an interaction between presequences or preproteins and the IMS domain of Tom22 using glutathione *S*-transferase fusion proteins and by the finding that the IMS domain of Tom22 did not inhibit in vitro protein import into mitochondria or *trans* site binding in outer membrane vesicles (data not shown). The latter experiments should have detected even labile interactions between the peptide and the preproteins.

Our data on protein import into mitoplasts suggest that the IMS domain of Tom22 may slightly increase the efficiency of presequence transfer from the *trans* site to the inner membrane for some preproteins. The import complexes of the two mitochondrial membranes are closely opposed during the import of preproteins into the matrix (3, 13, 32, 36). The IMS domain of Tom22 may be one of several structural elements involved in establishing an interaction between the two import complexes. Although the two machineries can act independently (15, 41), protein import may be most efficient when they are in proximity so that the preprotein can be transferred directly from the outer to the inner membrane import machinery. If this association is more labile in Tom22 mutant mitochondria, there may be a reduction in the transfer of especially those precursors which exhibit lower affinities for the inner membrane machinery. The recent identification of a number of components of the inner membrane import machinery (cf. references 3 and 33) will facilitate the experimental testing of these proposed interactions.

Recently, similar investigations on the IMS domain of *Saccharomyces cerevisiae* Tom22 have been reported (4, 27). Even though almost identical deletions were generated in the C terminus of Tom22, contradictory results were obtained. Nakai et al. (27) did not observe a growth defect in yeast cells expressing mutant Tom22. Deletion of the IMS domain of Tom22 had no effect on protein import into these mitochondria. In contrast, the Tom22 mutant cells generated by Bolliger et al. (4) exhibited poor viability. Furthermore, the simultaneous presence of wild-type and truncated Tom22 proteins resulted in a dominant negative growth phenotype. In support of a role of the IMS domain of Tom22 in protein import, an interaction of this domain with preproteins and presequences was reported. In addition, protein import into Tom22 mutant mitochondria was reduced three- to eightfold. Both our mutants harboring truncated versions of *Neurospora* Tom22 and our inability to detect stable interactions between presequences and the Tom22 C terminus support and extend the findings made by Nakai et al. (27).

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