Translocation of preproteins across the mitochondrial outer membrane is mediated by the translocase of the outer mitochondrial membrane (TOM) complex. We report the molecular identification of Tom6 and Tom7, two small subunits of the TOM core complex in the fungus Neurospora crassa. Cross-linking experiments showed that both proteins were found to be in direct contact with the major component of the pore, Tom40. In addition, Tom6 was observed to interact with Tom22 in a manner that depends on the presence of preproteins in transit. Precursors of both proteins are able to insert into the outer membrane in vitro and are assembled into authentic TOM complexes. The insertion pathway of these proteins shares a common binding site with the general import pathway as the assembly of both Tom6 and Tom7 was competed by a matrix-destined precursor protein. This assembly was dependent on the integrity of receptor components of the TOM machinery and is highly specific as in vitro-synthesized yeast Tom6 was not assembled into N. crassa TOM complex. The targeting and assembly information within the Tom6 sequence was found to be located in the transmembrane segment and a flanking segment toward the N-terminal, cytosolic side. A hybrid protein composed of the C-terminal domain of yeast Tom6 and the cytosolic domain of N. crassa Tom6 was targeted to the mitochondria but was not taken up into TOM complexes. Thus, both segments are required for assembly into the TOM complex. A model for the topogenesis of the small Tom subunits is discussed.

The import of proteins into mitochondria is mediated by multisubunit translocases in the outer (TOM1 complex) and inner (TIM complexes) mitochondrial membranes (for reviews see Refs. 1–4). The TOM complex contains components that expose domains to the cytosol and function as preprotein receptors. The major receptor is Tom20, which is involved, together with Tom22, in the translocation of most precursors (5–7). Another receptor that forms a binding site for a more restricted set of preproteins, most notably the mitochondrial carrier family, is Tom70 (8, 9). These receptors are loosely attached to the other components of the TOM machinery that form the core complex (10, 11). The subunits of the core complex (Tom40, Tom22, Tom7, Tom6, and Tom5) are embedded in the outer membrane and form the translocation pore (10, 11). Tom40 represents the major component of the translocation pore (12, 13), whereas Tom22 and Tom5 possibly transfer preproteins from the receptors to the pore (14, 15). The yeast Tom6 and Tom7 were suggested to modulate the stability of the association of the Tom components (16, 17).

Tom6 is a small protein that was first identified in yeast as a high copy number suppressor of a temperature-sensitive mutant of Tom40 (18). Tom6 was proposed to support the cooperation between the receptors, in particular Tom22, and the general insertion pore (10, 15, 16). The protein contains one putative transmembrane domain close to its C terminus and is oriented with its N terminus in the cytosol. Thus, it belongs to the class of membrane proteins with a C-terminal anchor. It has been suggested that the insertion of the protein into the outer membrane is independent of surface receptors or the function of Tom40 (19).

Tom7 in yeast consists of 59 amino acid residues. It’s topology in the mitochondrial outer membrane is unknown (17). A lack of Tom7 was reported to stabilize the interaction of the receptors Tom20 and Tom22 with the pore element, Tom40. These findings suggested that Tom7 plays a role opposite to that of Tom6 by exerting a destabilizing effect on the association of Tom components (17). At present, Tom6 and Tom7 have been investigated only in yeast, though homologues from other organisms have been identified (4). Information on the molecular environment of Tom6 and Tom7 in the TOM complex would be of special interest for understanding the molecular function of the TOM machinery.

As Tom6 is a tail-anchored protein, it can serve as a model protein for the study of membrane insertion and assembly into functional complexes for this group of membrane proteins. Currently, the information on how tail-anchored proteins are targeted to mitochondria and inserted into the mitochondrial outer membrane is very limited. In this report we describe the cloning of TOM6 and TOM7 from the fungus Neurospora crassa electrophoresis; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol.
and an analysis of the processes by which they are inserted into the mitochondrial outer membrane and assembled into the TOM core complex. We also investigated the molecular environment of the two proteins in their assembled state and found them to be in direct contact with Tom40, whereas Tom6 was also found to interact with Tom22 in a manner that depends on the presence of preproteins in transit.

**EXPERIMENTAL PROCEDURES**

Cloning of *N. crassa* Tom6 and Tom7—The TOM complex was isolated according to Künkele et al. (20), and its protein components were separated by urea-SDS polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane (20). The bands corresponding to Tom6 and Tom7 were isolated, and their N-terminal sequences were determined by Edman degradation. For Tom7, 25 amino acid residues were determined, and degenerate primers were synthesized for use in PCR reactions utilizing a cDNA library from *N. crassa* as the template. By this method a 70-base pair DNA fragment was amplified for use in PCR reactions utilizing a cDNA library from residues were determined, and degenerate primers were synthesized according to Tom6 and Tom7 were isolated, and their N-terminal sequences were separated by urea-SDS polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membrane and nitrocellulose membrane. One cDNA was completely sequenced and found to encode a protein of 60 amino acid residues. For determination of the genomic sequence a cosmid containing the TOM6 gene was isolated, and the TOM6-containing part was sequenced.

For Tom7, 25 residues were determined from the N terminus of the protein. A PCR-based strategy, similar to the one used for Tom6, revealed the entire sequence of both the cDNA and genomic versions of the gene.

Biochemical Procedures—Isolation of mitochondria and outer membrane vesicles (OMV) from *N. crassa* was performed as described (21).

For cross-linking experiments, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added to isolated OMV in either the absence or presence of various amounts of pSu9-DHFR. After incubation for 30 min at 25 °C, excess cross-linker was removed by centrifugation in tube and the mixture was analyzed on a 6–13% gradient blue native gel. The cathode and anode buffers for performing the electrophoresis were as described (22). Electrophoresis was started at 100 V until the samples were within the stacking gel and continued with voltage and current limited to 500 V and 15 mA. At the end of the electrophoresis excess dye was removed, the gel was blotted on a polyvinylidene difluoride membrane using a semi-dry apparatus, and immunodecoration was as described above.

Results

Cloning and Primary Sequence of *N. crassa* Tom6 and Tom7—The N-terminal sequences of isolated Tom6 and Tom7 were determined. Degenerate primers were constructed according to the sequences obtained, and the genomic DNAs cloned by a PCR-based procedure (see details under “Experimental Procedures”). Comparison of the amino acid sequence obtained by the PCR-amplified genomic DNA and cosmid containing TOM6 and TOM7, respectively with the corresponding protein and were used to determine the enrichment of the proteins in purified TOM complex as related to mitochondria or OMV (Fig. 2A).
Tom6 and Tom7 are integral parts of the N. crassa TOM core complex. A, presence of Tom6 and Tom7 in the isolated TOM complex. Intact mitochondria (Mit.), OMV, and purified TOM holo complex (TOM) (50 μg in each lane) were loaded on a high Tris urea SDS-PAGE gel, blotted, and decorated with antibodies against Tom6, Tom7, or Tom40. Apparent molecular masses are given on the left. B, analysis by BNGE of Tom6 and Tom7 in the TOM complex. OMV (20 μg) were solubilized in a buffer containing 1% of digitonin (Dig.), DDM, or Triton X-100 (Tx100) and analyzed by BNGE. For detection of Tom proteins antibodies against Tom6, Tom7, Tom22, and Tom40 were used. C, analysis of Tom6 and Tom7 in the TOM holo complex by co-immunoprecipitation (IP). OMV (50 μg per lane) were lysed in a buffer containing 1% digitonin and added to protein A-coupled Sepharose beads containing prebound antibodies against the indicated Tom components. Proteins bound to the beads were subjected to SDS-PAGE, blotted, and immunodecorated with the antibodies indicated at the left side. Contr., control. D, analysis of Tom6 and Tom7 in the TOM core complex. OMV (60 μg per lane) were lysed in a buffer containing 1% DDM, and clarifying spin was performed (1 h, 100,000 × g, 1.5 cm). Further treatment was as in C.

To analyze the association of Tom6 and Tom7 with the TOM complex in N. crassa, BNGE was conducted on OMV solubilized in the mild detergent digitonin. The conditions during BNGE cause the Tom20 and Tom70 receptors of both the yeast and N. crassa TOM complexes to dissociate from the remaining components that constitute the TOM core complex (10, 11, 23). Both Tom6 and Tom7 comigrate with Tom22 and Tom40 demonstrating their firm association with the core complex (Fig. 2B). The stability of the interaction was further demonstrated by solubilizing OMV with 1% of either β-dodecylmaltoside or Triton X-100. Yeast Tom6 and Tom7 were reported to dissociate from the TOM core complex after solubilization with Triton X-100 (10). In contrast, with N. crassa most of Tom7 and Tom22 and all of Tom6 and Tom40 migrated as a high molecular mass complex (Fig. 2B). This latter complex migrates faster than the complex solubilized with digitonin. This difference can be explained by variations in the lipid contents of the various complexes. We have previously reported a much higher phospholipid content in the complex isolated with digitonin as compared with the complex isolated with DDM (24).

The interactions between the various components of the N. crassa TOM complex were further investigated by co-immunoprecipitation. OMV were solubilized with either digitonin, a detergent that is known to keep the TOM holo complex intact (20), or DDM, which results in the formation of the TOM core complex (11). Tom6 and Tom7 were precipitated with antibodies against Tom22 and Tom40 (Fig. 2, C and D). Hence, both Tom6 and Tom7 are in close association with the other components of the TOM core complex. In agreement with previous observations (10, 11), antibodies against Tom70 precipitated only minor amounts of the other Tom components (Fig. 2, C and D), supporting the notion that Tom70 is loosely associated with the other components of the TOM machinery. In contrast to the observation in yeast (10) antibodies against Tom20 did precipitate with high efficiency the other components of the TOM complex from mitochondria dissolved in digitonin suggesting that each complex contains at least one molecule of Tom20. The observation that not all the Tom20 molecules residing in the outer membrane could be precipitated by antibodies against other components of the TOM complex suggests the existence of a subpopulation of Tom20 molecules that are not, or only loosely, attached to the complex. Antibodies against Tom20 could not, however, precipitate the other Tom components after solubilization of the OMV with β-dodecylmaltoside (Fig. 2D). These conditions are known to result in the formation of the TOM core complex that lacks both receptor proteins, Tom20 and Tom70 (11). Taken together, our data indicate that Tom6 and Tom7 are integral components of the N. crassa TOM core complex.

Tom6 and Tom7 are in Direct Contact with Other Members of the TOM Core Complex—Tom6 was previously found to be in the vicinity of Tom40, and their interaction was modified by the formation of specific translocation intermediates of a precursor protein (25). To determine whether both Tom6 and Tom7 are only in the vicinity of Tom40 or actually in direct contact with the protein, the cross-linking reagent EDC was added to outer membrane vesicles, and cross-linking products were analyzed by immunodecoration. Specific cross-linking adducts of Tom40 with both Tom6 and Tom7 could be identified (Fig. 3A). As EDC is a zero-spacer cross-linking reagent, it can be concluded that Tom40 is in direct contact with both proteins.

In addition, a cross-linking adduct between Tom6 and Tom22 was identified (Fig. 3B). The formation of this adduct was reduced gradually by adding increasing amounts of the precursor, pSu9(1–69)-DHFR, a chimeric preproteins consisting of the first 69 amino acids of subunit 9 of the mitochondrial F<sub>0</sub>-ATPase fused to mouse dihydrofolate reductase, before performing the cross-linking reaction. The Tom6-Tom22 adduct was also observed using other cross-linking reagents (not shown). Hence, Tom6 and Tom22 are in a direct contact, and their interaction is dynamically modulated by preproteins in
transit. A similar interaction between Tom22 and Tom7 was not observed (not shown).

Membrane Insertion and Assembly of Tom6 and Tom7—To determine whether in vitro-synthesized precursors of Tom6 and Tom7 can be imported and assembled into authentic TOM complexes we took advantage of the characteristic migration of the endogenous Tom6 and Tom7 on BNGE. Isolated mitochondria were incubated with precursors of Tom components at various temperatures, solubilized with digitonin, and their proteins were analyzed by BNGE. A significant amount of the precursors of Tom6 and Tom7, like those of Tom22 and Tom40, were assembled in a temperature-dependent manner into the endogenous pre-existing TOM core complex (Fig. 4A). This assembly was specific. In a control experiment the imported precursor of porin, another outer membrane protein, did not migrate with the endogenous TOM complex (not shown). The lower molecular weight bands in Fig. 4A are probably insertion intermediates or unproductively bound precursors, as shown previously when these low molecular weight bands were analyzed in detail following import of Tom40 (23). In this experiment typical precursor-product relationships were not observed for all Tom components. A significant portion of the precursor molecules that are absorbed to the surface of the mitochondria during the incubation at 0 °C dissociate from the mitochondria during the wash and centrifugation, which are performed before loading the material on BNGE. Hence, part of the molecules observed after incubation at 25 °C are products of early intermediates that cannot be observed with BNGE. Assembly of precursors of Tom components into the TOM core complex was also observed after solubilization of mitochondria with another detergent, DDM (Fig. 4B).

The assembly of newly synthesized Tom6 and Tom7 was further tested by co-immunoprecipitation. Radiolabeled precursors were incubated with mitochondria, and following the import reactions, mitochondria were solubilized with digitonin and subjected to immunoprecipitation with antibodies against various Tom components. Both precursors were precipitated with antibodies against Tom20, Tom22, and Tom40 (Fig. 4C).

Thus, both precursor proteins are imported into the mitochondrial outer membrane and assembled into TOM complexes.

The requirements for efficient insertion of Tom6 and Tom7 into the mitochondrial outer membrane were investigated. A previous report suggested that neither import receptors nor Tom40 were required for insertion of Tom6 and that other, unknown, proteins were involved (19). We studied assembly into the endogenous TOM complex as a criterion for correct insertion and addressed the question of whether Tom6 and Tom7 use the general insertion pore of the TOM complex for insertion. The protein conducting pore was blocked by accumulating chemical amounts of a translocation intermediate of the fusion protein pSu9(1–69)-DHFR in the presence of methotrexate (26, 27). When precursors of Tom6 and Tom7 where imported into these blocked mitochondria a strong reduction in the level of assembly of both was observed (Fig. 5A). Thus, precursors of a matrix-destined protein apparently compete with precursors of Tom6 and Tom7 for sites required for translocation. However, we cannot exclude the possibility that the
reduction in assembly upon blocking the pore results from induction of a conformational change in the complex, which masks a distal Tom6 binding site.

Does Tom6 require the receptor components for its proper assembly? Mitochondria were treated with trypsin resulting in the removal of the exposed parts of the surface receptors (Fig. 5B, inset). The ability of Tom6 precursor to assemble into the TOM complex was reduced by this procedure (Fig. 5B). This is in contrast to previous reports (17, 19) and suggests that the receptor proteins significantly enhance the assembly of Tom6. In summary, our results support the hypothesis that Tom6 and Tom7 follow an import pathway involving the TOM complex.

Targeting, Insertion, and Assembly Information within the Sequences of Tom6—Tom6, like all outer membrane proteins, does not contain a cleavable targeting sequence. To determine which portions of the protein contain information for targeting, insertion, and integration we constructed fusion proteins containing Tom6 variants (Fig. 6A). To improve the detection of the newly synthesized Tom6, we used a fusion protein where a DHFR domain was present at the N terminus of Tom6. A similar fusion construct in yeast was shown to be capable of functionally replacing the native Tom6 protein in vivo (19). Our constructs included a chimeric protein with the complete Tom6 protein (DHFR-Tom6) and two mutant variants lacking either residues 1–12 of Tom6 (DHFR-Tom6Δ12) or residues 1–36 (DHFR-Tom6Δ36). All constructs were targeted to mitochondria and inserted into the outer membrane as shown by their recovery in the membrane pellet following carbonate extraction (not shown). Co-immunoprecipitation and BNGE were employed to investigate the assembly of the variant Tom6 precursors into the endogenous TOM complex. Using BNGE we observed that only the wild type construct and the DHFR-Tom6Δ12 variant were assembled into TOM complexes whereas DHFR-Tom6Δ36 was found to be attached to mitochondria but not assembled (Fig. 6B). Assembly of DHFR-Tom6Δ36 was further analyzed by co-immunoprecipitation with antibodies against Tom22 and Tom40. Imported native Tom6 was efficiently co-immunoprecipitated, but only minor amounts of DHFR-Tom6Δ36 were precipitated by these two antibodies (Fig. 6C). These minor amounts of precipitated DHFR-Tom6Δ36 may represent translocation intermediates of the precursors that are attached to, but not assembled into, the TOM complex. Only background levels were precipitated by antibodies against Tom22 in a control experiment where OMV was first solubilized with digitonin, and only then was precursor added (not shown). These results indicate that amino acid residues 13–36 in the cytosolic domain of Tom6 contain essential information for either the correct assembly of the protein or for the overall folding of the protein, which then affects assembly.

The primary sequence of yeast Tom6 has high similarity to the N. crassa protein in the C-terminal 20 amino acid residues (Fig. 1A). To study further the requirements for specific assembly into the TOM complex, we constructed a hybrid precursor composed of the C-terminal domain of yeast Tom6 fused to the cytosolic domain of N. crassa Tom6 (Fig. 7A). We then asked whether in vitro-synthesized yeast Tom6 and the hybrid construct would be assembled into the N. crassa complex. Radio-labeled precursors were imported into mitochondria isolated from N. crassa, and specific assembly was investigated by BNGE. Both yeast Tom6-containing precursors migrated as low molecular weight bands indicating that the precursors were bound to mitochondria but not assembled (Fig. 7B). When the integration of these precursors into the yeast TOM complex was examined, the opposite integration behavior was observed (Fig. 7C). Yeast Tom6 was assembled into the yeast complex, but the N. crassa Tom6 and the hybrid precursor were only bound as non-assembled species. The results of importing the precursors into N. crassa mitochondria were verified by co-immunoprecipitation (Fig. 7D). Although the precursor of N. crassa Tom6 was efficiently precipitated by antibodies against Tom22 and Tom40, only minor amounts of yeast Tom6 were brought down by these antibodies. Hence, the Tom6 precursor from each organism contains specific information that allows it to assemble only into the TOM complex from the corresponding organism. Furthermore, this information cannot be localized exclusively to the more variable N-terminal, cytosolic domain, as the hybrid precursor containing the yeast C terminus also did not assemble into the N. crassa TOM complex.

Discusson

We have cloned Tom6 and Tom7 from N. crassa and investigated their insertion into the mitochondrial outer membrane, integration into the TOM core complex, and their interactions with other Tom components. These two small subunits, like the other components of the TOM complex, have significant sequence similarity to their yeast counterparts. Hence, their function can be predicted to be similar to the yeast homologues.

The components of the TOM complex, Tom22, Tom70 and
Tom40, use the pre-existing TOM complex for their own insertion (23, 28, 29). In contrast to previous observations (19), we report that Tom6 also utilizes the TOM complex for insertion. How can this discrepancy be explained? Although the temperature-sensitive tom40-3 allele used in the previous study (19) does not affect the import of Tom6, other tom40 temperature-sensitive alleles might do so. Indeed, the tom40-3 allele does not affect the import of porin, a major outer membrane protein, but two newly characterized alleles, tom40-2 and tom40-4, clearly do so (30). Moreover, we have used BNGE as a specific assay to directly monitor the assembly of in vitro imported Tom6 into pre-existing TOM complexes. The criterion of resistance to alkaline extraction used in the previous study (19) can be used to distinguish insertion into membranes but can be misleading when taken as a measure for correct assembly.

The highest sequence similarity between Tom6 from *N. crassa* and yeast resides in the C-terminal domain, which includes the putative transmembrane segment. Therefore, this region is likely to be important for the function and/or assembly of Tom6, and this part of yeast Tom6 was reported to be essential for targeting to mitochondria and for proper assembly (31). Using *N. crassa* Tom6 our study has validated these findings. In addition, we have demonstrated that the C-terminal domain is necessary for initial targeting and membrane

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**FIG. 6.** *A segment in the cytosolic domain of Tom6 is essential for assembly into the TOM complex.* A, sequences of DHFR fusions with Tom6 variants. The putative transmembrane segment of Tom6 is underlined. B, DHFR-Tom6 fusion proteins were incubated with isolated mitochondria for 20 min at the indicated temperatures and further treated as described in the legend to Fig. 5. For clarity of comparison the part containing the variant precursors is taken from longer exposure. Assembled TOM complex and non-assembled precursors (m) are indicated. C, DHFR-Tom6 and DHFR-Tom6A36 were incubated with isolated mitochondria at 25 °C for 30 min. At the end of the import reaction mitochondria were reisolated. One aliquot was directly analyzed by SDS-PAGE, and the rest was solubilized with Triton-containing buffer and split into three aliquots, which were subjected to immunoprecipitation with antibodies against Tom22 or Tom40 or with antibodies from control serum. The control serum did not precipitate the fusion proteins (not shown).

**FIG. 7.** *Precursors of Tom6 assemble exclusively into the TOM complex from the corresponding organism.* A, sequences of DHFR-Tom6 fusion constructs. Tom6 from *N. crassa* and the portion from *N. crassa* Tom6 in the hybrid construct are underlined. B, yeast Tom6 and a hybrid Neurospora-yeast Tom6 are not assembled into authentic *N. crassa* TOM complexes. DHFR-Tom6 constructs were incubated with isolated *N. crassa* mitochondria for 20 min at the indicated temperatures, and further treatment was as described in the legend to Fig. 6. Assembled TOM complex and non-assembled precursors (m) are indicated. C, *N. crassa* Tom6 and a hybrid Neurospora-yeast Tom6 are not assembled into authentic yeast TOM complexes. Import and analysis were as in panel B. D, precursors of DHFR-*N. crassa*-Tom6 or DHFR-yeTom6 were incubated with isolated *N. crassa* mitochondria at 25 °C for 30 min. Further treatment and analysis were as in Fig. 6C.
Tom6 and Tom7 in Neurospora crassa

References

Because chemical cross-linking did not reveal a direct contact between Tom40 and Tom22 (not shown), these results provide experimental support to the previously suggested role of Tom6 as a linking component between Tom40 and Tom22 (10, 25).

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