

# Structural Requirements of Tom40 for Assembly into Preexisting TOM Complexes of Mitochondria

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Tom40 is the major subunit of the translocase of the outer mitochondrial membrane (the TOM complex). To study the assembly pathway of Tom40, we have followed the integration of the protein into the TOM complex *in vitro* and *in vivo* using wild-type and altered versions of the *Neurospora crassa* Tom40 protein. Upon import into isolated mitochondria, Tom40 precursor proteins lacking the first 20 or the first 40 amino acid residues were assembled as the wild-type protein. In contrast, a Tom40 precursor lacking residues 41 to 60, which contains a highly conserved region of the protein, was arrested at an intermediate stage of assembly. We constructed mutant versions of Tom40 affecting this region and transformed the genes into a sheltered heterokaryon containing a *tom40* null nucleus. Homokaryotic strains expressing the mutant Tom40 proteins had growth rate defects and were deficient in their ability to form conidia. Analysis of the TOM complex in these strains by blue native gel electrophoresis revealed alterations in electrophoretic mobility and a tendency to lose Tom40 subunits from the complex. Thus, both *in vitro* and *in vivo* studies implicate residues 41 to 60 as containing a sequence required for proper assembly/stability of Tom40 into the TOM complex. Finally, we found that TOM complexes in the mitochondrial outer membrane were capable of exchanging subunits *in vitro*. A model is proposed for the integration of Tom40 subunits into the TOM complex.

## INTRODUCTION

Transport of proteins into and across the two mitochondrial membranes is achieved through the concerted action of translocation machineries: the TOM complex in the outer membrane and either of the two TIM complexes in the inner membrane (Glick and Schatz, 1991; Lill *et al.*, 1996; Schatz, 1996; Neupert, 1997; Pfanner *et al.*, 1997; Koehler *et al.*, 1999; Bauer *et al.*, 2000). Targeting and initial translocation of most preproteins that are destined to the mitochondrial matrix are dependent on amino-terminal, cleavable presequences (Hauke and Schatz, 1997; Neupert, 1997). In contrast, proteins of the outer membrane and a number of proteins of the inner membrane and the intermembrane space contain non-cleavable targeting signals (Shore *et al.*, 1995; Stuart and

Neupert, 1996; Neupert, 1997). Currently, the nature of most of these latter signals is obscure, though a few such as Tom70 (McBride *et al.*, 1992), Tom22 (Rodriguez-Cousino *et al.*, 1998), BCS1 (Fölsch *et al.*, 1996), and cytochrome *c* heme lyase (Diekert *et al.*, 1999) have been analyzed in detail.

The TOM complex contains import receptors for the initial recognition of preproteins (Tom20, Tom22, and Tom70) and membrane-embedded components that form the general import pore, which facilitates the translocation of preproteins across the outer membrane (Tom40, Tom5, Tom6, and Tom7). Tom40, a protein essential for viability of yeast and *Neurospora crassa* cells, was found to be the most abundant component of the TOM complex (Dekker *et al.*, 1998; Künkele *et al.*, 1998) and the core element of the preprotein-conducting pore (Hill *et al.*, 1998; Künkele *et al.*, 1998). The protein forms oligomers, with dimers as the basic structure, and interacts with polypeptide chains in transit (Vestweber *et al.*, 1989; Kiebler *et al.*, 1990; Rapaport *et al.*, 1997; Rapaport *et al.*, 1998). During preprotein translocation, the Tom40 oligomer undergoes conformational changes that affect both the structure of the Tom40 dimer and its interaction with other constituents of the TOM complex (Rapaport *et al.*, 1998). Tom40 has been predicted to traverse the outer mem-

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Abbreviations used: BNGE, blue native gel electrophoresis; OMV, outer membrane vesicles; RIP, repeat induced point mutation; TOM, translocase of the outer mitochondrial membrane.

brane as a series of 14 antiparallel  $\beta$ -strands that form a  $\beta$ -barrel (Court *et al.*, 1995; Mannella *et al.*, 1996). In contrast, all other TOM components are postulated to be anchored to the outer membrane by helical transmembrane segments. The import signals of these latter components were suggested to be located in the membrane anchor itself or in the sequences that flank the anchor (McBride *et al.*, 1992; Cao and Douglas, 1995; Rodriguez-Cousino *et al.*, 1998).

Tom20 and Tom70 act as receptors in the recognition of Tom40 precursors, whereas the translocation pore of the TOM complex is utilized for insertion (Keil *et al.*, 1993; Rapaport and Neupert, 1999). Previous studies have suggested the presence of a targeting signal in a yet undefined internal part of the Tom40 precursor and a signal required for assembly at the N-terminal region of the protein (Rapaport and Neupert, 1999). Understanding the biogenesis of the TOM complex requires more information on the mechanisms by which Tom40 is inserted into the membrane, how it achieves its final structure, and how it interacts with the other components in the assembled complex. In the present study we have addressed the question of Tom40 assembly and have identified a region at the N-terminus of the protein that is involved in the process. The region is highly conserved in Tom40 sequences from various species. We also give evidence that TOM complexes in the outer membrane can dynamically exchange subunits, and we discuss possible models for the insertion of Tom40 subunits into preexisting TOM complexes.

## MATERIALS AND METHODS

### Strains, Media, and Growth

Growth and handling of *N. crassa* strains was as described (Davis and De Serres, 1970). Race tubes were constructed in sterile 25-ml pipettes or 80-cm glass tubes as described (Davis and De Serres, 1970; White and Woodward, 1995). The extent of mycelial elongation was recorded every 24 h.

### Construction of *N. crassa tom40* Mutant Strains

Mutant alleles of *tom40* were created by site directed mutagenesis of single-stranded DNA derived from a plasmid containing the genomic version of *N. crassa tom40* and a bleomycin resistance gene in a Bluescript plasmid. After confirmation of the desired mutation, plasmids were transformed (Schweizer *et al.*, 1981; Akins and Lambowitz, 1985) into spheroplasts of a *tom40*<sup>RIP</sup> sheltered heterokaryon (to be described elsewhere) that was generated by the standard *N. crassa* genetic procedure (Metzenberg and Grotelueschen, 1992; Harkness *et al.*, 1994) of sheltered RIP (repeat induced point mutation). Rescue of the RIPed nucleus was confirmed by testing for biochemical requirements (see RESULTS), and integration of the mutant alleles was confirmed by DNA sequence analysis of PCR products from genomic DNA of the transformants.

### Isolation of the Suppressor Mutant of Yeast *tom40*<sup>ts</sup>

The suppressor mutant of *tom40*<sup>ts</sup> was isolated by performing random mutagenesis. Yeast strain KKY-Isp42-6 (Kassenbrock *et al.*, 1993), a haploid strain containing a complete deletion of the genomic *tom40* coding sequence and a mutated *tom40* gene on the centromere plasmid, pRS314, was mutagenized (Lawrence, 1991) with methanesulfonic acid ethylester and incubated on plates at the nonpermissive temperature (37°C). After several days, 77 suppressor-containing colonies were tested for plasmid-linked mutations in the *tom40*<sup>ts</sup> gene. For this purpose, the ability of plasmids isolated

from these colonies to confer the suppressor phenotype was tested. In 66 cases the suppressor phenotype was found to be plasmid linked. The strongest suppressor mutant, *tom40*<sup>ts</sup>Sup, had a back mutation at position 66 from a proline residue in the temperature-sensitive strain to the wild-type leucine residue. This back mutation restored the steady-state level and stability of the Tom40 protein.

### Import of Preproteins into Isolated Mitochondria

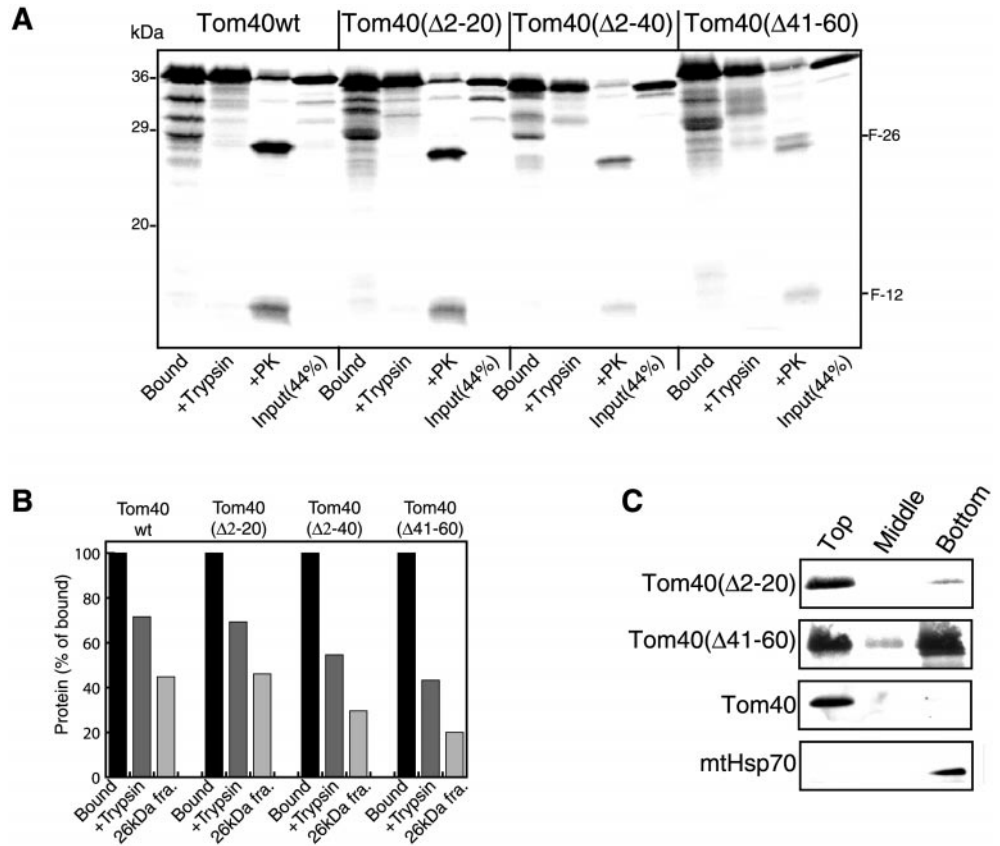
For import of Tom40 precursors in vitro, mitochondria from *N. crassa* were isolated as described (Mayer *et al.*, 1993). Radiolabeled preproteins were synthesized in rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine (ICN Biomedicals, Costa Mesa, CA) after in vitro transcription by SP6 polymerase from pGEM4 vectors containing the gene of interest. Import reactions were performed by incubation of radiolabeled preproteins with 30–50  $\mu$ g mitochondria in import buffer (0.5% BSA [wt/vol], 250 mM sucrose, 80 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM ATP, 10 mM MOPS-KOH, pH 7.2) at the indicated temperature. Proteinase K (PK) or trypsin treatment of samples was performed by incubation with the protease for 15 min on ice, followed by addition of 1 mM phenylmethylsulfonyl fluoride (PMSF) for 5 min. Import was analyzed by SDS-PAGE, and the gels were viewed by autoradiography or quantified using a phosphorimaging system (BAS 1500; Fuji Medical Systems, Stamford, CT). Immunodecoration was according to standard procedures and was visualized by the ECL method (Amersham).

Carbonate extraction was performed to determine if imported precursor proteins were inserted into membranes. We used sucrose flotation gradients to avoid the possibility of having nonintegrated protein aggregates pelleting with membranes. After import reactions, mitochondria were pelleted, resuspended in 100  $\mu$ l 0.1 M Na<sub>2</sub>CO<sub>3</sub>, and incubated for 30 min on ice. Then, a solution of 2.4 M sucrose, 0.1 M Na<sub>2</sub>CO<sub>3</sub> was added to a final concentration of 1.5 M sucrose (final volume, 266  $\mu$ l). This was overlaid first with 250  $\mu$ l of buffer containing 1.4 M sucrose, 0.1 M Na<sub>2</sub>CO<sub>3</sub> and then with 200  $\mu$ l of buffer containing 0.25 M sucrose, 0.1 M Na<sub>2</sub>CO<sub>3</sub>. The gradient was centrifuged for 2 h at 337,000  $\times$  g in a Beckman SW60 rotor (Fullerton, CA) at 2°C, which causes the membranes to float to the upper layer of the gradient. Gradients were analyzed by removing 250  $\mu$ l from the top zone, 150  $\mu$ l from the middle zone, and 200  $\mu$ l from the bottom zone of the gradient. Proteins in these fractions were precipitated with trichloroacetic acid and analyzed by SDS-PAGE and autoradiography or Western blotting.

### Construction of *tom40* Mutants for In Vitro Import

pGEM4-Tom40( $\Delta$ 2–20) DNA and pGEM4-Tom40( $\Delta$ 2–40) DNA were constructed by PCR amplification of the relevant DNA from pGEM4-Tom40, which contains the *N. crassa* wild-type *tom40* gene. For pGEM4-Tom40( $\Delta$ 2–20) and pGEM4-Tom40( $\Delta$ 2–40) the upstream primers 5'-AGAAAAGAATTCACCATGAGCCTTTC-CGATGCCTTC-3' and 5'-AGAAAAGAATTCACCATGCCCG-CACGATCGAGACC-3', respectively, were used. In both cases the downstream primer 5'-CTCTAAGCTTTTAAAAGGGGATGTT-GAGG-3' was used. Both PCR products were digested with *Eco*RI and *Hind*III and subcloned into pGEM4. pGEM4-Tom40( $\Delta$ 41–60) DNA was constructed by a method involving the simultaneous ligation of two inserts. The first insert, representing amino acid residues 1–40, and the second insert, representing amino acid residues 61–349, were constructed by PCR amplification of the relevant DNA from pGEM4-Tom40. The upstream primer for the first insert represents the sequence containing the *Nhe*I from the pGEM4 vector, whereas the downstream primer 5'-AAAAAATCATATGGTT-GGAAAGACCGAAGCTGTTT-3' contained an *Nde*I site. This PCR product was digested with *Eco*RI (site derived from the multiple cloning site of pGEM4) and *Nde*I. For the second insert, the upstream primer was 5'-AAA GAA TTC CAT ATG TTC TCT GGC CTC CGC GCC GAC-3', whereas the downstream primer was the same as used for the cloning of pGEM4-Tom40( $\Delta$ 2–20) and pGEM4-

**Figure 1.** Insertion of Tom40 variants into the mitochondrial outer membrane. (A) Radiolabeled precursors of Tom40 wild-type (Tom40wt) and the Tom40 deletion mutants were incubated with isolated mitochondria for 15 min at 25°C. After import, each sample was divided into three parts, and mitochondria in all parts were reisolated by centrifugation and resuspended in import buffer. One part was left untreated (Bound), and the other two parts were treated with 100 μg/ml either trypsin (+Trypsin) or Proteinase K (+PK). Samples were analyzed by SDS-PAGE and autoradiography. F-26 and F-12: characteristic N-terminal and C-terminal fragments of Tom40 formed after proteinase K treatment, respectively. (B) The results from A were quantified and presented as a percentage of total bound material. Bound, total bound precursor; +Trypsin, precursor resistant to trypsin treatment; 26-kDa fra., quantification of the N-terminal 26-kDa fragment (F-26) after correction for loss of [<sup>35</sup>S]methionine residues. (C) Radiolabeled precursors of Tom40 deletion mutants were imported into isolated mitochondria as for A. After import, the mitochondria were pelleted and then suspended in 0.1 M Na<sub>2</sub>CO<sub>3</sub>. After 30 min on ice, the samples were analyzed on sucrose flotation gradients where membranes float to the top of the gradient and soluble proteins remain at the bottom (see MATERIALS AND METHODS). The top two boxes show the fractionation pattern of the radiolabeled precursors. The bottom two boxes show immunodecoration of the endogenous Tom40 and mtHsp70 proteins.



Tom40(Δ2–40). This PCR product was digested with *NdeI* and *HindIII*. The digested products were ligated into pGEM4 that had been digested with *EcoRI* and *HindIII*.

Tom40 variants for in vitro import that contained smaller deletions and amino acid substitutions were generated by site-directed mutagenesis of a Tom40 cDNA cloned in the pGEM-7Zf(+) vector.

**Cross Linking and Coimmunoprecipitation**

For cross-linking experiments, radiolabeled precursors were incubated with isolated mitochondria under various conditions. After the import reaction mitochondria were isolated and resuspended in import buffer followed by addition of 440 μM of the cross-linking reagent disuccinimidyl glutarate (DSG; Pierce Chemical Co., Rockford, IL) for 40 min at 0°C. Excess cross-linker was quenched by the addition of 80 mM glycine, pH 8.0, and incubation for 15 min at 0°C. Aliquots were removed before and after addition of the cross-linking reagents. For coimmunoprecipitation, samples were dissolved in lysis buffer (0.5% digitonin, 150 mM NaCl, 10 mM Tris-HCl, pH 7.2). After a clarifying spin (15 min at 20,000 × g), the supernatant was incubated with antibodies that were coupled to protein A-Sepharose beads.

**Blue Native Gel Electrophoresis (BNGE)**

Mitochondria (50–100 μg) were lysed in 50 μl detergent-containing buffer (1% digitonin, 0.3% dodecylmaltoside, or 1% dodecylmalto-

side in 20 mM Tris-HCl, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM PMSF, pH 7.4). After incubation on ice for 10 min and a clarifying spin (20 min, 22,000 × g), 5 μl sample buffer (5% [wt/vol] Coomassie brilliant blue G-250, 100 mM Bis-Tris, 500 mM 6-aminocaproic acid, pH 7.0) were added, and the mixture was analyzed on a 6 to 13% gradient blue native gel (Schägger *et al.*, 1994; Schägger and von Jagow, 1991).

**RESULTS**

**A Tom40 Precursor Lacking Amino Acid Residues 41 to 60 Does Not Integrate into the TOM Complex**

The assembly pathway of Tom40 can be divided into three stages: binding to the mitochondrial surface, insertion into the membrane, and assembly into the TOM complex (Rapaport and Neupert, 1999). Here we have investigated Tom40 with respect to the structural features required for integration into the complex. Precursor proteins lacking residues 2 to 20, 2 to 40, or 41 to 60 were analyzed for their ability to be integrated into the TOM complex, because previous studies had indicated a role for the N-terminal region in the process (Rapaport and Neupert, 1999). All three mutant forms were targeted to mitochondria with efficiencies similar to wild-type (Figure 1, Bound). The ability of the variants to insert correctly into the

mitochondrial outer membrane was determined by assessing the acquisition of protection against added trypsin and the formation of proteinase K cleavage fragments characteristic of the inserted wild-type protein. Deletion of residues 2 to 20 did not affect proper insertion, whereas removing residues 2 to 40 had a moderate effect (Figure 1). The variant lacking residues 41 to 60 was ca. 50% less efficient in its ability to properly insert than the wild-type precursor. Treatment of mitochondria with proteinase K after import of the  $\Delta 41-60$  variant resulted in the formation of an additional cleavage fragment visible just above the usual F-26 fragment that is observed for wild-type Tom40. The additional fragment was likely a digestion product from the fraction of the Tom40 $\Delta 41-60$  molecules that did not insert properly.

Insertion of the precursor proteins into the mitochondrial outer membrane was further assessed by carbonate extraction after *in vitro* import (Figure 1C). The extraction products were analyzed on sucrose gradients, which results in flotation of membranes to the top of the gradient (see MATERIALS AND METHODS). Thus, integral membrane proteins (e.g., Tom40) will be found in the upper zone of the gradient, whereas soluble proteins (e.g., Hsp70) will be found in the bottom zone. Tom40 $\Delta 2-20$  was found in the upper zone of the gradient, demonstrating its insertion into mitochondrial membranes. About half of the Tom40 $\Delta 41-60$  molecules were integrated into the membrane, whereas the remaining half fractionated with the soluble proteins. These data are in agreement with the results of the protease treatment experiments (Figure 1, A and B), where about half of the protein molecules acquired the correct conformation. Taken together, these results demonstrate that the deletions did not impair membrane insertion or cause dramatic changes in the conformation of the inserted protein.

Integration of the variants into the endogenous TOM complex was studied by BNGE. The variant lacking the first 20 amino acid residues was found to integrate into the fully assembled complex with an efficiency similar to the wild-type form (see Figure 4D; shown as a control), whereas the variant lacking the first 40 residues assembled at slightly reduced efficiency (our unpublished results). However, the precursor lacking residues 41 to 60 accumulated at a stage shown previously to be an intermediate (Figure 2A, I) on the assembly pathway (Rapaport and Neupert, 1999) and was not assembled into an authentic TOM complex (Figure 2A). In the intermediate, both the wild-type and mutant forms were only loosely associated with the TOM complex and they dissociated from the complex upon solubilization of mitochondria with dodecylmaltoside (Figure 2A). The band between the monomer and intermediate bands (Figure 2A, lanes 1, 3, and 7) is unproductively bound material (Rapaport and Neupert, 1999).

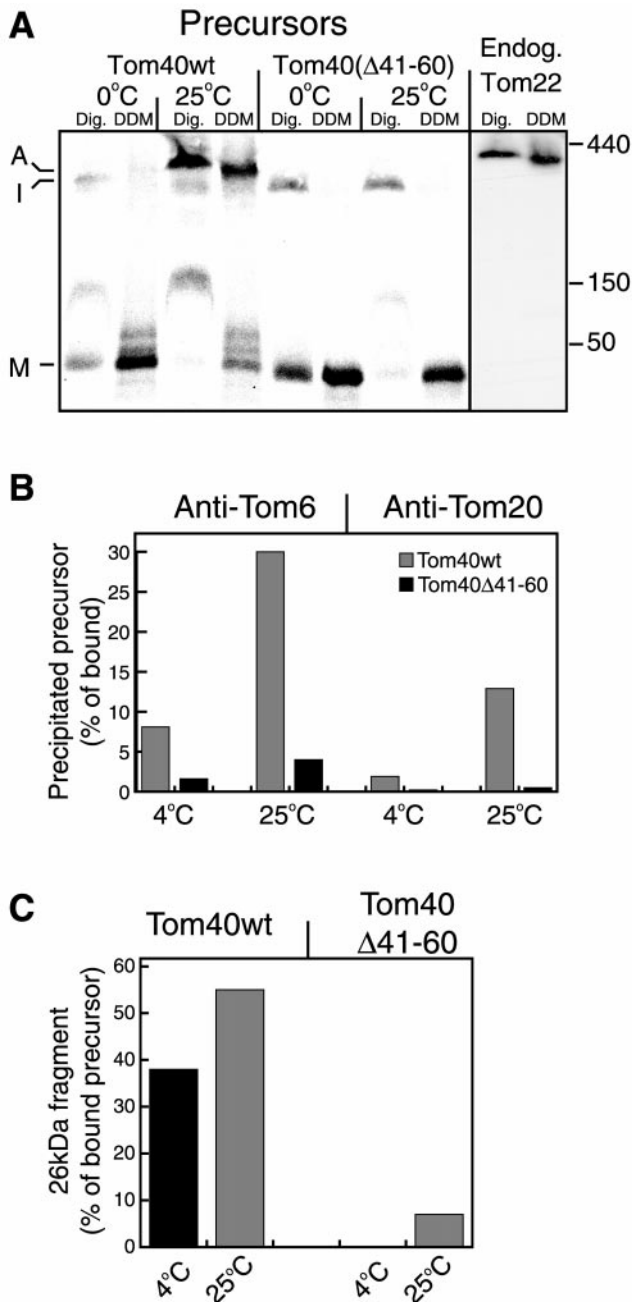
The efficiency of integration into the TOM complex was tested further by immunoprecipitation with antibodies against either Tom6 or Tom20. When full-length Tom40 was imported into mitochondria at 4°C, only low levels of the protein were assembled into the TOM complex. However, the efficiency was high when import was performed at 25°C (Figure 2B). In contrast, very low levels of assembly of Tom40 $\Delta 41-60$  were detected upon import at either temperature (Figure 2B). These levels might reflect association with the TOM complex as an insertion intermediate rather than actual assembly. The levels of integration of precursors lacking the first 20 or 40 residues

were similar to those of wild-type precursor (our unpublished results). Thus, the immunoprecipitation experiments confirm the results of the BNGE analysis and demonstrate the inability of Tom40 $\Delta 41-60$  to progress into fully assembled TOM complexes. Interestingly, significant levels of the characteristic 26-kDa proteinase K fragment from the wild-type precursor were observed after incubation at 4°C (Figure 2C). This implies that the Tom40 precursor readily acquires its native (or near native) folding even before it is stably integrated into the authentic TOM complex. In previous experiments we observed that folding did not occur in experiments performed at 0°C with short incubation times (Rapaport and Neupert, 1999).

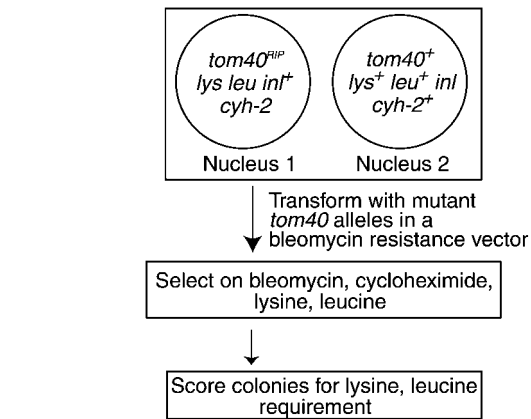
### **Mutations in Amino Acid Residues 40 to 50 of Tom40 Result in Growth Defects**

To investigate further the role of the N-terminal portion of the protein in assembly and function of Tom40 *in vivo*, we examined the ability of mutant derivatives of *tom40* to complement a nonfunctional RIP allele of the gene (Figure 3). Because Tom40 is an essential protein, we used the procedure of sheltered RIP (Metzenberg and Grotelueschen, 1992; Harkness *et al.*, 1994) to create a strain of *N. crassa* (to be described elsewhere), in which a nucleus lacking a functional *tom40* gene is maintained in a heterokaryon with a nucleus containing a wild-type version of the gene (Figure 3). A *tom40* gene encoding a protein lacking amino acid residues 2 to 60 was not able to restore viability to the nucleus harboring the RIPed version of *tom40*, supporting the *in vitro* findings (Rapaport and Neupert, 1999) that the first 60 residues of the N-terminal domain contain crucial information for Tom40 assembly. To identify regions in the N-terminal part of the protein that might play a role in the assembly process, we compared Tom40 sequences from various organisms. We observed no conservation of sequence between organisms prior to amino acid 38 of the *N. crassa* protein, which is in agreement with the results from *in vitro* experiments. However, we found a number of highly conserved residues in the region of residues 40 to 60 with the greatest level of similarity within residues 40 to 50 (Figure 4A). Three mutant derivatives of *tom40* affecting the conserved region were created (Figure 4A). One mutant ( $\Delta$ NPGT) had a deletion of the four highly conserved residues NPGT at positions 40 to 43 of the *N. crassa* sequence, whereas a second mutant (AAAA) had four alanine residues at those sites. The third mutant ( $\Delta 40-48$ ) had a deletion of residues 40 to 48, with an additional single amino acid change, R49A. Each of these mutant alleles was able to rescue the *tom40*<sup>RIP</sup> nucleus and give rise to homokaryons requiring lysine and leucine (Figure 3).

The  $\Delta$ NPGT and AAAA mutants displayed a complex growth phenotype. We analyzed 18 different lysine- and leucine-requiring transformants of the AAAA type and 13 of the  $\Delta$ NPGT type for growth characteristics on race tubes. Nine of the AAAA strains and five of the  $\Delta$ NPGT strains displayed a "stop-start" growth phenotype. These strains grew at near normal rates for a few days and then grew very slowly or not at all for 1 or 2 days before they resumed their initial growth rate. The strains exhibiting this behavior are remarkably consistent, because impaired growth was observed on the same day in up to six different race tubes of an individual strain. The rest of the strains analyzed from both groups grew slightly slower than control strains for 12 days



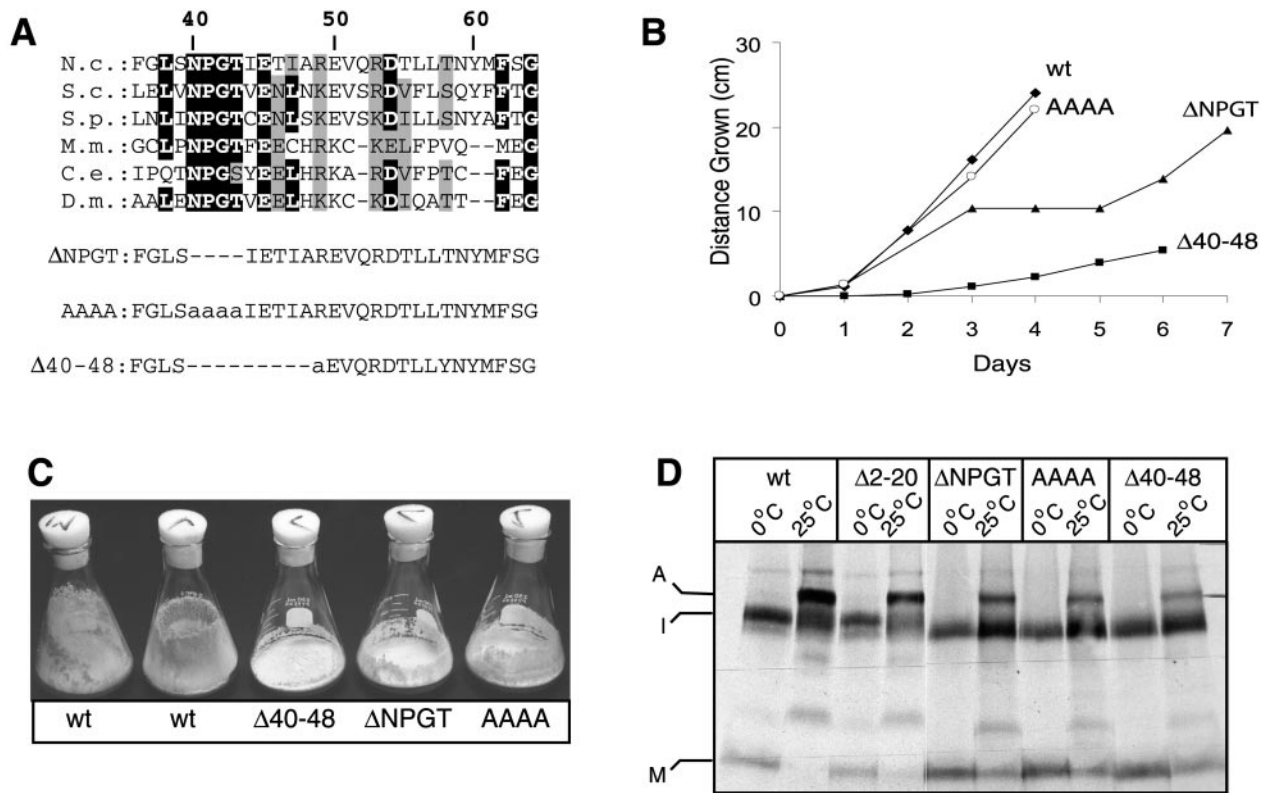
**Figure 2.** Tom40 variant lacking amino acid residues 41–60 is not integrated into the TOM complex. (A) Radiolabeled precursors were incubated at 0°C or 25°C with 50 μg mitochondria for 20 min. All samples were reisolated and solubilized in buffer containing either 1% digitonin (Dig.) or 0.3% dodecylmaltoside (DDM). After a clarifying spin, the solubilized mitochondria were loaded on a blue native gel. For detection of the endogenous TOM complex, antibodies against Tom22 were used. The three main stages of import are indicated: M, bound monomer; I, high-molecular-weight intermediate; and A, assembled TOM core complex. The positions of molecular weight markers (kDa) are indicated on the right side of A. (B) Tom40Δ41–60 is not stably associated with Tom20 and Tom6. Tom40 (Tom40wt) and Tom40Δ41–60 radiolabeled precursors were incubated with mitochondria for 20 min at 4°C or 25°C and mito-



**Figure 3.** Creation of *N. crassa* strains expressing Tom40 variants. A sheltered heterokaryon containing a nucleus with a *tom40<sup>RIP</sup>* allele (Nucleus 1) and a nucleus with a wild-type *tom40* allele (Nucleus 2) is depicted in the large box at the top of the figure. The heterokaryon was created by the procedure of sheltered RIP using strains described previously for linkage group V of *N. crassa* (Metzenberg and Grotelueschen, 1992). Additional markers in nucleus 1 impart cycloheximide resistance and requirements for lysine and leucine. Transformation of the strain with mutant alleles of *tom40*, carried on a bleomycin resistance plasmid, followed by selection on media containing bleomycin (3.7 μg/ml), cycloheximide (50 μg/ml), lysine, and leucine resulted in homokaryons expressing only the predicted mutant alleles of *tom40*.

without evidence of stopping. An example of each type of growth is shown in Figure 4B. Tom40 levels in all strains were similar, regardless of their growth phenotype (our unpublished results). Still, it is conceivable that subtle differences in expression, not identified in the analysis of Western blots, may explain the different growth characteristics. This could be due to locus-specific effects at different integration points of the transformed mutant alleles in the individual strains. Regardless, we have found no differences between the strains except for this behavior. For further analysis, one stopper type of the ΔNPGT strains and one normal growth type of the AAAA strains were chosen. The Δ40–48 strain had a slower growth rate that was easily distinguished from the other mutants (Figure 4B). The ability of all three mutant strains to climb the walls of flasks and to form conidia in these flasks was significantly reduced (Figure 4C). Thus, the data from the mutant strains suggest a crucial role for residues 40–49 in the function of Tom40.

chondria were reisolated. One aliquot from each import reaction was directly analyzed by SDS-PAGE and was taken as total bound precursor. A second aliquot was solubilized with 0.5% digitonin and split into three aliquots that were subjected to immunoprecipitation with antibodies against either Tom6, Tom20, or with antibodies from preimmune serum. The latter precipitated only background levels of the precursors (our unpublished results). The lower amounts of assembled precursor observed with Tom20 antibodies are likely due to the fact that Tom20 is only loosely bound to the TOM complex (Ahting *et al.*, 1999). (C) Tom40 can acquire its correctly folded state despite lack of integration into the TOM complex. A third aliquot of the import reactions described in B was treated with proteinase K, and the formation of the characteristic 26-kDa fragment was quantified.



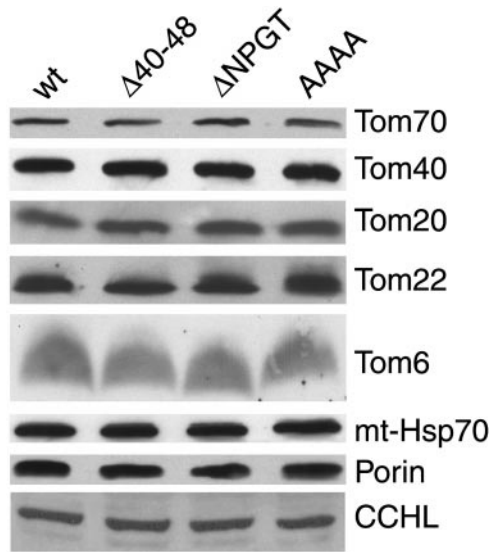
**Figure 4.** Tom40 mutants and their growth phenotypes. (A) Protein sequence alignment of *N. crassa* Tom40 (residues 36–64) with homologues from other organisms. Regions modified in the mutant variants are indicated below the alignment. Deleted residues are indicated by dashes, and altered residues are shown in lower case. N.c., *Neurospora crassa*; S.c., *Saccharomyces cerevisiae*; S.p., *Schizosaccharomyces pombe*; C.e., *Caenorhabditis elegans*; M.m., *Mus musculus*; D.m., *Drosophila melanogaster*. Identical residues occurring in four or more sequences are indicated in black highlight. Similar residues are indicated by gray highlights. (B) Mycelial elongation rate of wild-type (wt) and mutant strains as measured in race tubes. Representative strains of the AAAA and ΔNPGT strains are shown, but it should be noted that some strains containing the AAAA mutation grow in the manner shown for ΔNPGT, whereas some ΔNPGT strains grow in the manner depicted for AAAA (see text). (C) Strains containing mutations in the region encompassing residues 40 to 50 are inefficient at climbing the walls of flasks and in the formation of conidia compared with the wild-type strains (wt) that were the parents in the sheltered RIP cross that gave rise to the sheltered heterokaryon shown in Figure 3. (D) Radiolabeled Tom40 variants have reduced ability to assemble into authentic TOM complex. Radiolabeled precursors of wild-type Tom40 (wt) and mutant variants were incubated at 0°C or 25°C with 50 μg mitochondria for 20 min. All samples were reisolated and solubilized in buffer containing 1% digitonin. Further treatment was as described in the legend to Figure 2A. Molecular weights are as indicated in Figure 2A.

The fact that viable strains were obtained by transforming the *tom40<sup>RIP</sup>* nucleus with the AAAA, ΔNPGT, and Δ40–48 variants implies that these forms are at least partially capable of assembling into mature, functional TOM complex. This was confirmed by using BNGE to assess assembly after *in vitro* import of the mutant precursors. All three mutants show partial assembly into the TOM complex, whereas a significant proportion remains in the high molecular weight intermediate (Figure 4D). Interestingly, the Δ40–48 mutant appears to have the least amount of precursor in the fully assembled form, which correlates well with the more severe growth phenotype observed for strains bearing this mutation (Figure 4B).

#### Mutations in Residues 40 to 50 of Tom40 Result in a More Fragile TOM complex

The level of TOM complex components and other mitochondrial proteins from the ΔNPGT, AAAA, and Δ40–48 strains

was found to be similar to those in wild-type controls (Figure 5). The TOM complex in the mutants was further examined by BNGE and immunoblotting with antibodies directed against individual TOM complex components. When mitochondria were dissolved in 1% digitonin and subjected to BNGE and the blots decorated with antibody to Tom40, all three mutants were found to contain a TOM complex with slightly increased electrophoretic mobility (Figure 6). When the experiment was repeated with mitochondria dissolved in 1% dodecylmaltoside, the TOM complex in the three mutants was found to be more fragile than the wild-type strain, and at least some fraction of the Tom40 molecules in the mutants migrated as monomers (Figure 6). These results demonstrate that residues 40 to 49 play an important role in the stability of the TOM complex. Blots of blue native gels were also examined using antibodies directed against two other TOM core complex components (Ahting *et al.*, 1999), Tom6 and Tom22. In both cases, the samples solubilized in



**Figure 5.** Mitochondrial proteins in wild-type (wt) and mutant strains. Mitochondria isolated from each strain were subjected to SDS-PAGE, blotted to nitrocellulose membrane, and decorated with antisera to TOM complex proteins as well as the mitochondrial outer membrane protein porin, the intermembrane space protein cytochrome *c* heme lyase (CCHL), and the matrix protein mitochondrial Hsp70 (mt-Hsp70).

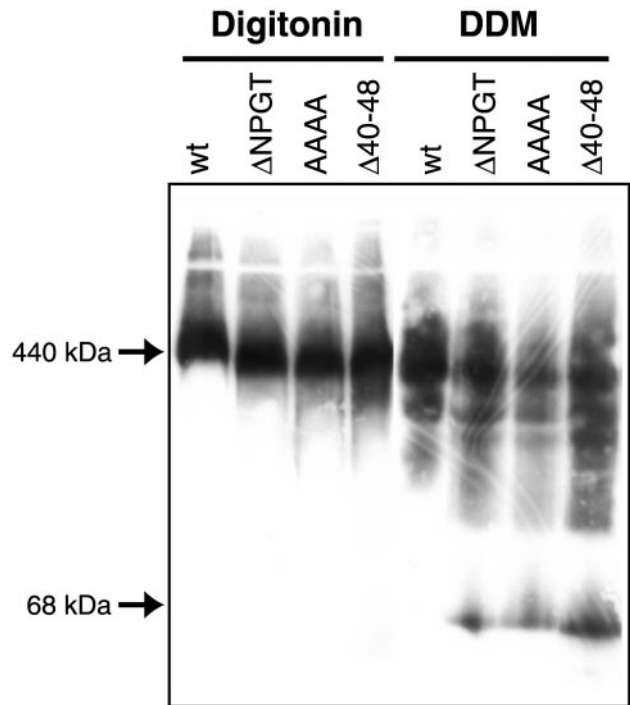
digitonin showed the same electrophoretic mobility alteration seen in the blots examined with Tom40 antibodies (our unpublished results). For samples dissolved in 1% dodecylmaltoside, the patterns were similar to those seen with Tom40 antibodies, except that only trace amounts of Tom6 monomers were released from the mutant complexes.

#### Reversion of a Yeast Tom40 Temperature-sensitive Mutant Restores an Amino Acid in the Conserved N-terminal Region

A study of temperature-sensitive strains of the yeast *Saccharomyces cerevisiae* further supports the notion that amino acid residues in the 40-to-50 region are important for the biogenesis of Tom40. Kassenbrock *et al.* (1993) isolated several temperature-sensitive strains carrying mutations in the *tom40* gene. In one of the strains (KKY-Isp42-6), DNA sequencing identified 10 mutations in the *tom40* gene. We isolated revertants of this temperature-sensitive strain and found that a single reversion in the temperature-sensitive strain, Pro66, back to the wild-type Leu66, was sufficient to allow the revertant strain to grow at the wild-type rate at the restrictive temperature (Figure 7). This result suggests a crucial role for Leu66 in the function of yeast Tom40. The yeast Tom40 Leu66 corresponds to the Ile residue at position 47 in the *N. crassa* protein (Figure 4A).

#### Integration of Tom Components into the TOM Complex

To gain further insight into the structure and assembly of the TOM complex, we wished to determine if newly incorporated precursors can integrate into preexisting complexes. In

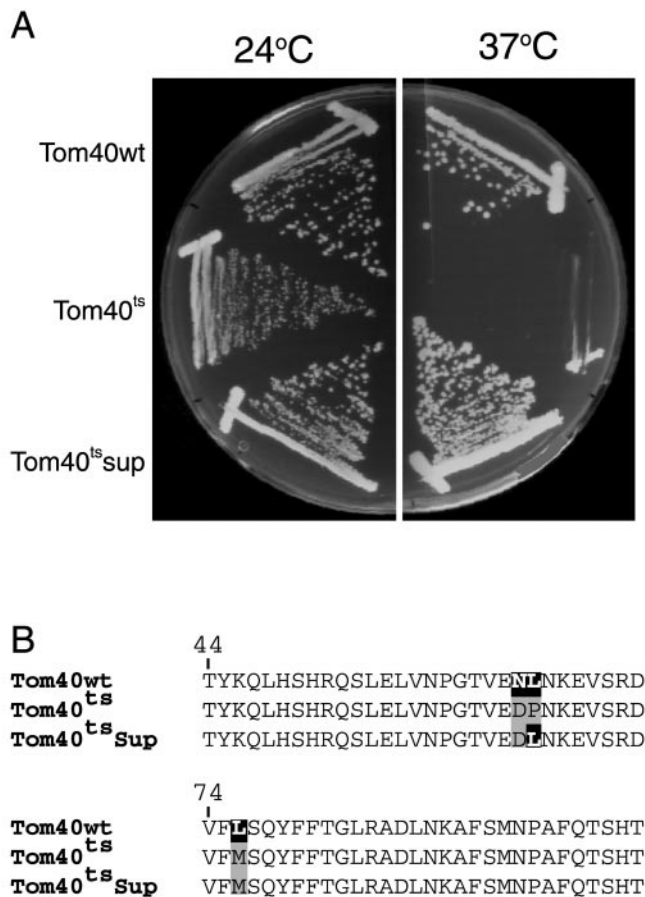


**Figure 6.** Stability of TOM complexes containing mutant Tom40 molecules. Mitochondria were dissolved in 1% digitonin or 1% dodecylmaltoside (DDM) and subjected to BNGE. The gels were blotted and stained with antiserum to Tom40. In the dodecylmaltoside experiments, the low-molecular-weight form of Tom40 in the lanes containing mutant mitochondria comigrated with Tom40 monomer released when mitochondria were dissolved in SDS (our unpublished results). The positions of molecular weight markers are indicated on the left.

a mutant strain of *N. crassa* that expresses only a truncated form of Tom40 lacking the C-terminal 20 amino acid residues, the TOM complex is expected to be ~13 to 17 kDa smaller than the wild-type complex, based on estimates of six to eight molecules of Tom40 per complex (Ahting *et al.*, 1999). We found that this size difference could be detected by BNGE (Figure 8). The difference was exploited to determine if imported TOM complex subunits can be inserted into preexisting complexes. When full-length precursors of Tom22 and Tom40 were imported into mitochondria isolated from the C-terminal deletion strain, they were rapidly integrated into complexes with the molecular weight characteristic of this strain (Figure 8). These observations support previously suggested models in which precursors are either taken up into a small pool of nearly completed complexes or integrated directly into existing functional complexes (Rapaport and Neupert, 1999).

#### Exchange of TOM Complex Subunits in Isolated Outer Membrane Vesicles (OMVs)

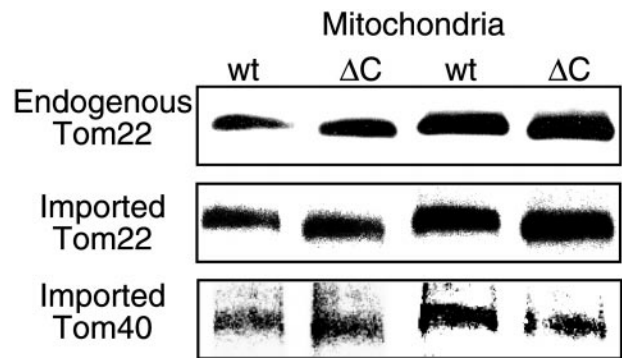
Because the stoichiometry of subunits in a functional TOM complex is likely to be constant, direct integration into functional complex would imply displacement of preexisting



**Figure 7.** A revertant suppresses the growth phenotype of the *tom40* temperature-sensitive mutant KKY-Isp42-6. (A) Growth on agar plates of wild-type cells (Tom40wt), the Tom40 temperature-sensitive strain (Tom40<sup>ts</sup>) and a revertant (Tom40<sup>ts</sup>sup). (B) The *tom40* gene in the revertant was sequenced and one back mutation from Pro66 to the original wild-type Leu66 was identified. Two additional amino acid substitutions in the temperature-sensitive protein that are located in the vicinity of residue 66 are indicated.

subunits. To determine if exchange of subunits can take place between different complexes in vitro, we mixed OMVs isolated from a wild-type *N. crassa* strain with OMVs from a strain whose only form of Tom22 contained a hexahistidiny tag. The two OMV samples were induced to undergo fusion by three cycles of freeze/thaw, which is known to induce fusion of lipid vesicles (Hincha *et al.*, 1998). The samples were then solubilized with digitonin and incubated with Ni-NTA sepharose beads to isolate TOM complex containing his-tagged Tom22. Complexes containing his-tagged Tom22 were also found to contain the wild-type form of the protein, indicating that mixing of the two original complexes had occurred (Figure 9A, lane 4). Optimal formation of mixed complexes required fusion of the vesicles, whereas simple mixing, without freeze/thaw cycles, resulted in low levels of mixed TOM complex (Figure 9A, lane 3) only slightly above background (Figure 9A, lane 1).

In a related set of experiments, antibodies against a C-terminal peptide of Tom40 were used as a tool to analyze exchange



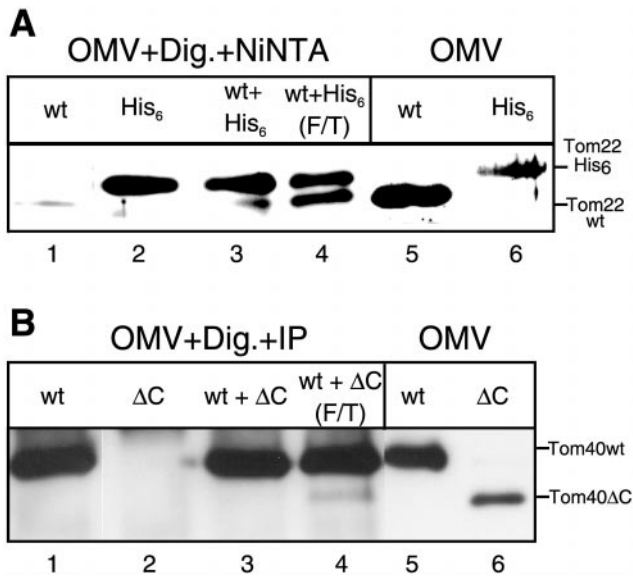
**Figure 8.** Precursors of Tom40 and Tom22 integrate into preexisting complexes. Radiolabeled precursors of either Tom22 or Tom40 were incubated for 20 min at 25°C with 50 μg mitochondria isolated from either a wild-type strain (wt) or a strain lacking the C-terminal 20 amino acid residues of Tom40 (ΔC). The mitochondria were then reisolated and solubilized in a buffer containing 1% digitonin. After a clarifying spin, the solubilized mitochondria were loaded onto a blue native gel. The endogenous TOM core complex was detected by antibodies against Tom22. The location of the imported precursors was detected by exposure of the blot to x-ray film. For easier observation of the small size differences, the same samples were loaded twice in alternating lanes.

of Tom40 subunits. OMVs from a wild-type strain and OMVs from the strain harboring Tom40 with the C-terminal deletion were fused, solubilized with digitonin, and subjected to immunoprecipitation with the C-terminal antibodies. Both forms of Tom40 were present in immunoprecipitates when OMVs underwent fusion but not in immunoprecipitates from controls where OMVs were mixed but not subjected to the fusion treatment (Figure 9B, lanes 3 and 4). Thus, both Tom22 and Tom40 subunits can be exchanged between complexes. The data suggest that Tom22 subunits can be exchanged more easily than Tom40 subunits. Tom40 molecules may be more tightly associated in the complex than Tom22 so that exchange of these subunits may be less frequent.

## DISCUSSION

We have examined the mechanisms by which the polytopic mitochondrial outer membrane protein Tom40 is inserted into the mitochondrial outer membrane and assembled into the TOM complex of *N. crassa*. The highly conserved region containing amino acid residues 41 to 60 of Tom40 was found to be expendable for binding receptors and membrane insertion but crucial for the assembly of the protein into the complex. Deletion of residues 41-60 completely abolished assembly in vitro, whereas smaller mutations resulted in the formation of TOM complexes with altered electrophoretic mobility and stability in vivo. The changes in mobility likely reflect changes in conformation of the TOM complex because all mutants analyzed displayed similar changes in mobility, whereas the molecular weight of Tom40 in the AAAA mutant is not significantly different from that of the wild-type form. Tom40 monomers were lost from the mutant strains after solubilization in dodecylmaltoside. Because the three mutants exhibited similar behavior with respect to loss of Tom40, this suggests that the NPGT sequence, which is affected in all three





**Figure 9.** TOM complexes can exchange subunits. (A) OMV from a wild-type strain or from a strain expressing a His-tagged version of Tom22 instead of native Tom22 (40  $\mu$ g each per lane) were incubated separately or together for 2 h at 25°C in a buffer composed of Tris-HCl (10 mM, pH 7.2). In one case, three cycles of rapid freezing in liquid N<sub>2</sub> followed by a 5-min thaw at 25°C were performed (F/T). The OMV were then centrifuged (20 min, 125,000  $\times$  g) and resuspended in buffer containing 1% digitonin and 10 mM imidazole. Ni-NTA sepharose beads were added for 20 min. The beads were washed once, and bound proteins were eluted with sample buffer. The proteins were analyzed by SDS-PAGE and immunodecoration with antibody against Tom22. For comparison, OMV from both strains were directly loaded on the gel (lanes 5 and 6). (B) OMV (80  $\mu$ g per lane) from a wild-type strain (wt) or from a strain expressing a C-terminal deletion of Tom40 ( $\Delta$ C) were incubated separately or together for 2 h at 25°C in a buffer composed of Tris-HCl 10 mM, pH 7.2. In one case three cycles of freeze/thaw (as above) were included in the incubation period (F/T). The OMV were centrifuged (20 min, 125,000  $\times$  g), resuspended in buffer containing 1% digitonin, and immunoprecipitated with antibodies against a C-terminal peptide of Tom40. The precipitated proteins were analyzed by SDS-PAGE and immunodecoration with antibody against an N-terminal peptide of Tom40. For comparison, OMV from both strains were directly loaded on the gel (lanes 5 and 6).

strains, may play a role in mediating proper assembly and stability of the TOM complex. The *N. crassa* strains expressing the Tom40 variants were altered in their ability to form conidia and exhibited growth defects. Furthermore, a suppressing back mutation of a yeast *tom40* temperature-sensitive allele occurred within the same amino acid residues, emphasizing the importance of the conserved region in a separate organism. Taken together, these observations show that the sequence containing residues 41–60 plays an important role in the assembly of Tom40. The sequence seems to function as an assembly signal only in its native context, because fusion proteins between this region of Tom40 and a cytosolic protein DHFR (Tom40(41–60)-DHFR) or Tom20 (Tom40(41–60)-Tom20) did not integrate into the Tom core complex (our unpublished results). Thus, these residues are necessary but not sufficient to achieve assembly.

Several observations support the view that the impaired assembly of Tom40 mutant variants reflects a specific function

of the affected amino acid residues rather than simple misfolding of the mutant forms. Tom40 in the TOM complex yields characteristic fragments upon treatment of mitochondria with proteinase K (Künkele *et al.*, 1998). The same fragments can be generated after integration of full-length Tom40 precursor into the membrane of isolated mitochondria at a stage when Tom40 is not yet assembled into the complex. Similarly, the  $\Delta$ 41–60 variant, which is not assembled into the complex, still gave rise to the characteristic proteolytic cleavage fragments after integration into the membrane. Thus, Tom40 appears to reach its final, or near final, conformation rather early in its assembly pathway, and the actual integration of Tom40 precursor into the core of the TOM complex may not induce major conformational changes.

Wild-type Tom40 precursors do not persist in a pool of monomers in the outer membrane but are quickly assembled into full-size complexes via a short-lived, high molecular weight intermediate. The relatively small number of radio-labeled molecules of either Tom40 or Tom22 taken up by mitochondria during *in vitro* import are rapidly integrated into complexes with preexisting subunits. Newly imported subunits could be directly assembled into the preexisting functional complexes by which they are imported. Alternatively, the newly imported subunits could be moved into the lipid phase of the outer membrane and quickly associate with a small pool of partially assembled complexes. In the first model, assuming a fixed stoichiometry of the complex, incorporation of new subunits must be coupled to the release of preexisting subunits. This suggests the existence of a pool of partially assembled TOM complexes made up of the released subunits. Thus, the two models are related in that both require a pool of partially assembled subunits that can interact with other subunits. The inability to detect such a pool implies that such pools would be small and formation of the functional complex rapid. Our observation of subunits being exchanged between existing TOM complexes is consistent with models suggesting that incorporation of new subunits into the complex could result in the displacement of preexisting subunits. Regardless of the mechanism of assembly of new subunits into the TOM complex, a dynamic equilibrium of completely assembled and partially assembled TOM complex may exist in the outer membrane.

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