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# Functions of the Small Proteins in the TOM Complex of *Neurospora crasssa*

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**The TOM (translocase of the outer mitochondrial membrane) complex of the outer mitochondrial membrane is required for the import of proteins into the organelle. The core TOM complex contains five proteins, including three small components Tom7, Tom6, and Tom5. We have created single and double mutants of all combinations of the three small Tom proteins of *Neurospora crassa*. Analysis of the mutants revealed that Tom6 plays a major role in TOM complex stability, whereas Tom7 has a lesser role. Mutants lacking both Tom6 and Tom7 have an extremely labile TOM complex and are the only class of mutant to exhibit an altered growth phenotype. Although single mutants lacking *N. crassa* Tom5 have no apparent TOM complex abnormalities, studies of double mutants lacking Tom5 suggest that it also has a minor role in maintaining TOM complex stability. Our inability to isolate triple mutants supports the idea that the three proteins have overlapping functions. Mitochondria lacking either Tom6 or Tom7 are differentially affected in their ability to import different precursor proteins into the organelle, suggesting that they may play roles in the sorting of proteins to different mitochondrial subcompartments. Newly imported Tom40 was readily assembled into the TOM complex in mitochondria lacking any of the small Tom proteins.**

## INTRODUCTION

Import of mitochondrial precursor proteins from the cytosol to the various subcompartments of the organelle requires the action of several multiprotein complexes housed in the mitochondrial inner and outer membranes (Paschen and Neupert, 2001; Pfanner and Wiedemann, 2002; Endo *et al.*, 2003; Hoppins *et al.*, 2004; Koehler, 2004). The TOM complex (translocase of the outer mitochondrial membrane) is responsible for the initial recognition of virtually all mitochondrial precursors synthesized in the cytosol. The holo-TOM complex contains seven proteins. Tom70 and Tom20 contain large hydrophilic domains that extend into the cytosol and act as receptors for precursor proteins. The five additional proteins, Tom40, Tom22, Tom7, Tom6, and Tom5, make up the TOM core complex which acts as the general insertion pore (Dekker *et al.*, 1998; Ahting *et al.*, 1999; Meisinger *et al.*, 2001).

Tom40 and Tom22 of the core complex have been studied extensively in both *S. cerevisiae* and *Neurospora crassa*. Tom40 is essential for viability in both organisms (Baker *et al.*, 1990; Taylor *et al.*, 2003) and is known to be the pore forming component of the complex (Hill *et al.*, 1998; Ahting *et al.*, 2001). The protein also plays a role in binding precursors at both the *cis* (cytosol facing side) and *trans* (intermembrane space facing side) sites of the TOM complex during the translocation process (Rapaport *et al.*, 1997, 1998b). Tom22 is also essential in *N. crassa* (Nargang *et al.*, 1995), but yeast strains lacking the protein can grow slowly on fermentable carbon sources (van Wilpe *et al.*, 1999). Tom22 has several roles including a receptor function (Mayer *et al.*, 1995), passage of precursor proteins from the receptors to the pore (Kiebler *et al.*, 1993; van Wilpe *et al.*, 1999), binding precursors at the *trans* site (Nakai *et al.*, 1995; Court *et al.*, 1996; Moczko *et al.*, 1997; Kanamori *et al.*, 1999), and as a general organizer of the complex (van Wilpe *et al.*, 1999).

A single study on the *N. crassa* Tom6 and Tom7 proteins focused on their assembly into the TOM complex (Dembowski *et al.*, 2001). However, the effects of knocking out the genes encoding the proteins have only been investigated in *Saccharomyces cerevisiae*. Disruption of the gene encoding yeast Tom6 resulted in a slightly reduced growth rate on nonfermentable carbon sources at 37°C (Kassenbrock *et al.*, 1993; Alconada *et al.*, 1995). Import of the ADP-ATP carrier protein (AAC) of the mitochondrial inner membrane and precursors destined to the mitochondrial matrix was reduced (Alconada *et al.*, 1995). Mitochondria lacking Tom6 had a destabilized TOM complex that gave rise to subcomplexes containing Tom40 but not Tom22 in digitonin (DIG)-solubilized mitochondria. However, because subcomplexes of Tom40 and Tom22 that lack Tom6 were observed from wild-type mitochondria solubilized in Triton X-100, it was suggested that Tom6 is required to promote assembly of Tom40 and Tom22, but is not required for the maintenance of the interaction (Alconada *et al.*, 1995; Dekker *et al.*, 1998).

Yeast strains with a deletion of the gene encoding Tom7 were found to grow very slowly at 30°C on nonfermentable carbon sources and were inviable on any carbon source at 37°C (Hönlinger *et al.*, 1996). Tom7 appears to be involved in

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Abbreviations used: AAC, ADP-ATP carrier protein; BNGE, blue native gel electrophoresis; DIG, digitonin; DDM, *n*-dodecyl-β-D-maltoside; $F_1\beta$, beta subunit of the $F_1$ ATP synthase; RIP, repeat induced point mutation; TOM, translocase of the outer mitochondrial membrane.

**Table 1.** Strains used in this study

| Strain | Genotype | Origin or source |
|---|---|---|
| NCN251 | *A* | Fungal Genetics Stock Center 2489 (74-OR23–1VA) |
| 7626 | *his-3 mtrR a* | R. L. Metzenberg |
| HI | *nic-2 cyh-1 inl inv mei-2* | Fungal Genetics Stock Center 7251 |
| Tom5RIP-Hyg-6 | *nic-2 cyh-1 inl inv mei-2* plus a single ectopic copy of *tom5* and hygR | Transformation of HI with plasmid pTom5RIP-Hyg |
| *tom5*$^{RIP}$ (strain used: Tom5Rip-7) | *nic-2 inl inv tom5*$^{RIP}$ | RIP cross. NCN251 by Tom5RIP-Hyg-6 |
| Tom6RIP-Hyg-1 | *his-3 mtrR a* plus a single ectopic copy of *tom6* and hygR | Transformation of 7626 with plasmid pTom6RIP-Hyg |
| *tom6*$^{RIP}$ (strain used: Tom6Rip-12) | *his-3 tom6*$^{RIP}$ | RIP cross. NCN251 by Tom6RIP-Hyg-1 |
| Δ*tom7* (strain used: Tom7KO-35) | Δ*tom7 hygR* | Tom7 disruption in NCN251 |
| *tom5*$^{RIP}$ *tom6*$^{RIP}$ (strains used: Tom5/6Rip-9 and Tom5/6Rip-10) | *nic-2 tom5*$^{RIP}$ *tom6*$^{RIP}$ | Cross between *tom5*$^{RIP}$ and *tom6*$^{RIP}$ |
| *tom5*$^{RIP}$ Δ*tom7* (strain used: LV10–24) | *nic-2 tom5*$^{RIP}$Δ*tom7 hygR* | Cross between Δ*tom7* and *tom5*$^{RIP}$ *tom6*$^{RIP}$ |
| *tom6*$^{RIP}$ Δ*tom7* (strains used: LV10–11 and LV10–2) | *nic-2 tom6*$^{RIP}$Δ*tom7 hygR* | Cross between Δ*tom7 and tom5*$^{RIP}$ *tom6*$^{RIP}$ |

preprotein sorting as import into mitochondria lacking the protein was only slightly affected for preproteins destined to interior compartments of the mitochondria, whereas the import of porin to the outer membrane was greatly reduced (Hönlinger *et al.,* 1996). Absence of Tom7 resulted in more stable associations between Tom40, Tom20, and Tom22 so that the protein seems to play a role opposite that of Tom6 with respect to TOM complex stability (Hönlinger *et al.,* 1996). Tom7 has also been found to have a function that overlaps with that of the C-terminal intermembrane space domain of Tom22. Both are involved in *trans* site binding and passage of precursors to the TIM23 complex (Esaki *et al.,* 2004).

Mutant strains lacking Tom5 have been studied in both *S. cerevisiae* and *N. crassa*. Yeast strains lacking Tom5 were unable to grow at 37°C and had growth defects at 30°C. Furthermore, deletions of *tom5* were found to be synthetically lethal in combination with any of the other TOM complex proteins that were known to be viable as single deletions (Tom6, Tom7, Tom20, Tom70; Dietmeier *et al.,* 1997). Import of all classes of preproteins into yeast mitochondria lacking Tom5 was strongly reduced and it was proposed that the cytosolic domain of the protein played a role in the passage of preproteins from the TOM complex receptors to the general insertion pore (Dietmeier *et al.,* 1997). The protein was also found to be a specific receptor for the small Tim proteins of the intermembrane space, which do not require the cytosolic domains of Tom20 or Tom70 for their import (Kurz *et al.,* 1999). However, it has subsequently been shown that replacement of the cytosolic domain of Tom5 with either GFP (Horie *et al.,* 2003) or the cytosolic domain of Fis1 (Habib *et al.,* 2003), could restore the growth defects of *tom5*Δ strains. Thus, the role of the cytosolic domain of yeast Tom5 is unclear.

Surprisingly, the recent characterization of an *N. crassa* strain lacking Tom5 revealed no growth or import defects (Schmitt *et al.,* 2005). Furthermore, no abnormalities in the TOM complex of the *N. crassa* mutant strain could be discerned, and the function of the protein in the organism was unclear. Further investigation of the TOM complex from a *S. cerevisiae* strain lacking Tom5 revealed altered structural characteristics. The structural abnormalities could be rescued by a Fis1-Tom5 fusion protein lacking the Tom5 N-terminus. Thus, it was suggested that the role of Tom5 in *S. cerevisiae* is to maintain the structure of the TOM complex rather than directly acting in preprotein transfer (Schmitt *et al.,* 2005).

The role of each of the small Tom proteins in the assembly of newly imported Tom40 into the TOM complex has also been studied in yeast. Tom40 assembles via a complex pathway involving two intermediates and many factors including the TOB complex (topogenesis of outer membrane β-barrels), which is also known as the SAM complex (sorting and assembly complex; Kozjak *et al.,* 2003; Paschen *et al.,* 2003; Wiedemann *et al.,* 2003; Gentle *et al.,* 2004; Ishikawa *et al.,* 2004; Milenkovic *et al.,* 2004; Waizenegger *et al.,* 2004), the Mdm10 protein (Meisinger *et al.,* 2004), and the Tom13 or Mim1 protein (Ishikawa *et al.,* 2004; Waizenegger *et al.,* 2005). Tom5 was found to act at an early stage in the pathway because formation of the earliest intermediate in assembly was inhibited in mitochondria lacking the protein (Model *et al.,* 2001). Tom6 was thought to function late in assembly because the early intermediates formed efficiently, whereas progression to the final assembled form was inhibited. The role of Tom7 in assembly was consistent with its action in destabilizing the interaction between Tom40 and the receptor proteins. Thus, the antagonistic roles of Tom6 and Tom7 were suggested to result in an ongoing cycling of the fully assembled TOM complex and a late assembly intermediate to promote efficient incorporation of newly imported Tom subunits into the complex (Model *et al.,* 2001; Pfanner and Wiedemann, 2002).

In this study, we have investigated the characteristics of *N. crassa* mutants lacking Tom6 and Tom7. In addition, we constructed double mutants lacking each of the possible pairs of Tom5, Tom6, and Tom7. Our results show many differences with respect to the functions of these proteins in *N. crassa* when compared with the functions assigned to their *S. cerevisiae* homologues.

## MATERIALS AND METHODS

### *Growth of N. crassa and Strains Used*

Growth and handling of *N. crassa* followed standard procedures (Davis and De Serres, 1970). The strains used in this study are listed in Table 1. The mutants used in this study were derived from three parental strains: HI, NCN251 and 76–26 (Table 1). Comparison of TOM complex stability, mitochondrial protein import, and Tom40 assembly between these three parental strains revealed no differences. We chose NCN251 to be used as a control in experiments with the mutants.

Growth rates were measured by spotting 10 μl of suspensions containing $2 \times 10^5$, $2 \times 10^4$, $2 \times 10^3$, and $2 \times 10^2$ conidia in water onto plates containing standard sorbose medium with the appropriate supplements. The plates were incubated until growth was seen in the lowest dilution spot of the control strain.

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

The *tom5*$^{RIP}$ mutant used in these studies was derived by crossing the *tom5* duplication strain Tom5RIP-Hyg-6, described previously (Schmitt *et al.*, 2005), with strain NCN 251. In this cross, the *tom5* duplication served as a substrate for repeat induced point mutation (RIP), which affects duplicated sequences in a single nucleus that is undergoing the sexual phase of the *N. crassa* life cycle (Selker, 1990). Mitochondria were isolated from the progeny of the cross and examined for the presence of Tom5 by Western analysis. Strain Tom5Rip-7 was devoid of Tom5 and was used for further work. It should be noted that the *tom5*$^{RIP}$ strain used in this work was not the same one used in a previous study in which Tom5RIP-Hyg-6 was used in a sheltered RIP cross with strain MI to produce progeny lacking Tom5 (Schmitt *et al.*, 2005).

The *tom6*$^{RIP}$ mutant was also generated via RIP mutagenesis. We wanted to create a plasmid carrying a copy of *tom6* for transformation into an appropriate strain to generate a duplication to serve as a RIP substrate. We were unable to clone *tom6* into a high copy plasmid. Therefore, a linker containing a *Not*I site flanked by *Eco*RI sticky ends was synthesized and inserted into the *Eco*RI site of the low copy plasmid pBR322 to give pBR322-*Not*I. To allow eventual selection in *N. crassa* transformations, the hygromycin resistance gene from plasmid pCSN44 (Staben *et al.*, 1989) was then inserted into the *Sal*I site of this vector to yield pBR322-*Not*I-HygR. Primers with *Not*I sites at their 5′ ends were then used to amplify a 2.5-kb *tom6* containing fragment from *N. crassa* genomic DNA by PCR. The fragment was inserted into the *Not*I site of pBR322-*Not*I-Hyg to yield plasmid pTom6RIP-Hyg. A strain carrying a duplication of *tom6* was generated by transforming strain 7626 with the latter plasmid. Strain Tom6RIP-Hyg-1 was identified by Southern analysis to contain one extra copy of the *tom6* sequence inserted into the genome. This strain was crossed with NCN251 to generate progeny containing *tom6*$^{RIP}$ alleles. Strains lacking the Tom6 protein were identified by Western blot analysis of isolated mitochondria.

The Δ*tom7* strain (Tom7KO-35) was made by replacing the *tom7* gene with a hygromycin resistance gene via homologous recombination using a split marker approach (Colot *et al.*, unpublished results). Because no antibody is available for the *N. crassa* Tom7 protein, the presence of the replacement was demonstrated by PCR using primers outside the *tom7* gene that resulted in amplification of either the wild-type *tom7* allele or the larger, disrupted Δ*tom7* allele containing the hygromycin resistance gene. The absence of Tom7 in one of our mutant strains (*tom5*$^{RIP}$ Δ*tom7*) was proven by isolation of purified TOM complex and silver staining of the components following SDS-PAGE (S. Schmitt, personal communication).

To obtain mutants lacking different combinations of the small Tom proteins, the *tom5*$^{RIP}$ strain (Tom5Rip-7) was crossed to the *tom6*$^{RIP}$ strain (Tom6Rip-12) and mitochondria from the progeny of this cross were screened for the absence of the Tom5 and Tom6 proteins by Western analysis. Two strains lacking both Tom5 and Tom6 were chosen (Tom5/6Rip-9 and Tom5/6Rip-10) and were shown to be sensitive to hygromycin. These were crossed to the Δ*tom7* strain, in which the hygromycin resistance marker had replaced the *tom7* gene. Ascospores from the cross were plated onto medium containing hygromycin to select for strains containing the deletion of *tom7*. After 1- to 3-d growth on the plates, the spores were transferred to hygromycin containing slants. After growth and conidiation on these slants, the strains were subcultured to slants without hygromycin. The strains were then grown in liquid medium and mitochondria were isolated. These were screened for the absence of the Tom5 and Tom6 proteins by Western blot analysis. The absence of Tom7, which was selected on the basis of hygromycin resistance, was confirmed by PCR analysis of eight separate isolates.

### *In Vitro Import of Radiolabled Proteins into Isolated Mitochondria*

For in vitro import studies, mitochondria were isolated (Mayer *et al.*, 1993) and import of mitochondrial preproteins was performed as described (Harkness *et al.*, 1994). Preproteins were produced by transcription and translation in rabbit reticulocyte lysate (Promega TnT reticulocyte lysate system, Madison, WI) in the presence of [$^{35}$S]methionine (ICN Biomedicals, Costa Mesa, CA). Incubation time points were 1, 3 and 5 min. Import reactions were analyzed by SDS-PAGE and viewed by autoradiography or phosphorimaging. Quantification of the image from the latter was done using the imagequant program (version 5.2, Molecular Dynamics, Sunnyvale, CA). The precursors of the β-subunit of the $F_1$ ATPase ($F_1\beta$), the ATP-ADP carrier protein (AAC), and porin were used in import studies.

### *Blue Native Gel Electrophoresis*

Mitochondria (50 μg) were solubilized in 50 μl of buffer containing detergent (either 1% DIG or 1% *n*-*D*odecyl-β-D-maltoside [DDM] in 20 mM Tris-Cl, pH 7.4; 0.1 mM EDTA; 50 mM NaCl; 1% glycerol; 1 mM phenylmethylsulfonyl fluoride). After gentle rocking at 4°C for 15 min and a clarifying spin (30 min, 4°C, 13,000 rpm), the supernatant was added to 5 μl of sample buffer (5% Coomassie Brilliant Blue G-250 in 100 mM Bis-Tris, 500 mM 6-aminocaproic acid, pH 7.0) and gently mixed at 4°C. Samples were analyzed on 6–13% gradient blue native gels as previously described (Schägger and von Jagow, 1991; Schägger *et al.*, 1994) except that electrophoresis was performed overnight (~16–20 h) at 4°C between 40 and 60 V before the excess Coomassie Blue was electrophoresed out for 1–1.5 h at 500 V.

### *Other Techniques*

Standard techniques for agarose gel electrophoresis, transformation of *Escherichia coli*, isolation of bacterial plasmid DNA, cloning, restriction digests of plasmid DNA, and PCR using *Taq* polymerase were performed as described (Ausubel *et al.*, 1992; Sambrook and Rusell, 2001). Separation of mitochondrial proteins by SDS-PAGE (Laemmli, 1970), Western blotting (Good and Crosby, 1989), genomic DNA isolation from *N. crassa* (Wendland *et al.*, 1996), mitochondria isolation (Mayer *et al.*, 1993), preparation of genomic DNA from conidiaspores for PCR analysis (Descheneau *et al.*, 2005), and electroporation of *N. crassa* conidiaspores (Tanton *et al.*, 2003) were as described previously.

The following procedures were performed as recommended by the supplier: Western blot detection by light emission using LumiGLO chemiluminescent substrate (KPL, Mandel, Guelph, Ontario, Canada), protein determination by the Coomassie dye binding assay (Bio-Rad, Hercules, CA), automated sequencing using a BigDye Terminator Cycle sequencing kit (version 3.1) with a Model 373 stretch sequencer separation system (Applied Biosystems, Foster City, CA), bacterial DNA plasmid isolation with Qiagen mini-prep spin kits (Qiagen, Santa Clarita, CA), and site-directed mutagenesis based on the Muta-Gene system (Bio-Rad).

Antiserum to Tom40 was raised in rabbits against a peptide composed of the C-terminal 12 amino acid residues. Tob55 antiserum was raised in rabbits against a hexahistidinyl-tagged DHFR fusion protein containing the N-terminal 69 residues of Tob55. All other antisera were a generous gift from the laboratory of W. Neupert.

## RESULTS

### *Isolation and Growth Characteristics of N. crassa Strains Lacking the Small Tom Proteins*

The Tom5, Tom6, and Tom7 proteins of *S. cerevisiae* have been assigned various roles based primarily on the study of mutants lacking the proteins. A recent study of a *N. crassa* strain lacking Tom5 revealed no detectable phenotype (Schmitt *et al.*, 2005), in striking contrast to the severe phenotype of yeast cells lacking the protein (Dietmeier *et al.*, 1997). Therefore, it was of interest to determine the phenotype of *N. crassa tom6* and *tom7* mutants. As described in *Materials and Methods*, a mutant lacking Tom6 was constructed by RIP mutagenesis. A *tom7* knockout mutant (Δ*tom7*) was isolated by replacing the Tom7 coding sequence with a gene for hygromycin resistance (Colot *et al.*, unpublished results). Neither of these strains exhibited a growth phenotype (Figure 1A).

In an attempt to reveal synthetic defects in cells lacking two or more of the proteins, we created strains carrying different combinations of the three small Tom mutant alleles by genetic crossing. A cross of a *tom5*$^{RIP}$ strain with the *tom6*$^{RIP}$ strain produced *tom5*$^{RIP}$*tom6*$^{RIP}$ double mutants, which had no obvious growth phenotype (Figure 1A). We then crossed the Δ*tom7* mutant with the *tom5*$^{RIP}$*tom6*$^{RIP}$ double mutant and plated the resulting ascospores on medium containing hygromycin to select directly for spores carrying the Δ*tom7* allele (see *Materials and Methods*). When germinated spores were picked to slants containing hygromycin, "fast-growing" and "slow-growing" isolates were identified, based on the time required to cover the surface of the slant with mycelium. To ensure that plating on hygromycin had selected for spores carrying Δ*tom7*, four isolates of each growth class were chosen randomly for analysis by PCR to determine whether the wild-type or disrupted allele of *tom7* was present (see *Materials and Methods*). All eight were found to contain the Δ*tom7* allele (unpublished data). We then examined 30 of the fast growing strains by Western blot analysis for the presence of Tom5 and Tom6. This revealed that 18 strains were Δ*tom7* single mutants and 12 were *tom5*$^{RIP}$Δ*tom7* double mutants. We also examined 20 slow growing strains for the presence of Tom5 and Tom6 and all were found to be *tom6*$^{RIP}$Δ*tom7* double mutants. Thus, the

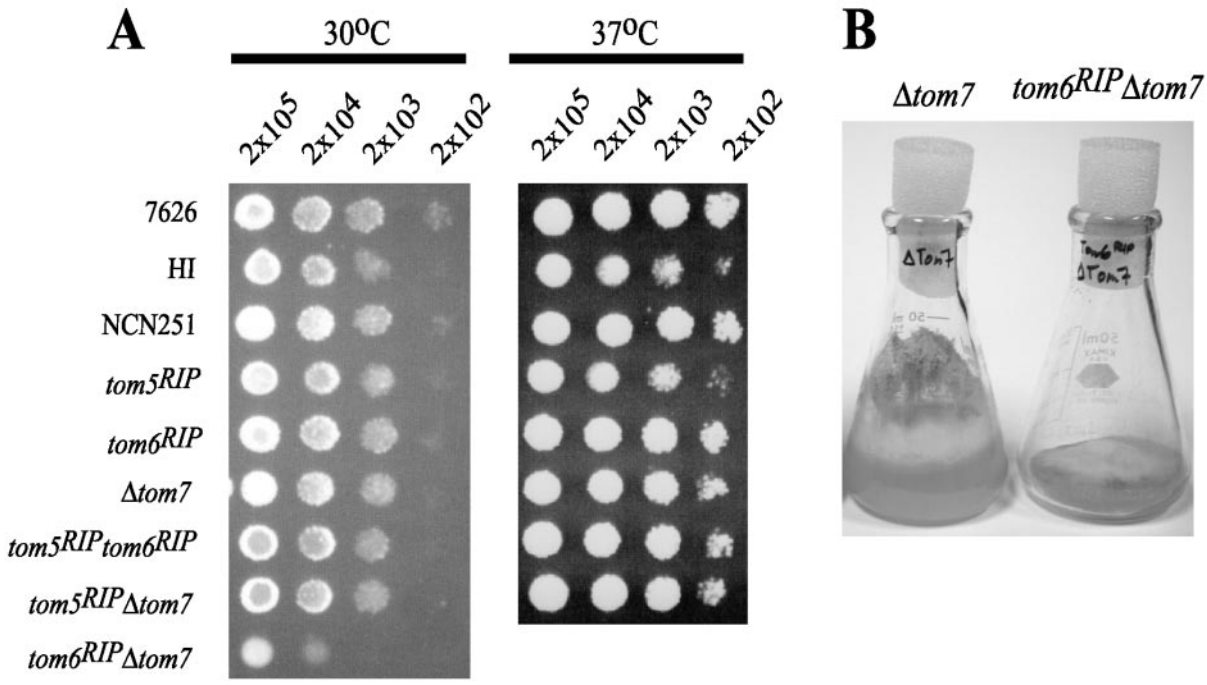


**Figure 1.** Growth of strains lacking the small Tom proteins. (A) A 10-μl suspension containing the indicated number of conidia from each strain was applied to sorbose-containing medium. Plates were incubated at 30 or 37°C. We were unable to obtain sufficient numbers of conidia to test the *tom6*$^{RIP}$Δ*tom7* strain at both temperatures. (B) Growth of the Δ*tom7* strain (left) in flasks containing solid medium is indistinguishable from any other strain used in this study except for the *tom6*$^{RIP}$Δ*tom7* strain (right).

predicted *tom5*$^{RIP}$*tom6*$^{RIP}$Δ*tom7* triple mutant was not present in either class. Because all three small Tom genes are located on separate chromosomes (Galagan *et al.*, 2003), the triple mutants should have represented 25% of the Δ*tom7*, hygromycin-resistant progeny. The absence of triple mutants among the progeny strongly suggests that loss of all three small Tom proteins is lethal in *N. crassa*.

The growth rate of each class of single and double mutant at 30 and 37°C is shown in Figure 1A. All mutant strains were indistinguishable from controls except for the *tom6*$^{RIP}$Δ*tom7* double mutant which had a severe growth rate defect. Unlike all controls and other mutants examined in this study, the *tom6*$^{RIP}$Δ*tom7* double mutants are also characterized by an inability to climb the walls of growth flasks containing solid medium and an inability to produce conidia (Figure 1B). Both the *tom5*$^{RIP}$ strain and its parent HI, displayed a minor growth rate defect at 37°C. Further genetic analysis proved that the growth defect did not segregate with the Tom5 deficiency (unpublished data).

### *The TOM Complex in the Small Tom Mutants*

Because the small Tom proteins are known to be part of the TOM core complex (Dekker *et al.*, 1998; Ahting *et al.*, 1999; Meisinger *et al.*, 2001), it was conceivable that the level of other components of the complex might be affected by the absence of one or more of the small proteins. We examined the levels of various mitochondrial proteins in each of the mutant strains by Western blot analysis. Unless directly affected by the mutated gene, the levels of Tom70, Tom40, Tom22, Tom20, Tom6, and Tom5 are unaltered in the individual mutants, the *tom5*$^{RIP}$*tom6*$^{RIP}$ double mutant and the *tom5*$^{RIP}$Δ*tom7* double mutant (Figure 2). The loss of both Tom6 and Tom7 dramatically reduced the levels of Tom5, Tom20, and Tom22. Interestingly, the amount of Tob55/Sam50, the major component of the β-barrel assembly complex of the outer membrane, appears to be slightly increased in the *tom6*$^{RIP}$Δ*tom7* double mutant. Conceivably, this could be due to a response to compensate for the presence of a TOM complex, which is deficient in several of its usual components. For unknown reasons we consistently observe a double band in Western blots for Tob55/Sam50. Two observations suggest that both bands represent the Tob55/Sam50 protein. First, both bands disappear in *Neurospora crassa tob55* mutants. Second, when a *tob55* mutant is rescued with a gene encoding a His-tagged version of the protein, an antibody to the tag also detects two bands on Western blots (S. C. Hoppins and F. E. Nargang, unpublished results). The ratio of the higher molecular-weight band to the lower one appears to be increased in the *tom6*$^{RIP}$Δ*tom7* mitochondria. The possible significance of this is not known. The levels of Tim23 of the inner membrane, CCHL of the intermembrane space, and Hsp60 of the matrix were not affected by the absence of any of the small Tom proteins. We were unable to measure effects caused by the lack of Tom5 or Tom6 on the level of Tom7 because an antibody to the *N. crassa* protein is not available.

We examined the stability of the TOM complex in the mutants by lysing mitochondria in the presence of DIG or DDM and determining the location of Tom40 after blue native gel electrophoresis (BNGE; Figure 3, A and B). In control mitochondria, Tom40 is present with other TOM components in a 400-kDa complex after treatment with 1% DIG. However, this complex breaks down into smaller complexes after treatment with 1% DDM. As reported previously (Schmitt *et al.*, 2005), the absence of Tom5 had no effect on the stability of the *N. crassa* TOM complex. The absence of Tom6 resulted in some Tom40 dissociating from the 400-kDa complex into a smaller subcomplex in 1% DIG and caused

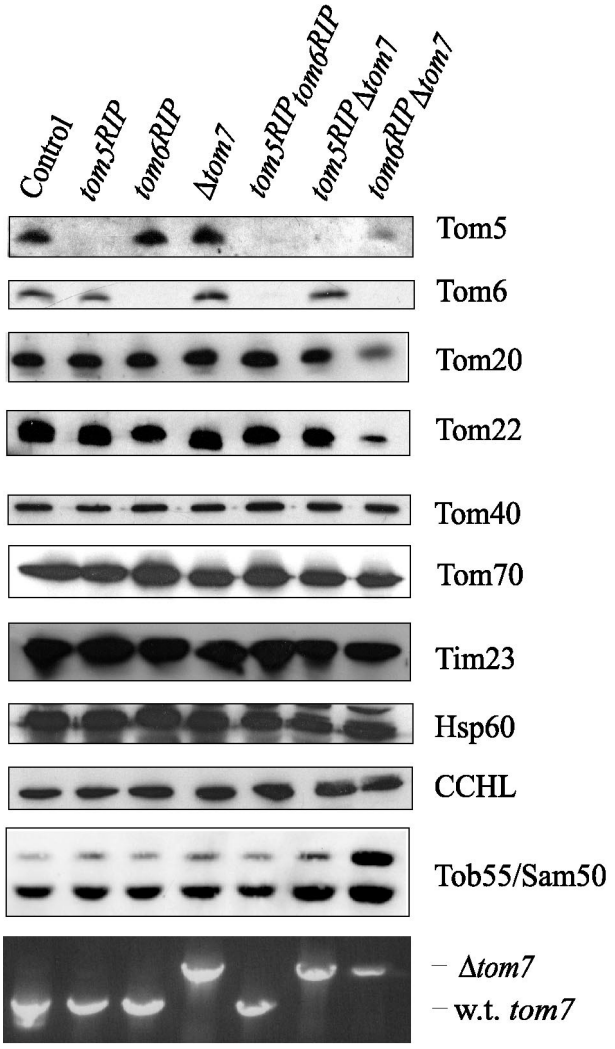


**Figure 2.** Levels of TOM complex and other mitochondrial proteins in strains lacking the small Tom proteins. Mitochondria, 30 μg, isolated from each strain was subjected to SDS-PAGE and transferred to nitrocellulose. Western blots were analyzed for each of the TOM complex proteins and for the matrix protein mitochondrial Hsp60, Tim23 of the inner membrane, and cytochrome *c* heme lyase (CCHL) of the intermembrane space. The presence or absence of Tom7 was determined by PCR analysis because no antibody to *N. crassa* Tom7 is available. Genomic DNA was isolated from each strain and PCR was performed using primers outside the *tom7* gene that amplify both the disrupted allele (Δ*tom7*), carrying the gene encoding hygromycin resistance, and the wild-type *tom7* gene (w.t. *tom7*). Because the hygromycin resistance gene is larger than the gene encoding Tom7, the PCR product is larger for the Δ*tom7* allele. (The control is NCN251.)

the TOM complex to completely dissociate to smaller Tom40 containing forms in 1% DDM. The stability of the *N. crassa* TOM complex appears to be less dependent than its yeast counterpart on the presence of Tom6 because yeast mitochondria lacking Tom6 show severe instability even in the presence of the mild detergent DIG (Dekker *et al.*, 1998). The absence of *N. crassa* Tom7 resulted in a slight loss of Tom40 from the 400-kDa complex and its appearance in smaller subcomplexes in both detergents. The loss of Tom40 from the complex in the $tom5^{RIP}$ $tom6^{RIP}$ double mutant was slightly enhanced compared with the $tom6^{RIP}$ single mutant in both DIG and DDM, suggesting a role for *N. crassa* Tom5 in TOM complex stability. In the $tom5^{RIP}$ Δ*tom7* double mutant the behavior of the TOM complex was similar to the single Δ*tom7* mutant in DIG but was slightly less stable in DDM. The absence of both Tom6 and Tom7 resulted in severe destabilization of Tom40 in the TOM complex in both DIG and DDM (Figure 3, A and B). Similar results were observed when blue gels were examined for the position of Tom22, the other essential component of the core TOM complex in *N. crassa* (Nargang *et al.*, 1995). In DIG (Figure 3C), at least some Tom22 was lost from the TOM complex of all single and double mutants except the mutant lacking Tom5 alone. In DDM (Figure 3D), most of Tom22 disassembles from the complex in the single $tom6^{RIP}$ mutant and the $tom5^{RIP}$ $tom6^{RIP}$ double mutant. In contrast to what was seen with Tom40, no high molecular-weight subcomplex containing Tom22 remains after lysis in DDM, demonstrating that the loss of Tom6 weakens the interactions between Tom40 and Tom22. Absence of Tom7 also resulted in loss of Tom22 from the TOM complex in the presence of DDM. The most severe effects were seen in the mutant lacking both Tom6 and Tom7, in which virtually all of Tom22 was seen in low-molecular-weight forms after lysis in either detergent.

### *Import of Mitochondrial Precursor Proteins*

The ability of the small Tom mutant strains to import mitochondrial proteins was tested by in vitro import of radiolabeled preproteins into isolated mitochondria from each of the strains. In agreement with previous findings (Schmitt *et al.*, 2005), we could not distinguish any difference between controls and the $tom5^{RIP}$ mutant for import of any precursor tested, including $F_1\beta$, AAC, porin, and Tim10 (unpublished data). Mitochondria lacking Tom6 had a minor defect in importing $F_1\beta$ into the matrix but was similar to the control with respect to the import of AAC and porin, to the inner and outer mitochondrial membranes, respectively (Figure 4, A–C). Mitochondria lacking both Tom5 and Tom6 imported preproteins in a manner similar to those devoid of only Tom6 although the defect in $F_1\beta$ import was more pronounced (Figure 4). The strains lacking Tom7 or both Tom7 and Tom5 were defective in importing porin and $F_1\beta$, but import of AAC was unaffected (Figure 5). We were unable to obtain enough conidia from the $tom6^{RIP}$ Δ*tom7* double mutant to grow cultures for analysis of mitochondrial protein import (see Figure 1B).

### *Effects of the Small Tom Proteins on Tom40 Assembly into the TOM Complex*

As shown previously, in both yeast and *N. crassa*, a newly imported Tom40 molecule is first observed in a 250-kDa intermediate, progresses to a 100-kDa intermediate, and then appears in the final assembled 400-kDa TOM core complex (Model *et al.*, 2001; Taylor *et al.*, 2003). Mitochondria from each of the small Tom mutant strains were examined for their ability to assemble newly imported Tom40. In the control strain, Tom40 accumulates in the 250-kDa intermediate when import is performed at 0°C (Figure 6). This represents a monomer of Tom40 associated with the TOB/SAM complex, which is required for the assembly of β-barrel proteins such as Tom40 into the mitochondrial outer membrane (Kozjak *et al.*, 2003; Paschen *et al.*, 2003). When import is performed at 25°C with control mitochondria, Tom40 can progress past the TOB/SAM complex to the 100-kDa intermediate, and then to the fully assembled 400 kDa complex. Membrane integration occurs at the 100-kDa form and assembly of the incoming precursor to the 400-kDa complex occurs within the membrane (Model *et al.*, 2001;

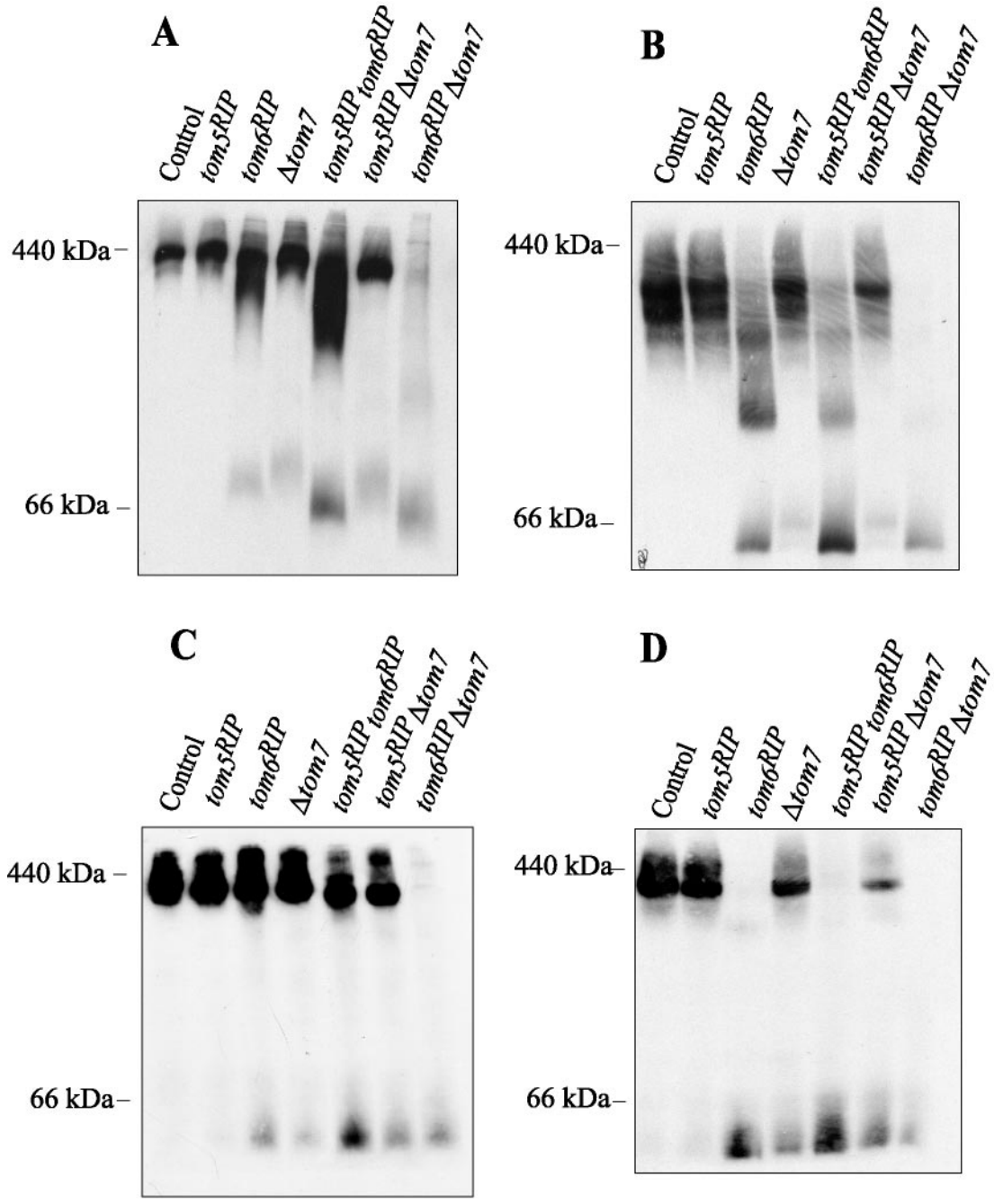


**Figure 3.** Stability of the TOM complex in strains lacking the small Tom proteins. Mitochondria (50 $\mu$g) lacking different small Tom proteins were dissolved in 1% DIG (A and C) or 1% DDM (B and D), subjected to BNGE, and blotted to PVDF membrane. Membranes were decorated with $\alpha$Tom40 antiserum (A and B) or $\alpha$Tom22 antiserum (C and D) and processed for detection of bands by light emission. The positions of 440- (apoferritin) and 66-kDa (bovine serum albumin) marker proteins are shown on the left. Although equal amounts of mitochondrial protein were loaded in all lanes, there is a striking loss of material in the *tom6*$^{RIP}$Δ*tom7* lanes. We have previously suggested that this may be due to aggregation of disassembled components (Taylor *et al.*, 2003; the control is NCN251.)

Rapaport, 2002). Each of the single and double mutant strains was able to assemble Tom40 into the 400-kDa complex at 25°C. In all cases this appeared to be somewhat more efficient than in the control strain (Figure 6). Mitochondria lacking either Tom5 or Tom7 even appeared to assemble a small amount of Tom40 into the 400-kDa form after 20 min of import at 0°C. Furthermore, very little Tom40 was observed in the 250-kDa intermediate following 20 min of import at 25°C in mitochondria from any of the mutant strains except the *tom6*$^{RIP}$ single mutant. These data suggest that loss of the small Tom proteins affects the kinetics of progression of Tom40 assembly through the TOB/SAM complex. To further investigate this possibility we performed time course assays of Tom40 import at 25°C using mitochondria isolated from each of the single mutants (Figure 7). In each case it is apparent that Tom40 assembly into

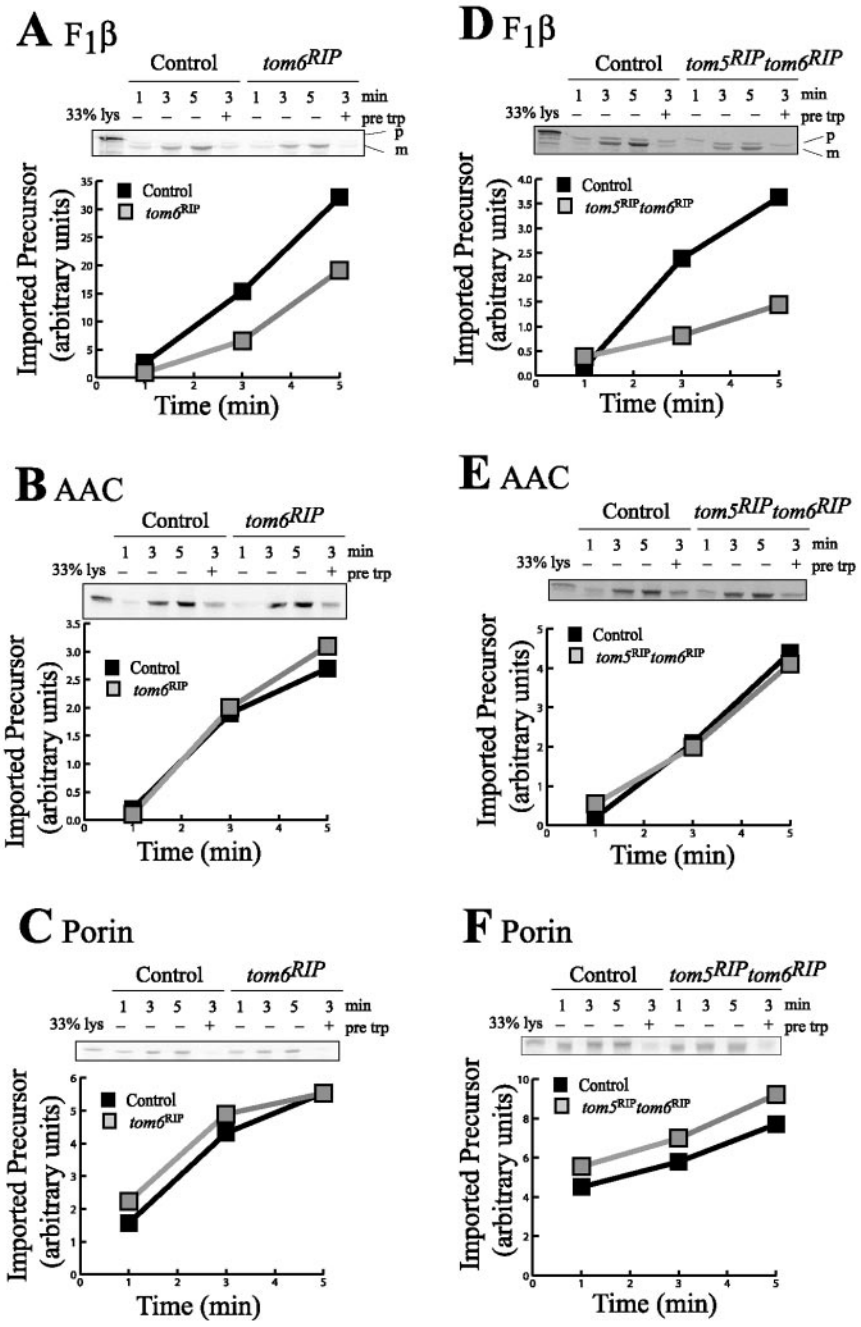


**Figure 4.** In vitro import of preproteins into mitochondria lacking Tom6 or both Tom5 and Tom6. Radiolabeled mitochondrial preproteins $F_1\beta$ (A and D) and AAC (B and E) synthesized in rabbit reticulocyte lysate were incubated with mitochondria isolated from a control strain (NCN251) or from strains without Tom6 (*tom6*$^{RIP}$) or both Tom5 and Tom6 (*tom5*$^{RIP}$*tom6*$^{RIP}$), for 1, 3, and 5 min at 25°C. Mitochondria were incubated at 15°C with lysate containing the labeled precursor of porin (C and F). A sample of mitochondria from each strain was treated with trypsin before import (pre trp), to demonstrate that import was receptor dependent. After the import reaction, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Mitochondria were then reisolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and exposed to x-ray film (insets) and then a Phosphorimager screen for visualization and quantification. "33% lysate" shows a lane in which one-third of the amount of radiolabeled lysate used in each import reaction was electrophoresed.

the final 400-kDa complex occurs more rapidly than in the control. Unlike the wild-type control, significant amounts of Tom40 have reached the 400-kDa stage after only 1 min of import in all three mutant strains. Assembly to the final form in the mutants continues to be more efficient than in the control at each time point. This was particularly striking in the mitochondria lacking Tom7. The rapid disappearance of Tom40 from the initial 250-kDa intermediate in mitochondria from the *tom5*$^{RIP}$ and the Δ*tom7* strains was also confirmed in the time course experiments.

## DISCUSSION

Our study has revealed that the roles of the small Tom proteins in *N. crassa* differ in a variety of ways from the roles assigned to these proteins from studies in *S. cerevisiae*. It was previously shown that *tom5* mutants of *N. crassa* displayed no observable phenotype, whereas yeast cells lacking the protein have growth defects, import defects, and decreased TOM complex stability (Dietmeier *et al.*, 1997; Schmitt *et al.*, 2005). Our findings support the conclusion that Tom5 plays a minor role in the *N. crassa* TOM complex compared with the more crucial role of the protein in *S. cerevisiae*. For example, yeast *tom5* mutants display synthetic lethality when present in double mutants with knockouts of any other Tom protein (Dietmeier *et al.*, 1997). In *N. crassa*, we observed no synthetic effects on growth when the *tom5*$^{RIP}$ mutation was combined with either the *tom6*$^{RIP}$ or Δ*tom7* mutation. Like the single mutants for each gene, the growth

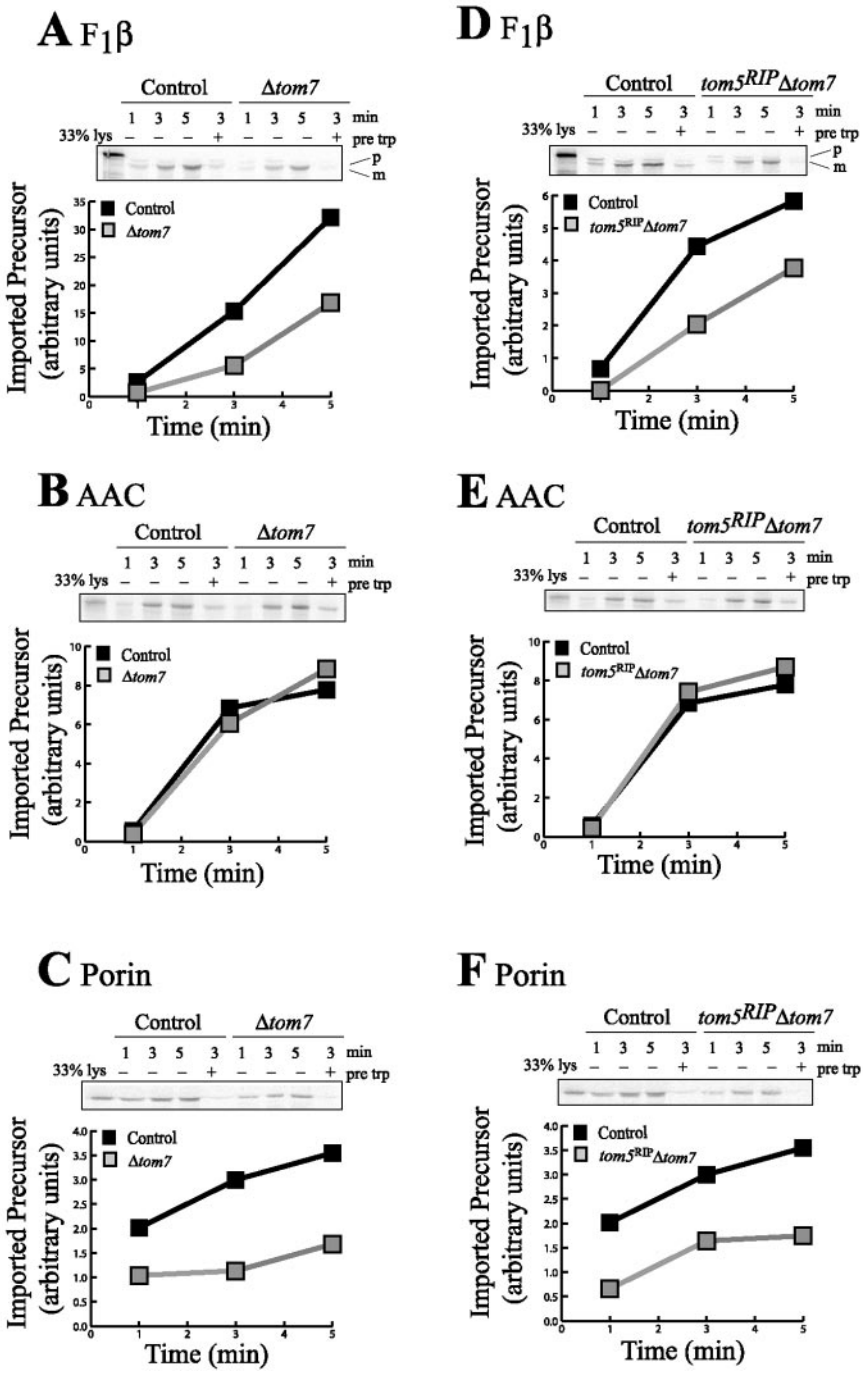


**Figure 5.** In vitro import of preproteins into mitochondria lacking Tom7 or both Tom5 and Tom7. As in Figure 4, except that mitochondria were isolated from a control strain (NCN251) and from strains without Tom7 (Δ*tom7*) or both Tom5 and Tom7 (*tom5*$^{RIP}$Δ*tom7*).

rate of these double mutants was indistinguishable from controls. However, the slight increase in loss of Tom40 and Tom22 from the TOM complex observed in the *tom5*$^{RIP}$*tom6*$^{RIP}$ double mutant compared with the *tom6*$^{RIP}$ mutant alone offers the first insight into a function for *N. crassa* Tom5 and suggests that the protein plays a minor role in maintaining complex stability. This notion is supported by the fact that we were unable to isolate a *tom5*$^{RIP}$*tom6*$^{RIP}$Δ*tom7* triple mutant. Thus, the function of Tom5 in complex stability appears to be necessary for viability in the *tom6*$^{RIP}$Δ*tom7* double mutant, which already has a severely destabilized complex and a severe growth phenotype. All three small Tom proteins may have overlapping or redundant roles in maintaining TOM complex stability in *N. crassa*.

Some aspects of Tom6 function are similar in yeast and *N. crassa*. The absence of Tom6 in both organisms leads to destabilization of the TOM complex. In addition, the subcomplexes of Tom40 formed in the *N. crassa tom6* mutant in the presence of DDM do not contain Tom22, suggesting that Tom6 serves to stabilize the interaction between the two proteins. These findings are consistent with previous suggestions for the role of Tom6 in modulating the interactions of the Tom proteins during preprotein translocation (Rapaport *et al.*, 1998a; Dembowski *et al.*, 2001). *N. crassa* mitochondria lacking Tom6 showed a mild defect in the import

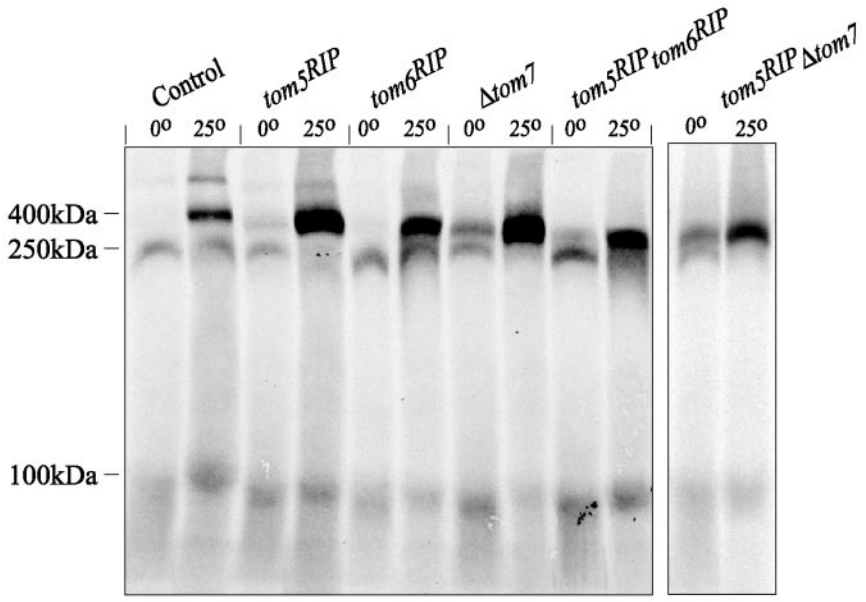


**Figure 6.** Tom40 assembly in mitochondria lacking the small Tom proteins. Radiolabeled Tom40 protein was incubated with isolated mitochondria from a control (NCN251) and each of the indicated small Tom protein mutant strains for 20 min at either 0 or 25°C. After the incubation, mitochondria were pelleted, dissolved in 1% DIG, subjected to BNGE, transferred to PVDF membrane, and exposed to x-ray film. The positions of the 400-kDa assembled TOM complex, the 250-kDa intermediate, and the 100-kDa intermediate in the control mitochondria are indicated on the left.

of $F_1\beta$ to the matrix, whereas the import of AAC to the inner membrane and porin to the outer membrane was not affected. Import of precursors destined for the mitochondrial matrix, and of AAC, were reduced in yeast mitochondria devoid of Tom6. It seems likely that this difference between the organisms is due to the greater instability of the yeast TOM complex imparted by loss of Tom6.

The role of Tom7 appears to be quite different in *N. crassa* compared with its *S. cerevisiae* counterpart. In *N. crassa* the absence of Tom7 partially destabilizes the TOM complex. In contrast, the presence of yeast Tom7 is thought to have a destabilizing affect on the Tom complex so that loss of the protein results in stronger interactions between TOM complex components (Hönlinger *et al.*, 1996). In keeping with this function, absence of yeast Tom7 provides a partial stabilizing effect on the subcomplexes present in solubilized yeast mitochondria lacking the stabilizing protein, Tom6 (Dekker *et al.*, 1998). However, the opposite is observed in *N. crassa* in which the TOM complex is destabilized to a much greater degree in the *tom6*$^{RIP}$ Δ*tom7* double mutant compared with either single mutant. In yeast mitochondria lacking Tom7, the import of mitochondrial precursors to all subcompartments is reduced, with the effects greatest on the outer membrane protein porin. A severe effect on porin import is also seen in *N. crassa* mitochondria lacking Tom7 and a slight decrease in the import of $F_1\beta$ to the matrix is

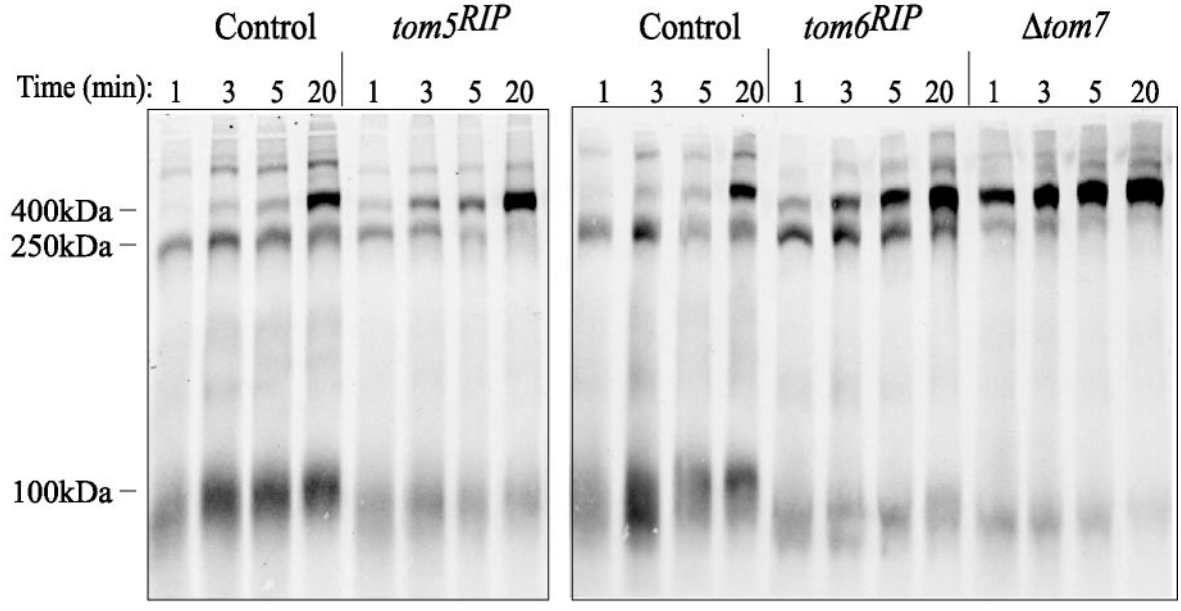


**Figure 7.** Time course of Tom40 assembly in mitochondria lacking the small Tom proteins. The experiment was performed as in Figure 6 except that no import was done at 0°C and imports at 25°C were done for each of the indicated time points.

also observed. However, no effect was seen on import of AAC to the inner membrane. A decrease in the import of porin compared with the apparently increased efficiency of Tom40 import and assembly in the Δ*tom7* mutant suggests that the protein has different roles with respect to the precursors of these two β-barrel proteins. Tom7 may be more directly involved in porin import at the level of recognition or in the passage of the protein to post-TOM complex stages of import. On the other hand, absence of Tom7 may increase the ability of Tom40 to assemble into the 400-kDa core TOM complex. In support of this interpretation, Tom7 in yeast has been shown to have a role in forming the trans binding site along with the C-terminus of Tom22 on the intermembrane space side of the outer membrane (Esaki *et al.*, 2004). Mutants lacking these trans site components were found to have a significant deficiency of porin, whereas Tom40 levels were unaffected.

The effects of the small Tom proteins on TOM complex stability make it difficult to establish whether they have a direct role in the import of mitochondrial preproteins or if the effects are the result of destablilization of other components. Regardless of the reason, absence of Tom6 or Tom7 has a differential effect on the import of different classes of precursor. As mentioned above, the role of yeast Tom7 in the *trans* site suggests a more direct role in import for this protein (Esaki *et al.*, 2004).

Differences in the assembly of Tom40 into the TOM complex are also apparent in the small Tom mutant strains of *N. crassa* and *S. cerevisiae*. In yeast (Model *et al.*, 2001), Tom5 is needed for Tom40 to reach the initial 250-kDa intermediate. Tom6 is needed for Tom40 to reach the fully assembled complex of 400 kDa, but the 250- and 100-kDa intermediates are efficiently formed. Tom7 is required for the Tom40 precursor to be released from the receptor subunits. Thus, when Tom40 is imported into yeast mitochondria lacking Tom7, a series of additional intermediates is observed that represent complexes containing extra receptor protein subunits. However, in *N. crassa*, import and assembly of Tom40 into mitochondria lacking one or more of the small Tom proteins appears to be more efficient as the appearance of an incoming Tom40 monomer in the final 400-kDa assembled complex occurs more rapidly in the mutants than wild-type mitochondria. Furthermore, the time course experiments revealed that the duration of association of the Tom40 precursor with the TOB/SAM complex is decreased in the $tom5^{RIP}$ and the Δ*tom7* strains. A decreased level of Tom40 accumulation in the 250-kDa intermediate is reminiscent of the situation in human mitochondria in which the Tom40 precursor does not accumulate at the 250-kDa TOB/SAM complex intermediate stage, even though the complex is required for Tom40 assembly (Humphries *et al.*, 2005). It was suggested that in yeast, the requirement for newly imported Tom40 to interact with the Tom5 and Tom6 proteins, which may be absent from the mammalian TOM complex, might be the reason for Tom40 accumulation at the 250-kDa stage (Humphries *et al.*, 2005). Because the 250-kDa intermediate is observed in *N. crassa*, but the requirement for the small Tom proteins in Tom40 assembly does not appear to be crucial, it is conceivable that the function of these proteins in *N. crassa* may be evolving away from their important roles in yeast toward a state in which they may be eventually lost, as may have happened in mammalian cells.

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