Mitochondrial preproteins synthesized in the cytosol are imported through the mitochondrial outer membrane by the translocase of the outer mitochondrial membrane (TOM) complex. Tom40 is the major component of the complex and is essential for cell viability. We generated 21 different mutations in conserved regions of the *Neurospora crassa* Tom40 protein. The mutant genes were transformed into a *tom40* null nucleus maintained in a sheltered heterokaryon, and 17 of the mutant strains gave rise to viable strains. All mutations reduced the efficiency of the altered Tom40 molecules to assemble into the TOM complex. Mitochondria isolated from seven of the mutant strains had defects for importing mitochondrial preproteins. Only one strain had a general import defect for all preproteins examined. Another mutation resulted in defects in the import of a matrix-guided preprotein and an outer membrane β-barrel protein, but import of the ADP/ATP carrier to the inner membrane was unaffected. Five strains showed deficiencies in the import of β-barrel proteins. The latter results suggest that the TOM complex distinguishes β-barrel proteins from other classes of preprotein during import. This supports the idea that the TOM complex plays an active role in the transfer of preproteins to subsequent translocases for insertion into the correct mitochondrial subcompartment.

The TOM\(^4\) complex (Translocase of the Outer mitochondrial Membrane) is responsible for the recognition of mitochondrial preproteins and for the first stages of their import into mitochondria (1–6). The complex contains hydrophilic receptors that extend into the cytosol for interaction with mitochondrial preproteins synthesized on cytosolic ribosomes. These receptors recognize preproteins with cleavable N-terminal mitochondrial targeting presequences as well as those with targeting information within the mature protein sequence. After recognition, the preproteins are passed to the TOM core complex for passage through the outer membrane. The core complex contains five different subunits: Tom40, Tom22, Tom7, Tom6, and Tom5 (7–12).

Following the initial recognition and translocation of mitochondrial preproteins through the outer membrane, most preproteins interact with one of three sorting translocase complexes for targeting and assembly to the correct mitochondrial subcompartment. Proteins destined for the mitochondrial matrix interact with the TIM23 complex (Translocase of the Inner mitochondrial Membrane), although carrier proteins of the inner membrane interact with the TIM22 complex (3, 5, 13–16). β-Barrel preproteins of the outer membrane are inserted into the membrane via the TOB/SAM (Topogenesis of mitochondrial Outer membrane β-barrel proteins or Sorting and Assembly Machinery) complex (17–19). In addition, an oxidative folding mechanism involving the Mia40 and Erv1 proteins traps small proteins that contain characteristic paired cysteine residues in the intermembrane space, once they have traversed the outer membrane via the TOM complex (20–25).

Tom40 is the major component of the TOM complex and forms the pore through which preproteins traverse the outer membrane (9, 26–28). The protein is essential for viability in yeast and *Neurospora crassa* (29, 30). Based on computer analysis, Tom40 is predicted to exist as a β-barrel within the mitochondrial outer membrane (31–34). However, computer predictors differ regarding the number and position of the β-strands. Furthermore, there are conflicting reports about the proportion of the protein that exists as β-sheet as well as the position of the β-strands within the protein. When yeast or rat Tom40 was expressed in *Escherichia coli*, purified, and refolded from inclusion bodies (26, 35), measurements of β-sheet content by circular dichroism studies were in the range of 60%. In contrast, when Tom40 was purified; AAC, ADP/ATP carrier; CCHL, cytochrome c heme lyase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; RIP, repeat induced point mutation; MOPS, 4-morpholinepropanesulfonic acid.

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[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[5] The abbreviations used are: TOM, translocase of the outer mitochondrial membrane; TIM, translocase of the inner mitochondrial membrane; TOB, topogenesis of outer membrane β-barrel proteins; SAM, sorting and assembly machinery; DIG, digitinin; DDM, n-dodecyl-β-D-maltoside; BNGE, blue native gel electrophoresis; F1β, β-subunit of the F1-ATP synthase; AAC, ADP/ATP carrier; CCHL, cytochrome c heme lyase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; RIP, repeat induced point mutation; MOPS, 4-morpholinepropanesulfonic acid.
Tom40 Mutants

TABLE 1
Strains used in this study

| Strain   | Genotype                        | Origin or source                                      |
|----------|---------------------------------|-------------------------------------------------------|
| NCN251   | A cylb-2 lys-2 leu-5 mei-2       | Fungal Genetics Stock Center no. 2489 (74-OR23-1VA)   |
| HV       | A am132 int inv mei-2           | Fungal Genetics Stock Center no. 7255                 |
| MV       | a cylb-2 lys-2 leu-2 mei-2. Carries an ectopic copy of tom40. Hygromycin-resistant | Fungal Genetics Stock Center no. 7265                 |
| 40Dupl   | tomb40+1am132 int inv mei-2     | Transformation of the host V strain (Fungal Genetics Stock Center no. 7255) with the tomb40 plasmid pRIP-4 (30) |
| RIP40het | Sheltered heterokaryon (tomb40+1am132 int inv mei-2), mating type unknown. Both nuclei also contain an ectopic RIPed copy of tom40 | Cross of MateV (Fungal Genetics Stock Center no. 7265) × 40Dupl (30) |

Strains expressing only mutant versions of Tom40 as listed in Table 2

| Strain   | Genotype                        | Origin or source                                      |
|----------|---------------------------------|-------------------------------------------------------|
| Strains containing two distinct nuclei. The first carries a wild type copy of tom40, the second contains a null allele of tom40 as listed in Table 2. | Bleomycin-resistant                                      |

Chordia (33). Two additional proteins, Mim1/Tom13 (44, 45) and Mdm10 (46), play a role in assembly of Tom40 following interaction with the TOB/SAM complex. Thus, efficient assembly of Tom40 requires interaction with several assembly factors as well as different subunits of the TOM complex.

It is clear that Tom40 fulfills many functions and has interactions with several proteins. Past investigations have shown that it may be possible to assign roles to various residues or regions of the protein that are responsible for one or more of these functions. The observation that a single amino acid change in the primary sequence of Tom40 affected only transfer of preproteins to the TIM23 complex, but not to the TIM22 complex, suggested that Tom40 plays an active role in sorting preproteins to the next mitochondrial translocase (47). Furthermore, a series of conserved residues near the N and C termini of the protein have been shown to be required for Tom40 assembly but not for receptor recognition (30, 33, 48). In this study we describe the effects of changing several regions containing conserved residues of Tom40 on the assembly, stability, and function of the TOM complex.

EXPERIMENTAL PROCEDURES

Growth of N. crassa—Growth and handling of N. crassa followed standard practices as described previously (49). The strains used are listed in Table 1. Measurement of growth rates was done by spotting 10 μl of suspensions containing 5 × 10^6, 5 × 10^5, 5 × 10^4, and 5 × 10^3 conidia/ml onto plates of standard sorbose medium with the appropriate supplements and incubating at 30 °C.

Creation of Mutant Tom40 Alleles and Strains—Mutant alleles of tom40 were created by site-directed mutagenesis of single-stranded DNA from a plasmid containing the genomic version of tom40 and a bleomycin resistance gene. These were linearized and used to transform conidia from the RIP40het strain by electroporation (30). RIP40het is a heterokaryon containing two distinct nuclei. The first carries a wild type copy of tom40, an inositol requirement, and a leucine requirement. The second contains a null allele of tom40 created by repeat-induced point mutation (RIP), leucine and lysine requirements, and cycloheximide resistance. Mutant tom40 alleles that maintain sufficient Tom40 function should rescue the nucleus containing the nonfunctional tom40^RIP allele. Selection and purification of transformants was as described (30). Nutritional testing for the lysine and leucine requirements of the original...
Tom40 Mutants

| Species | Sequence Information |
|---------|---------------------|
| Nc      | -------- | -------- | -------- | -------- | -------- | -------- | -------- | -------- | -------- |
| Sc      | -------- | -------- | -------- | -------- | -------- | -------- | -------- | -------- | -------- |
| Mm      | -------- | -------- | -------- | -------- | -------- | -------- | -------- | -------- | -------- |

Nc: *N. crassa*, Sc: *S. cerevisiae*, Mm: *M. musculus*

In Vitro Import of Radiolabeled Proteins into Isolated Mitochondria—For *in vitro* import studies, mitochondria were isolated as described (52), and import of mitochondrial preproteins was basically as described (53). The preproteins of porin and Tom40 were imported at 15 °C, and all other preproteins were imported at 25 °C. Preproteins were produced by coupled transcription and translation in rabbit reticulocyte lysate (Promega Tnt T7 reticulocyte lysate system, Madison WI) in the presence of [35S]methionine (ICN Biomedicals, Costa Mesa CA). Incubation time points were as indicated in figure legends. Import reactions were analyzed by SDS-PAGE and viewed by autoradiography or a PhosphorImager system. Quantification of the image from the latter was done using the ImageQuant program (version 5.2; Amersham Biosciences).

In some cases, Tom40 import reactions were examined by BNGE and autoradiography. For these experiments, *in vitro* import of Tom40 proteins labeled with [35S]methionine was as described (48).

Time Course Pulse Import of Tom40 Variant Preproteins—For pulse imports with mutant Tom40 preproteins, an import mixture consisting of 50 μg of isolated mitochondrial preproteins, an import mixture containing 50 μl of mitochondrial suspension buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2), 2 μl of a lysate containing the desired variant of Tom40 labeled with [35S]methionine, and 100 μl of import buffer (0.5% bovine serum albumin (w/v), 250 mM sucrose, 80 mM KCl, 5 mM MgCl2, 2 mM ATP, 10 mM MOPS-KOH, pH 7.2) was assembled. The amount of each component of the import mixture was multiplied by the number of time points to be analyzed. The reaction was incubated at 25 °C for 2 min. Mitochondria were re-isolated by centrifugation at 2 °C for 15 min at 15,000 rpm. The mitochondria were then resuspended in 100 μl of fresh import buffer, without Tom40 lysate, and incubated at 25 °C. Aliquots were taken at each time point and added to 500 μl of ice-cold mitochondrial suspension buffer. Samples were centrifuged at 2 °C for 15 min at 15,000 rpm, and the pellets were kept on ice until all time points could be processed for BNGE.

Other Techniques—Agarose gel electrophoresis, transformation of *E. coli*, isolation of bacterial plasmid DNA, cloning, restriction digests of plasmid DNA, and PCR were carried out using standard protocols (54, 55). The following techniques were performed as described previously: separation of mitochondrial preproteins by PAGE (56), Western blotting (57),
TABLE 2  
Mutations created in tom40

| Residues affected by mutation | First residue affected | Name |
|------------------------------|------------------------|------|
| DGLRAD                       | 64                     | Δ64  |
| GLRAD to AAAAA               | A64                    |      |
| ΔASHQ                        | 82                     | Δ82  |
| SHQ to AAA                   | A82                    |      |
| ΔGNLD                        | 109                    | Δ109 |
| GNLD to AAA                  | A109                   |      |
| ΔATK                         | 131                    | Δ131 |
| TK to AA                     | A131                   |      |
| ΔQFHE                        | 145                    | Δ145 |
| QFHE to AAAAA                | A145                   |      |
| ΔNP                          | 163                    | Δ163 |
| NP to AA                     | A163                   |      |
| ΔVTP                         | 183                    | Δ183 |
| VTP to AAA                   | A183                   |      |
| ΔP                           | 185                    | Δ185 |
| ΔKK                          | 237                    | Δ237 |
| KK to AA                     | A237                   |      |
| ΔEKR                         | 297                    | Δ297 |
| EKR to AAA                   | A297                   |      |
| ΔVDH                         | 313                    | Δ313 |
| VDH to AAA                   | A313                   |      |

genomic DNA isolation (58), isolation of mitochondria (52), electroporation of *N. crassa* conidia (59, 60) with modifications (30), and PCR of genomic DNA isolated from conidia (61).

The following procedures were performed as recommended by the supplier: Western blot detection using LumiGLO chemiluminescent substrate (Kirkegaard & Perry Laboratories), protein determination by the Coomassie dye binding assay (BioRad), 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), and bacterial DNA plasmid isolation with Qiagen mini-prep spin kits (Qiagen Inc., Santa Clarita, CA). Nonrelevant regions/lanes of autoradiographs were digitally removed.

RESULTS

**Creation of Tom40 Mutant Strains**—The specific regions in the Tom40 protein that are important for its targeting to mitochondria, assembly into the TOM complex, interactions with other subunits, and its activity as a pore are not well defined. We reasoned that the residues responsible for some or all of these activities might be localized by functional analysis of specific mutations affecting the protein. Alignments of Tom40 proteins from various species show several regions with a high degree of conservation that may be important to the structure and function of the protein (30, 47). We chose 10 of these regions for site-directed mutagenesis of a cloned genomic version of *tom40*. Mutations (shown in Fig. 1 and listed in Table 2) were made in pairs with the targeted residues either deleted or changed to Ala residues. The deletion mutations were named “Δ” followed by the number of the first residue of the region that was deleted. The Ala substitutions were named “A” followed by the number of the first residue of the region that was changed to an Ala residue. We also made one additional mutation by deleting the Pro residue at position 185 (Δ185) to give a total of 21 *tom40* mutant alleles (Table 2). Plasmids encoding these variants were transformed into the sheltered heterokaryon RIP40het (Table 1). The lysine-leucine-requiring nucleus of this heterokaryon carries a null allele of *tom40* (30).

Only 4 of the 21 *tom40* variants that were transformed into RIP40het were unable to rescue the *tom40* null nucleus. All of these were deletion mutants (Δ82, Δ183, Δ237, and Δ297). Seventeen of the *tom40* mutant alleles were able to rescue the nucleus bearing the null allele and gave rise to homokaryotic strains with lysine and leucine requirements. These strains were examined with respect to their growth rate (Fig. 2). The Δ64 and Δ145 mutants had the most obvious growth defect, whereas the growth of the Δ109, Δ131, and Δ163 strains was slightly reduced. All the other mutant strains were indistinguishable from controls. Regardless of their growth rate, when inoculated into Erlenmeyer flasks for production of conidiospores, hyphae of all the mutant strains had a reduced ability to climb the walls of the flasks compared with the control strains (not shown).

To determine the steady state levels of mitochondrial proteins in each of the *tom40* mutant strains, mitochondria were isolated from each strain and subjected to Western blot analysis using antibodies directed against components of the TOM complex, porin, and mitochondrial Hsp70. No differences relative to the control strain were observed except in the A183 and Δ185 strains (supplemental Fig. 1). In these strains, the level of Tom40 and other TOM complex components examined was slightly reduced, with the exception of Tom70. Thus, the alterations in the A183 and Δ185 Tom40 proteins appear to result in decreased levels of the protein that, in turn, leads to decreased assembly of other subunits into the TOM complex. An unexpected observation was the increase in apparent molecular weight of the A145 Tom40 protein where the residues QFHE were changed to five Ala residues. A similar change was not seen in the Δ145 Tom40 protein where these same residues...
Tom40 Mutants

**FIGURE 3. Stability of the TOM complex in tom40 mutant strains.** Mitochondria were isolated from each of the mutant strains and the control (A, 40Dupl). Samples (50 µg) were dissolved in 1% DIG or 1% DDM and electrophoresed on blue native gels. Proteins were transferred to polyvinylidene difluoride membrane and immunodecorated with antiserum to Tom40. The mutants were classified into three groups (B–D) based on the stability of the TOM complex in the two detergents (see text). The position of molecular weight markers is shown on the right of each row.

were deleted. The substitution of Ala residues at this position may impart a change in the shape of the Tom40 protein giving rise to the altered electrophoretic ability.

**TOM Complex Stability**—Alterations in the different Tom40 variants could affect the interactions between Tom40 subunits or between Tom40 and other proteins in the TOM complex. Solubilization of the TOM complex in different detergents, or different concentrations of the same detergent, has been used previously to define the more tightly interacting components of the TOM core complex or to assess the stability of the complex in mutant strains (7, 8, 10, 11). In an attempt to measure the stability of the TOM complex in our tom40 mutants, the strains were examined qualitatively by BNGE following solubilization of isolated mitochondria with either 1% DIG or 1% DDM.

When wild type mitochondria are dissolved in 1% DIG, Tom40 migrates in a complex with an apparent molecular mass of ~400 kDa. When dissolved in 1% DDM, most of the wild type Tom40 appears in a complex of slightly less than 400 kDa with a small amount in lower molecular weight forms (Fig. 3A). The behavior of the TOM complexes containing the different mutant versions of Tom40 fell into one of three categories. The first category was defined as “mild or no defects.” Mutants in this category (A109, A131, A145, A163, A163, A183, and A185) were indistinguishable from wild type in the presence of DIG. In DDM they were either indistinguishable from wild type (A109, A131, A183, and A185) or they showed slightly decreased TOM complex stability (ΔA131, A145, A163, and A163), with a fraction of Tom40 appearing in complexes that were smaller than those observed in wild type (Fig. 3B). The second category was characterized as having “moderate defects.” Mutants in this category (A64, ΔA109, ΔA145, A237, A297, ΔA313, and A313) were similar to wild type following DIG treatment but were severely destabilized in DDM (Fig. 3C). Mutants in the third category were defined as having “severe defects.” These mutants (ΔA64 and A82) had a severely destabilized TOM complex following solubilization in either DIG or DDM (Fig. 3D).

**Import of Mitochondrial Preproteins**—The effect of each of the mutations on the ability of the TOM complex to import precursors was studied using *in vitro* import assays with three different preproteins that interact with different translocase complexes following their import into mitochondria via the TOM complex. The preprotein of the β subunit of the F₁-ATP synthase (F₁β) contains an N-terminal targeting presequence and is transferred to the matrix via the TIM23 complex. The preprotein of the ADP/ATP carrier (AAC), which belongs to the carrier family and contains an internal targeting sequence, is inserted into the inner membrane via the TIM22 complex. The preprotein of porin, a β-barrel protein, is inserted into the outer mitochondrial membrane via the TOB/SAM complex. Of the 17 strains tested, seven were found to be defective in the import of one or more of these preproteins. Mitochondria containing the ΔA145 Tom40 variant showed a general import defect that affected each of the three preproteins tested (Fig. 4). Strain ΔA64 was found to be defective in the import of F₁β and porin but not AAC (Fig. 4). The other five strains, A64, ΔA109, ΔA131, ΔA163, and A163, were deficient in porin import (Fig. 5), but the import of F₁β and AAC was unaffected (not shown).

It was surprising that several mutants were specifically defective for porin import, and we considered another possible explanation for these results. Previous work has shown that mutants of the TOM complex in *N. crassa* can have fragile mitochondria whose outer membrane is damaged during isolation, leading to reduced levels of *in vitro* porin import (62). We tested for the integrity of the outer membrane by examining *in vitro* import assays for the presence of the intermembrane space protein cytochrome c heme lyase (CCHL). As part of the *in vitro* import procedure, mitochondria are subjected to proteinase K treatment to remove nonimported preproteins. Thus, breaches in the outer membrane would allow access of the proteinase to the intermembrane space components. Figs. 4 and 5 show import blots probed with antiserum to CCHL. Compared with wild type, there is no loss of CCHL in most of the mutants. However, mutant ΔA145 does show reduced levels...
of CCHL compared with the control. Thus, we cannot rule out the possibility that the observed defect in porin import in Δ145 is because of damaged mitochondria rather than to an inefficient TOM complex in this strain.

It was of interest to determine whether the decreased import of porin was specific for this β-barrel protein or if it was indicative of a general defect in the import of all β-barrel preproteins. Therefore, the Δ163 and ΔA163 mutants were examined for their ability to import two other β-barrel proteins, Tob55/Sam50 and Tom40. As shown in Fig. 6, the import of these proteins was also reduced.

Assembly of Altered Tom40 Proteins into the TOM Complex—The mutations listed in Table 2 were also made in a cloned cDNA version of tom40. The mutant versions were then transcribed and translated in vitro, imported into wild type mitochondria, and subjected to BNGE so that assembly of the mutant Tom40 protein into the TOM complex could be examined. All 21 mutations were tested. The assembly of the different Tom40s was assessed qualitatively with respect to the formation and ratio of the three major forms that have been described previously during Tom40 assembly assays examined by BNGE (30, 39, 40). These include the 250-kDa intermediate that represents an incoming molecule of Tom40 bound at the TOB/SAM complex, the 100-kDa intermediate in which the incoming molecule has integrated into the membrane and associated with a pre-existing Tom40 molecule and Tom5, and the 400-kDa complex in which the newly incorporated Tom40 molecule is assembled into the core TOM complex.

Assays of assembly were performed for 15 min at 0 and at 25 °C. No differences were seen at 0 °C, but at 25 °C variations in the patterns of assembly were observed and we grouped these into five general classes (A–E) with wild type considered as class A (Fig. 7). Class B con-

**FIGURE 4. Import defects in strains containing the Δ145 and Δ64 Tom40 variants.** Radiolabeled mitochondrial preproteins (F1, β, AAC, and porin) were imported into mitochondria isolated from strains Δ145 (A–C) and Δ64 (D–F) for 1, 3, and 5 min at 25 °C (F1, β and AAC) or 15 °C (porin). Following the import reactions, samples were treated with proteinase K to remove nonimported preproteins. Mitochondria were re-isolated by centrifugation and subjected to SDS-PAGE. Proteins were blotted to a nitrocellulose membrane that was exposed to a PhosphorImager screen for quantification of import. The precursor (p) and mature (m) forms of F1, β are indicated. A sample of mitochondria from each strain was pretreated with trypsin (pre trp) to show the amount of receptor-independent import. The 33% lys lane shows one-third of the amount of labeled precursor that was used in each import reaction. Import blots decorated with antiserum to CCHL are shown below C and F for Δ145 and Δ64, respectively. The control strain was Dupl40.
tained mutants that were similar to wild type, but the variant forms of Tom40 showed some tendency to accumulate at the 250-kDa intermediate so that the ratio of the 250–400-kDa form was increased relative to the ratio in the control Tom40 assembly pattern (Fig. 7B). Mutants in this category include A82, A109, A131, A163, A237, and A313. Class C contained those Tom40 mutants that stall at the 250-kDa intermediate stage, so that very little of the 100- and 400-kDa forms are evident following the 15-min import assay (Fig. 7C). Mutants in this category include A64, A109, A163, A237, and A297. Accumulation of Tom40 molecules at the 250-kDa stage, as observed to varying extents for the class B and C mutants, suggests that these mutant forms of the protein cannot be efficiently inserted into the outer membrane by the TOB/SAM complex. A slightly different assembly pattern was seen with class D mutants (Δ145, Δ183, A183, and Δ185). These variant Tom40 molecules also tend to accumulate at the 250-kDa stage, but there is also a large amount of material that forms a smear below the position of the 100-kDa intermediate (Fig. 7D). The nature of the smear is not known, but might represent Tom40 in different conformations and/or in association with different subunits of the TOM complex. Class E contains mutants A297 and A313. These mutant Tom40 molecules accumulate at both the 250- and the 100-kDa stages (Fig. 7E).

Accumulation at the 100-kDa intermediate had not been observed with any previous Tom40 mutants. Therefore, the A297 and A313 variants were examined in a pulse version of the assembly assay to determine whether there was a slower rate of

FIGURE 5. Decreased ability to import porin in mutant strains. Import of the radiolabeled precursor of porin was performed as described in the legend for Fig. 4 using mitochondria isolated from the A64, Δ109, A109, Δ163, and A163 mutant strains in A–E, respectively. Import blots decorated with antiserum to CCHL are shown below each porin import panel for the respective mutants. pre trp, pretreated with trypsin.
assembly into the 400-kDa core complex. Labeled versions of the Tom40 variants were added to isolated wild type mitochondria for 2 min. The mitochondria were then washed by re-isolation, resuspended in import buffer, and incubated for additional times ranging from 0 to 6 h at 25 °C (Fig. 8). The A297 variant did continue to assemble after longer times, with a steady increase of the 400-kDa complex and a slight decrease in the 100-kDa intermediate. Some assembly of the A313 Tom40 occurred after 1 h, but this did not increase upon further incubation. In both mutants the 250- and 100-kDa intermediates were still present after 6 h. Thus, it appears that both of these Tom40 variants have a reduced ability to progress along the Tom40 assembly pathway. The assembly defect appears to affect both the conversion of the 250-kDa form to the 100-kDa form and conversion of the 100-kDa intermediate to the 400-kDa fully assembled form. These mutants may be inefficient in developing interactions with other TOM components and/or assembly factors.

**DISCUSSION**

We have created 21 new mutant alleles of *tom40* by altering residues in 10 regions of the protein. Various assays designed to test properties of the Tom40 mutant proteins were carried out, and the findings are summarized in Table 3. Only four of the mutant *tom40* genes were unable to rescue a nucleus containing a null allele of *tom40* that is maintained in a sheltered heterokaryon. Of the 17 strains obtained with mutations in Tom40, only 5 have a growth rate defect although the hyphae of all the viable strains were defective in climbing the walls of Erlenmeyer flasks. Thus, each mutation has at least some in vivo effect. Given that *tom40* is an essential gene and that conserved regions of the protein were altered, it is surprising that most of the mutations do not result in a more severe phenotype.

Decreased stability of the TOM complex in the presence of detergents was observed in many of the mutant strains. The regions of Tom40 affected in these mutants may be involved in interactions with the other components of the TOM complex, including other Tom40 molecules. For the Δ131/A131, Δ163/A163, and Δ313/A313 pairs of mutants, the effects of both the deletion and the Ala substitutions are the same with respect to TOM complex stability. This suggests that the residues altered in these mutants are important for interactions between components of the complex under the conditions of the assay. On the other hand, for the Δ64/A64, Δ109/A109, and Δ145/A145 pairs, the deletion has a more severe effect on complex stability. In these cases, spacing of domains or conformational changes may have a more severe effect than simple substitutions in the altered regions. Interestingly, there is no obvious correlation relating the severity of stability defects to additional defects in TOM complex function. Some alterations may not show effects in vivo because of their specific environment within the outer membrane.

All of the changes introduced into Tom40 in this study had some effect on the ability of the altered Tom40 to be assembled into the TOM complex in isolated mitochondria. The variant forms of Tom40 that were unable to rescue the null *tom40* allele (Δ82, Δ183, Δ237, and Δ297) were able to assemble to the 250-kDa stage in vitro. Thus, the residues deleted in these variants are not critical for targeting Tom40 to mitochondria. However, all four of these variants were very inefficient in progressing past the 250-kDa assembly stage. Although it is possible that the assembly defects result in the inability of these mutants to res-

![Image](22561)

**FIGURE 6.** Import of the precursors of Tob55/Sam50 and Tom40 into isolated mitochondria of the Δ163 and A163 mutants. A and B show Tob55/Sam50 and Tom40 into isolated mitochondria of the Δ163 strain, and C and D show import of the same precursors into mitochondria of the A163 mutant. Tob55/Sam50 import was as described for F1 and AAC in Fig. 4, and Tom40 was imported as described for porin. When Tom40 is properly assembled into the membrane it is cleaved during the post-import proteinase K treatment to give a 26- and a 12-kDa fragment. The 12-kDa fragment is not shown on the gel insets, and the uncleaved 40- and 26-kDa bands are indicated. The 26-kDa fragment was used for quantification of import. The 51-kDa Tob55/Sam50 protein is also cleaved to produce a 45-kDa fragment. The 51- and 45-kDa bands are indicated. The 45-kDa band was used for quantification. pre trp, pretreated with trypsin.
cue the null nucleus, it is also conceivable that some assembly does occur in vivo, but the mutant molecules are nonfunctional in the complex.

For the mutant tom40 mutant alleles that give rise to viable strains, a very general correlation exists between the most pronounced in vitro assembly defects and some in vivo phenotype. Specifically, with the exception of strain A145, all of the variants displaying a severe assembly phenotype (class C or D) are either unable to restore viability to the tom40 null nucleus or they give rise to strains with an import defect or reduced levels of TOM complex components. This wide range of in vivo effects for mutants with similar Tom40 assembly patterns suggests that once assembled some variants (e.g. A145) function relatively normally, whereas others (e.g. Δ64) affect additional aspects of Tom40 function. Our assay measured the assembly of mutant Tom40 molecules into wild type mitochondria. Some Tom40 mutants may assemble more or less effectively with Tom40 molecules bearing similar alterations, as would occur in vivo for a given strain, than they do with wild type Tom40 molecules as measured in our assay.

The A297 and A313 variants of Tom40 showed an in vitro assembly defect of being slow to progress past the 100-kDa intermediate stage. The Tom40 preprotein is thought to be integrated into the membrane at the 100-kDa stage and to be associated with another pre-existing Tom40 subunit (39).
There is good correlation between effects on import and growth rate alterations. The \( \Delta 64 \) and \( \Delta 145 \) strains have the altered in this mutant. Because the alanine-substituted version of this deletion does not show import defects, it is likely that the effects on import caused by the deletion are because of structural changes in the protein rather than a specific role for the residues themselves. As judged by the accessibility of the intermembrane space protein CCHL to externally added proteinase K, the outer membrane of mitochondria from the \( \Delta 145 \) mutant is more readily broken during the isolation procedure than the outer membrane of control mitochondria. Because mitochondria with broken outer membranes do not import porin efficiently (62), we cannot determine whether the defects in porin import are entirely because of broken outer membranes or if the altered TOM complex also contributes to the import deficiency of porin. For the \( \Delta 145 \) mutant it seems likely that the altered TOM complex accounts for at least some of the porin import deficiency because other preproteins are also affected.

Mitochondria containing the \( \Delta 64 \) Tom40 variant were defective for import of the matrix preprotein F\(_1\)K and the \( \beta \)-barrel protein porin but imported the preprotein of AAC normally. The deletion of the GLRAD residues in the \( \Delta 64 \) mutant must result in alterations of Tom40 that affect both matrix-destined preproteins and \( \beta \)-barrels but do not have a general effect on import. This import phenotype is similar to that observed in \textit{N. crassa} mitochondria lacking Tom7 (12). Mitochondria containing Tom40 variants A64, \( \Delta 109 \), \( \Delta 131 \), \( \Delta 163 \), and A163 had a defect in the import of porin, whereas the F\(_1\)K and AAC pre-proteins were translocated normally. These alleles thus comprise a new class of Tom40 import mutants that are specific for defects in porin import. For mutants \( \Delta 163 \) and A163, we also tested the import of the Tom40 and Tob55/Sam50 preproteins into the mutant mitochondria. Import of these \( \beta \)-barrel proteins was also reduced in the mutants suggesting that a decrease in porin import is diagnostic for a general defect in \( \beta \)-barrel protein import.

There is good correlation between effects on import and growth rate alterations. The \( \Delta 64 \) and \( \Delta 145 \) strains have the

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**TABLE 3**

Summary of properties of the tom40 mutants

| Mutant | \( \Delta 64 \) | \( \Delta 64 \) | Growth rate | Complex stability | Import defect | Assembly defect at 25 °C (class from Fig. 7) |
|---|---|---|---|---|---|---|
| \( \Delta 64 \) | + | Moderate | Severe | F\(_1\)K, porin | Accumulates at 250 kDa, very little 400-kDa formed (class C) |
| A64 | + | None | Moderate | Porin | Accumulates at 250 kDa, very little 400-kDa formed (class C) |
| \( \Delta 82 \) | No | ND\(^a\) | ND | ND | Accumulates at 250 kDa, very little 400-kDa formed (class C) |
| A82 | + | None | Severe | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| \( \Delta 109 \) | + | Mild | Moderate | F\(_1\)K, AAC, porin | Accumulates at 250 kDa, very little 400-kDa formed (class C) |
| A109 | + | None | None | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| \( \Delta 131 \) | + | Mild | Mild | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| A131 | + | None | None | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| \( \Delta 145 \) | + | Moderate | Moderate | F\(_1\)K, AAC, porin | Accumulates in smear below 100 kDa (class D) |
| A145 | + | None | Mild | None | Accumulates in smear below 100 kDa (class D) |
| \( \Delta 163 \) | + | None | Mild | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| A163 | + | Mild | Mild | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| \( \Delta 183 \) | No | ND | ND | ND | Accumulates in smear below 100 kDa (class D) |
| A183 | + | None | None | None | Accumulates at 250 kDa, very little 400-kDa formed (class C) |
| \( \Delta 185 \) | + | None | None | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| \( \Delta 237 \) | No | ND | ND | ND | Accumulates at 250 kDa, very little 400-kDa formed (class C) |
| A237 | + | None | Moderate | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| \( \Delta 297 \) | No | ND | ND | ND | Accumulates at 250 kDa, very little 400-kDa formed (class C) |
| A297 | + | None | Moderate | None | Accumulates at 250 and 100 kDa (class E) |
| \( \Delta 313 \) | + | None | Moderate | None | Accumulates at 250 kDa, some 400-kDa formed (class B) |
| A313 | + | None | Moderate | None | Accumulates at 250 and 100 kDa (class E) |

\(^{a}\) ND indicates not determined.
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slowest growth rates, and these mutants have the most severe import defects. Three of the five mutants deficient in porin import (Δ109, Δ131, and A163) have mild growth defects.

Our data showing that certain mutations in Tom40 can result in import defects that are specific for certain classes of preprotein extend a previous observation in yeast where a single amino acid change (W243R) in Tom40 was found to affect import of only matrix preproteins (47). Taken together, these observations support the notion that the TOM complex is not a passive channel through which all preproteins simply traverse the outer membrane. Rather, it appears that different structures or functions within the complex are responsible for ensuring proper interactions between preproteins and subsequent components of the import machinery that result in proper sorting to the correct mitochondrial compartment. Import defects could result from alterations in many of the activities of the TOM complex, including movement of preproteins through the translocation pore (9, 26), interactions at the cis or trans binding sites (36, 63), interactions between preproteins emerging from the TOM complex and subsequent components of the import machinery (15, 41, 42, 64–67), or alterations in the ability of the TOM complex to act as a chaperone or unfolding activity (37, 38). Further work on the mutants with import defects may reveal the specific activities of the TOM complex affected.

All of the mutations we have constructed have resulted in effects on some aspect of Tom40 function. The smallest effects were seen with mutants A109 and A131, which have only a mild assembly defect. The results with some of the mutants suggest that individual features of Tom40 function may be discerned by alterations in specific regions of the protein. For example, the A183 and Δ185 mutations seem to define a region of the protein required for efficient assembly of the complex because they show a pronounced defect in the process in vitro and also have reduced levels of TOM complex components in vivo. Many other mutations have pleiotropic effects on the protein. For example, mutants Δ64 and Δ145 have defects in stability, assembly, and import as measured by our assays. More information about the functional domains of Tom40 may be obtained with analysis of additional mutants. However, deeper insight will come when it is possible to correlate the effects of known mutations with information on Tom40 structure.

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