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Characterization of the TOM complex in *Neurospora crassa*

by

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This thesis is dedicated to
my understanding husband Adrian
and my wonderful parents

Abstract

Mitochondria are ubiquitous organelles in eukaryotic cells. Although they contain their own genome, most mitochondrial proteins are encoded in the nucleus, translated in the cytosol and imported into mitochondria. Translocation across the outer mitochondrial membrane is achieved by the TOM complex (translocase of the mitochondrial outer membrane). This complex is composed of seven proteins. Tom70, Tom40, Tom22, Tom20, Tom7, Tom6 and Tom5, where the number in each protein's name indicates its molecular weight.

Tom40 is known to form the pore of the TOM complex. In an attempt to determine the residues important for translocation of proteins or for the assembly of Tom40 into the TOM complex, 10 regions of conserved residues in the Tom40 protein were mutated. The effects of these changes were studied in *Neurospora crassa* and mutations were identified which were lethal, caused defects in import of mitochondrial preproteins or resulted in destabilization of the TOM complex.

The roles of the small Tom proteins were also examined in *N. crassa* using mutants lacking each protein or combinations of the proteins. It was found that Tom5 has a very minor role in the TOM complex while Tom6 and Tom7 affect the stability of the TOM complex and the ability to import proteins. Only the double mutation of Tom6 and Tom7 showed more defects than the single mutations. The combination of all three mutations appeared to be lethal.

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List of Abbreviations

AAC	ATP/ADP carrier
AIF	apoptosis inducing factor
ALDH	aldehyde dehydrogenase
ATP	adenosine triphosphate
BNGE	blue native gel electrophoresis
CCHL	cytochrome c heme lyase
DDM	dodecylmaltoside
DIG	digitonin
ETC	electron transport chain
$F_1\beta$	F_1beta subunit of the ATP synthase
Fe-S	iron sulfur
GIP	general import pore
hr	hour
IMS	intermembrane space
kDa	kilo Dalton
MCC	mitochondrial inner membrane conductance channel
MIM	mitochondrial inner membrane
min	minute
MIP	mitochondrial intermediate peptidase
MOM	mitochondrial outer membrane
MPP	matrix processing peptidase
MSF	mitochondrial import stimulation factor
mtDNA	mitochondrial DNA
OMV	outer membrane vesicle
PAGE	polyacrylamide gel electrophoresis
PAM	presequence translocase-associated protein import motor
PiC	phosphate carrier
PTP	permeability transition pore
PVDF	polyvinylidene fluoride
RIP	repeat induced point mutation
ROS	reactive oxygen species
SAM	sorting and assembly machinery
SOD	superoxide dismutase
sec	second
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Su9DHFR	subunit 9 of the ATPase (residues 1-69) and dihydrofolate reductase
TCA	tricarboxylic acid cycle
TIM	translocase of the mitochondrial inner membrane
TOB	topogenesis of mitochondrial outer membrane β-barrel proteins
TOM	translocase of the mitochondrial outer membrane
TPR	tetratricopeptide repeat
VDAC	voltage dependent anion channel

1. INTRODUCTION

1.1. Mitochondrial functions in the cell

Mitochondria are a nearly ubiquitous organelle in eukaryotic cells and are considered the 'power house' because of their role in energy production (Attardi and Schatz 1988; Gray 1989; Roger *et al.* 1998). Mitochondria contain their own limited genome. In most eukaryotes mtDNA encodes mitochondrial ribosomal RNAs, mitochondrial tRNAs and a subset of proteins used in the formation of oxidative phosphorylation complexes (Attardi and Schatz 1988). Several human diseases, as well as the process of aging, have been associated with mitochondrial dysfunction (Morgan-Hughes and Hanna 1999; Schapira 1999; Schapira 1999; Tatton and Olanow 1999; Wallace 1999; Wallace *et al.* 1999; Wallace and Murdock 1999; Duchen 2004; Trifunovic *et al.* 2004).

The best known function of mitochondria is the generation of ATP (Attardi and Schatz 1988; Capaldi 2000; Duchen 2004). This is achieved by the process of oxidative phosphorylation where electrons donated by NADH and $FADH_2$, which are generated in cellular reactions such as the TCA cycle, are passed along the electron transport chain. The latter consists of various small molecules and four protein complexes housed in the mitochondrial inner membrane (Saraste 1999). The energy of the electrons is harvested by complexes I, III and IV and is used to pump protons from the matrix to the intermembrane space. Complex V, the F_1F_0 ATP synthase then uses this gradient to convert ADP and inorganic phosphate to ATP.

Ca^{2+} uptake/release is another important role of the mitochondria (Duchen 2004; Rizzuto *et al.* 2004). It has been suggested that most cellular calcium signaling involves some mitochondrial Ca^{2+} accumulation. This accumulation in the mitochondria can modulate the signals by decreasing the cytosolic Ca^{2+} causing the signal to have reduced amplitude and can also cause a slower propagation rate of intracellular calcium waves (Duchen 2004; Gunter *et al.* 2004; Rizzuto *et al.* 2004). It has also been shown that Ca^{2+} levels in the mitochondria regulate mitochondrial metabolism and stimulate ATP production, therefore, defects in mitochondrial Ca^{2+} accumulation can play an important role in mitochondrial dysfunction (Jouaville *et al.* 1999; Duchen 2004). While the outer membrane is permeable to Ca^{2+}, the inner membrane uses a uniporter to uptake Ca^{2+}.

Accumulation in mitochondria requires a membrane potential and a lower concentration of Ca^{2+} in the matrix relative to the cytosol. This balance is partly maintained by the Na^{+}/ Ca^{2+} exchanger, which regulates matrix Ca^{2+} efflux. The high concentration of Ca^{2+} in the cytosol can be partly due to the close association of mitochondria with the ER so that when Ca^{2+} is released from the ER, a microdomain of high Ca^{2+} concentration is created (Rizzuto *et al.* 1998; Szabadkai and Rizzuto 2004). Prolonged Ca^{2+} overload in the mitochondria, caused by the release of Ca^{2+} from the ER, has a role in stimulating mitochondrial mediated apoptosis, and it has been demonstrated that if the amount of Ca^{2+} able to be released by the ER is decreased, then apoptosis can be inhibited (Szabadkai and Rizzuto 2004).

As mentioned, mitochondria also play an important role in regulating apoptosis (Marzo *et al.* 1998; Bernardi 1999; Harris *et al.* 2000; McStay and Halestrap 2002). Control of apoptosis occurs by permeablizing the membranes of the mitochondria through the permeability transition pore complex (PTP) that includes the voltage dependent anion channel (VDAC) in the outer membrane and the adenine nucleotide translocator (ANT) in the inner membrane. Apoptosis is triggered when the PTP opens, which is partly regulated by members of the Bcl-2 protein family in the outer membrane and high concentrations of Ca^{2+}. This causes the inner membrane permeability to increase causing the membrane potential to be disrupted and the matrix to swell. Ultimately this results in the outer membrane bursting. This allows pro-apoptotic proteins to be released from the intermembrane space, such as the apoptosis inducing factor (AIF) and cytochrome *c* (Liu *et al.* 1996; Gross *et al.* 1999; Lorenzo *et al.* 1999).

Mitochondria also have other functions in the cell. They contain the enzymes for β-oxidation of fatty acids, which are a major source of energy for heart and skeletal muscles (Eaton 1996). Mitochondria are also the site of Fe-S cluster assembly (Lill and Kispal 2000). Fe-S clusters are important for the respiratory complexes in the mitochondria, but they also have roles elsewhere in the cell (Imsande 1999). For example, metabolic enzymes such as xanthine dehydrogenase and phophoribosylpyrophosphate amidotransferase required for purine metabolism contain Fe-S clusters, and the iron response protein (IRP-1) involved in iron uptake and storage uses Fe-S clusters as well.

Several mechanisms proposed to control life span directly or indirectly involve processes that happen in the mitochondria. One of the byproducts of oxidative phosphorylation is the generation of reactive oxygen species (ROS) as a result of the ETC when electrons are donated to oxygen (Martindale and Holbrook 2002; Wei and Lee 2002). A traditional hypothesis of aging is the oxidative stress theory, which originally proposed that ROS cause damage to DNA, proteins and lipids and that this damage is not fully repaired in the cell (Harman 1988). This accumulation of damage eventually leads to aging. One type of ROS, superoxide radicals, is made into hydrogen peroxide by superoxide dismutase (SOD), which is then further neutralized to water and oxygen by catalase (Raha and Robinson 2000). The roles of these enzymes in aging have been researched and several studies have implicated them in extending life span. In *Drosophila,* transgenic flies overexpressing SOD and catalase have increased life spans (Parkes *et al.* 1998) and mice heterozygous for SOD mutations have accelerated aging (Kokoszka *et al.* 2001). Several other studies have also demonstrated the effects of the ETC and ROS on life span. Changes and mutations to components of the ETC have produced longer life spans and it is thought that the underlying mechanism is less ROS production (Feng *et al.* 2001; Dillin *et al.* 2002; Miyadera *et al.* 2002). As well, it is known that calorie restriction can significantly increase the life span in a diverse range of animals. This could cause changes in metabolism and thereby affect the production of ROS, which might in turn reduce ROS damage. However, the mechanism(s) responsible for increased life span due to calorie restriction are not fully understood (Lee and Yu 1990).

Many diseases are the result of mitochondrial dysfunction. Some of the diseases are a result of mutation in the mtDNA, which can cause a more progressive type of disease due to the heterogeneous population of mitochondria in a cell, with a mutation arising in a wild type population of mitochondria and eventually the number of mitochondria with the mutation increasing. Recently, a direct link between mtDNA mutation and disease has been demonstrated using an error-prone mtDNA polymerase in mice, which causes a mtDNA mutator phenotype. These mice display premature onset of age-related diseases such as osteoporosis, anemia, heart enlargement and hair loss (Trifunovic *et al.* 2004). Disease can also occur from mutations that affect

mitochondrial proteins encoded in nuclear genes. Mitochondrial dysfunction can cause symptoms such as deafness, neurodegenerative disorders such as Alzheimer's, muscle atrophy, epileptic seizure, cardiomyopathies and vision loss. It has also been suggested that many other unexplained symptoms or diseases could be attributed to mitochondrial impairment (Morgan-Hughes and Hanna 1999; Schapira 1999; Schapira 1999; Tatton and Olanow 1999; Wallace 1999; Wallace *et al.* 1999; Wallace and Murdock 1999; Duchen 2004).

1.2. Mitochondrial morphology and maintenance

Mitochondria have a double membrane and can be divided into four subcompartments: the mitochondrial outer membrane (MOM), the intermembrane space (IMS), the inner membrane (MIM), which contains many folds called cristae, and the matrix, the inner compartment. The model for morphology of mitochondria has recently been revised from the traditional baffle model that depicts large openings in the cristae to the IMS. Advances in EM tomography revealed that cristae are pleiomorphic tubular extensions of the inner membrane with narrow openings to the intermembrane space and can be very diverse, based on the cell type (Mannella *et al.* 1994).

Mitochondria are maintained through a continual process of mitochondrial membrane fission and fusion (Frey and Mannella 2000; Jensen *et al.* 2000; Osteryoung 2000; Griparic and van der Bliek 2001; Osteryoung 2001; Shaw and Nunnari 2002; Meeusen *et al.* 2004). These events are tightly regulated and some of the proteins involved in both processes have been identified. Fission and fusion of the inner and outer membranes occur independently and probably have separate apparatus. Fzo1, a guanosine triphosphatase, is one protein needed for the fusion of the outer membrane. Inner membrane fission requires an electrical potential. When genes involved in outer membrane fission are mutated the result is a network of connected mitochondria and when there is a loss of fusion genes, the mitochondria become fragmented and lose their DNA.

1.3. Mitochondrial evolution and the mitochondrial genome

The theory of the origin of mitochondria dates back over a century when it was first proposed that they originated from free-living cells that were engulfed by a host cell. This endosymbiont theory of mitochondrial origin suggests that an ancestral anaerobic eukaryotic cell engulfed an ancient relative of bacteria. The engulfed cell remained intact and over time genes from the bacteria were transferred to the host cell nucleus. (Margulis 1975; Gray *et al.* 1999; Gray *et al.* 2001; Martin *et al.* 2001).

The endosymbiont theory is the favored model since the discovery that mitochondria contain their own, generally circular, genome (Sagan 1967; Sagan 1993). Sequencing of mitochondrial DNAs (mtDNAs) showed the closest living relative of the mitochondria are the obligate parasites of the α-proteobacteria such as *Rikettsia* (Yang *et al.* 1985). Analysis of the rRNA sequence and protein coding genes in mtDNA show that there was probably only a single evolutionary event of endosymbiosis. Some of the evidence that supports this idea comes from the fact that the genes found in the mtDNAs of different species are a subset of genes found in the mtDNA of *Reclinomonas americana*, which retains the most protein coding and ancestral traits of the bacterial genome (Gray *et al.* 1998). Although mtDNA gene arrangement is poorly conserved, a few mtDNAs still retain a similar order to some *E. coli* clusters such as ribosomal genes, and that mtDNAs from several species often have the same deletions, supporting an earlier common mitochondrial ancestor that contained the deletions (Lang *et al.* 1997; Andersson *et al.* 1998).

There are two main theories of the driving forces for endosymbiosis (Kurland and Andersson 2000). The aerobic mutualism theory suggests that the original anaerobic host engulfed an aerobic bacterium in order to deal with the toxic effects of the increasing concentration of oxygen in the atmosphere. The symbiotic cell would have been able to use the oxygen, as in a respiratory chain, and convert the oxygen to water, neutralizing the toxic effects of oxygen on the host cell (Andersson and Kurland 1999; Karlberg *et al.* 2000). The other theory proposes that the α-proteobacterium cell that would become the symbiont would have produced H_2 and CO_2 as waste products from fermentation, which the host cell would use for its own energy and carbon source, much like modern methane-producing archaean cells. Eventually, the host would become dependent on the

symbiont, start surrounding it and then have to start supplying the future symbiont with the necessary substances so the symbiont could keep producing H_2 and CO_2 for the host (Martin and Muller 1998). In this way, host cells, which are better at supplying the symbiont, could evolve.

Reduced nuclear genomes, as seen in mitochondria's closest neighbor *Rikettsia,* can be considered the consequence of the parasitic lifestyle they live, and genes that tend to be easily lost are those involved in processes that can easily be compensated for by the host, such as amino acid biosynthesis (Andersson *et al.* 1998). Although mitochondrial genomes are substantially smaller than their closest neighbor's genomes, there is no evidence yet that mitochondria originated from an ancestor whose genome was already reduced (Gray 1998).

The most recent debate in the endosymbiosis theory is whether the host cell was a eukaryote before the association with the endosymbiont. A recent theory suggests that a chimeric prokaryote arising from fusion of a bacterium and an archaebacterium, which retained glycolosis, subsequently engulfed the mitochondrial ancestor bacterium and this energy producing symbiont allowed the evolution of eukaroytic features (Emelyanov 2003). It was once thought that evidence against this theory was the existence of amitochondriate eukaryotes, suggesting that eukaryotes existed before the acquisition of mitochondria and some never obtained mitochondria. There are two types of amitochondriate eukaryotic cells: those that contain hydrogenosomes, which are double membrane bound organelles that produce ATP and H^+ but do not use oxygen or contain a genome (Martin *et al.* 2001), and those that contain mitosomes, which are a mitochondrial remnant double membrane bound organelle that do not make ATP or contain a genome (Martin *et al.* 2001; Embley *et al.* 2003; Tovar *et al.* 2003; Leon-Avila and Tovar 2004). Since hydrogenosomes and mitosomes have been shown to be related to mitochondria, it appears that all eukaryotic cells may have contained an ancestral mitochondrion supporting the idea that eukaryotic cells may not have existed without mitochondria (Embley *et al.* 2003; Embley *et al.* 2003).

1.4. Introduction to mitochondrial protein import

Mitochondria contain a limited genome, which encodes a small fraction of the proteins needed for proper mitochondrial function. Thus, the majority of mitochondrial proteins are encoded in the nucleus, translated in the cytosol, and transported into the correct mitochondrial subcompartment (reviewed in Pfanner and Neupert 1990; Neupert 1997; Bauer *et al.* 1999; Pfanner and Geissler 2001; Prokisch *et al.* 2002; Rapaport 2003; Hood and Joseph 2004; Hoppins *et al* 2004; Rehling *et al.* 2004). Five mitochondrial complexes achieve protein import and sorting into the proper compartment (Fig. 1). The Translocase of the Outer mitochondrial Membrane (TOM complex) recognizes all preproteins and transports them either across or into the outer membrane. β-barrel proteins destined for the outer membrane are further assisted by the SAM complex (Sorting and Assembly Machinery) also known as the TOB complex (Topogenesis of mitochondrial outer membrane β-barrel proteins). Once proteins destined for an inner compartment have passed the outer membrane they can enter the intermembrane space or be further transported by one of the two TIM complexes (Translocase of the Inner mitochondrial Membrane). The TIM23 complex primarily passes preproteins through the inner membrane to the matrix but also inserts a few into the inner membrane and the intermembrane space through a stop/transfer pathway. Associated with the TIM23 complex is the import motor, also known as the PAM complex (presequence translocase-associated protein import motor), which is needed to translocate proteins into the matrix. The TIM22 complex is responsible for inserting a subset of hydrophobic proteins into the inner membrane. This includes proteins with internal targeting sequences, such as proteins of the carrier family, like the ATP/ADP carrier (AAC). The OXA complex of the MIM inserts mitochondrial-encoded proteins and a few nuclear-encoded proteins from the matrix into the mitochondrial inner membrane. Each protein in a given complex is named for that complex and they are distinguished from each other by their weight in kDa (eg, Tom70, the 70 kDa component of the TOM complex).

The majority of work on mitochondrial protein import has been done in the yeast *Saccharomyces cerevisiae* and the filamentous fungus *Neurospora crassa*. Many homologues have been found in higher eukaryotes showing that these two organisms are suitable models for the study of mitochondrial protein import (Goping *et al.* 1995; Bömer

et al. 1996; Komiya and Mihara 1996; Iwahashi *et al.* 1997; Bauer *et al.* 1999; Jin *et al.* 1999; Saeki *et al.* 2000; Suzuki *et al.* 2000; Yano *et al.* 2000).

1.4.1. Post-translational import of mitochondrial precursor proteins

Most mitochondrial precursor proteins are imported after being completely translated in the cytosol (reviewed in (Neupert 1997; Pfanner and Geissler 2001; Rehling *et al.* 2004). It has been shown that preproteins are released from cytosolic ribosomes before they are targeted to the mitochondria and pulse-chase experiments showed accumulated labeled precursor in the cytosol before they were seen in the mitochondria (Hallermayer *et al.* 1977; Reid *et al.* 1982). However, some evidence suggests that certain precursors are imported in a co-translational manner (reviewed in Neupert 1997; Pfanner and Geissler 2001; Beddoe and Lithgow 2002). For example, two study shows import could only occur efficiently with the presence of ribosomes and without translational inhibitors (Fujiki and Verner 1991; Fujiki and Verner 1993). In the unique case of the fumarase protein in yeast, it is co-translationaly processed because of its quick folding kinetics which necessitates the need to be imported as it is being translated. It is then processed in the matrix and released back to the cytosol (Knox *et al.* 1998). Mitochondrial preproteins can be found on both cytosolic ribosomes bound to mitochondria and on free cytosolic ribosomes (Ades and Butow 1980). mRNAs encoding some mitochondrial preproteins are targeted to the mitochondria to be translated on ribosomes found on the organelle surface (Egea *et al.* 1997; Ricart *et al.* 1997).

1.4.2. Precursor targeting

The most well characterized mechanism of mitochondrial precursor targeting occurs with precursors carrying an N-terminal cleavable presequence, also called the matrix-targeting signal or presequence. This signal is found on matrix precursor proteins, is usually 20 to 60 residues long, and contains a high number of positively charged, hydrophobic and hydroxylated residues (reviewed in Neupert 1997; Pfanner and Geissler

2001; Rehling *et al.* 2004). It is sufficient to direct proteins into the mitochondrial matrix where it is cleaved off the mature protein by MPP (matrix processing peptidase) (Gillespie *et al.* 1985; Horwich *et al.* 1985; Horwich *et al.* 1986; von Heijne 1986). The structure of the presequence is an amphipathic α-helix with one surface having positive charges and the other being hydrophobic. Both sides are important for precursor recognition by the mitochondrial receptors (von Heijne 1986; Hammen *et al.* 1996; Brix *et al.* 1997; Abe *et al.* 2000).

There are also variations of the cleavable presequences (stop-transfer signals) (Pfanner and Geissler 2001). These contain the positively charged presequence domain followed by a hydrophobic membrane anchor domain, which halts the protein in the membrane. Some inner membrane proteins have a cleavable presequence that will target the protein to the matrix but a downstream hydrophobic domain will halt it at the TIM23 complex (Gärtner *et al.* 1995). Another class of preproteins, such as cytochromes b_2 and c_1 of the inner membrane and intermembrane space respectively, have a cleavable presequence that is removed in the matrix when the protein is halted in the inner membrane at the TIM23 complex by the downstream hydrophobic sorting signal. A protein in the IMS releases the mature domain of the protein into the IMS by cleaving off the sorting signal. (Glick *et al.* 1992; Bomer *et al.* 1997). Another class of inner membrane proteins contains a hydrophobic transmembrane domain followed by an internal positively charged presequence-like domain. These two domains fold into a hairpin structure that will direct the preprotein to the inner membrane (Fölsch *et al.* 1996).

Internal non-cleavable targeting signals are found in certain inner membrane, outer membrane, and IMS proteins. The metabolite carrier proteins of the inner membrane contain multiple internal signals throughout the protein that work together to interact with the TOM complex receptors (Smagula and Douglas 1988; Sirrenberg *et al.* 1998; Rehling *et al.* 2003). Outer membrane proteins have internal signals as well but are more clearly defined and are often called 'signal-anchored' proteins because the anchoring transmembrane domain is also used as a sorting signal. N-terminally anchored outer membrane proteins such as Tom20 and Tom70 require the transmembrane domain with additional flanking positive residues for proper sorting and targeting (Kanaji *et al.*

2000; Suzuki *et al.* 2002). Tail-anchored proteins, including Tom22, the other small Tom proteins and Bcl-2, also require the transmembrane domain and flanking positive residues (Nguyen *et al.* 1993; Dembowski *et al.* 2001; Kaufmann *et al.* 2003). In these Tom proteins, a single proline residue in the transmembrane segment has been shown to be important for efficient targeting (Allen *et al.* 2002). The signals for both of these types of proteins are not conserved at the sequence level and the information is most likely in the structure of the region. It is not completely clear how they are recognized by the mitochondria, as some do not require the TOM complex receptors and seem to use the interface between TOM complex and lipids for insertion, while others do require the receptor proteins and the TOM complex (Schneider *et al.* 1991; Lan *et al.* 2000; Motz *et al.* 2002; Rapaport 2003). The signals in the outer membrane β-barrel proteins, such as in Tom40 and porin, are still poorly understood. The signals are likely encoded in structural elements of the proteins and insertion into the membrane follows a more complicated pathway that uses both the TOM complex and the SAM complex (Rapaport and Neupert 1999; Model *et al.* 2001; Rapaport *et al.* 2001; Rapaport 2003).

1.4.3. Cytosolic requirements of import

It has been known from early import studies that the translocation of mitochondrial preproteins requires ATP and an inner membrane potential (Eilers *et al.* 1987; Pfanner *et al.* 1987). These studies showed that the ATP was needed to keep the preproteins in an unfolded import competent state because when ATP was present, preproteins were more sensitive to proteases and the amount of ATP required was dependent on the preprotein being tested. Other studies confirmed this idea by showing that artificially unfolded proteins did not require ATP for import (Pfanner *et al.* 1988; Ostermann *et al.* 1989; Pfanner *et al.* 1990). The inner membrane potential is not needed for translocation across the outer membrane but is required for further translocation across the inner membrane (Eilers *et al.* 1987).

Several molecular chaperones are also needed by mitochondrial preproteins for import (reviewed in Komiya *et al.* 1996; Beddoe and Lithgow 2002). Members of the Hsp70 family were the first chaperones discovered to be involved in import by acting post-translationally to keep the preprotein in an import competent state (Deshaies *et al.*

1988; Deshaies *et al.* 1988; Murakami *et al.* 1988; Beckmann *et al.* 1990; Frydman *et al.* 1994). There are also two Hsp40-Hsp70 partnerships along with the nascent-associated polypeptide complex and the ribosome associated complex. Mutants of the proteins in these complexes cause an accumulation of preproteins in the cytosol because of a decrease in mitochondrial import. However, these proteins are not essential in yeast (Caplan *et al.* 1992; Wiedmann *et al.* 1994; George *et al.* 1998; Gautschi *et al.* 2001). Another chaperone, MSF (Mitochondrial import Stimulation Factor), can restore aggregated preproteins to an import competent state using ATP, unlike the previous chaperones that only maintain preproteins in an import competent state after translation (Murakami and Mori 1990; Hachiya *et al.* 1993; Hachiya *et al.* 1994; Komiya *et al.* 1997). There has also been evidence that some chaperones help in targeting preproteins to mitochondria in mammalian cells. Hsp90 and Hsp70, are needed to deliver carrier class preproteins to Tom70 and are part of the recognition process (Young *et al.* 2003). Mtf1p was found to bind the amphiphilic targeting presequence in mitochondrial presequences (Cartwright *et al.* 1997). Much still needs to be learned of how cytosolic chaperones might have a role in targeting preproteins to the mitochondrial receptors.

1.4.4. Translocase of the mitochondrial outer membrane

The TOM complex is responsible for the initial recognition of mitochondrial preproteins present in the cytosol (Fig. 1, Fig. 2) (reviewed in Pfanner and Neupert 1990; Neupert 1997; Bauer *et al.* 1999; Pfanner and Geissler 2001; Prokisch *et al.* 2002; Hood and Joseph 2004; Rehling *et al.* 2004). The complex can be divided into two parts, the receptors and the general import pore (GIP). The receptor proteins recognize the preprotein targeting signals and then pass the preprotein to the GIP, which is made up of several membrane embedded components.

The TOM complex contains three receptor proteins (Kiebler *et al.* 1990; Künkele *et al.* 1998). The major function of the Tom70 receptor is to recognize preproteins of the metabolite carrier class such as the ATP/ADP carrier (AAC) that contain internal targeting signals (Hines *et al.* 1990; Söllner *et al.* 1990; Ryan *et al.* 1999). Yeast also contains a Tom70 homologue called Tom71, which is present in low abundance and loss of which does not affect import of preproteins (Schlossmann *et al.* 1996). Tom20 has

been called 'the master receptor' and works with Tom22 to recognize preproteins with cleavable presequences. After recognition, the receptors pass the preprotein to the GIP via Tom22 (Kiebler *et al.* 1993; Mayer *et al.* 1995). The GIP consists of the main pore forming component Tom40, Tom22 and the small Tom proteins, Tom5, Tom6 and Tom7, which are involved in stability of the complex and delivery of the preproteins to the pore (Hill *et al.* 1998; Künkele *et al.* 1998a; Künkele *et al.* 1998b; Ahting *et al.* 2001).

The TOM complex has been isolated and can be found in two different forms, depending on the isolation conditions. One form, the TOM holo complex, isolated from *N. crassa* in the mild non-ionic detergent digitonin (DIG), contains all the Tom proteins and is between 550 and 600 kDa in size (Künkele *et al.* 1998a; Künkele *et al.* 1998b). The stoichiometry of the components was determined to be 1.5:8:3.1:2 for Tom70:Tom40:Tom22:Tom20. Protein ratios for the small Toms were undetermined. The other form of the TOM complex, the core complex (400 kDa) is found when the complex is isolated in the stronger detergents dodecylmaltoside (DDM) or triton-X 100 and consists of Tom40, Tom22 and the small Tom proteins (Dekker *et al.* 1998; Ahting *et al.* 1999; Meisinger *et al.* 2001). The stochiometry in the core complex was determined to be 8:4:2:2 for Tom40:Tom22:Tom6:Tom7. When soluble isolated TOM complex was incubated with a presequence-containing preprotein, the presequence was protected from cleavage by exogenously added matrix processing peptidase (MPP). This shows that isolated TOM complexes can recognize, bind, and protect the N-terminus of mitochondrial preproteins without the presence of chaperones or a lipid bilayer (Stan *et al.* 2000).

Electrophysiology, electron microscopy, and size exclusion studies have shown that the pore is cation-selective and voltage gated with a diameter of 20 to 26 Å, (Hill *et al.* 1998; Künkele *et al.* 1998a; Künkele *et al.* 1998b; Schwartz and Matouschek 1999; Ahting *et al.* 2001). Based on EM tomography, there was debate as to whether the TOM complex contained two or three pores, as isolated holo complex contained both a two and a three pore form, while the core complex contained only the two pore form (Hill *et al.* 1998; Künkele *et al.* 1998; Künkele *et al.* 1998; Ahting *et al.* 2001). This was later resolved, as isolated complex without Tom20 has only two pores but has three when Tom20 is present (Model *et al.* 2002).

The TOM complex is proposed to contain several binding sites for the incoming preprotein. One site, termed the *cis* site, is on the cytosolic side of the outer membrane where the presequence binds (Mayer *et al.* 1995). Studies using outer membrane vesicles (OMVs) showed the *cis* site requires the presence of the protease sensitive cytosolic domains of Tom20 and Tom22 (Mayer *et al.* 1995) while crosslinking showed that Tom40 is also involved (Rapaport *et al.* 1997). The interaction of the presequence with the *cis* site is probably electrostatic as the interaction is sensitive to salt (100 mM KCl) (Rapaport *et al.* 1998). However, there may be different stages of interactions at the *cis* site binding since the interaction of the amphipathic presequence and Tom20 seems to be based on hydrophobic interactions and is not salt sensitive (Brix *et al.* 1997; Abe *et al.* 2000). The second binding site is located on the IMS side of the outer membrane and is called the *trans* site (Mayer *et al.* 1995). The interactions at this site are probably hydrophobic because they are resistant to relatively high salt concentrations (600 mM KCl) (Rapaport *et al.* 1998). Binding at the *trans* site does not require the cytosolic receptor domains and the presequence is accessible to cleavage by MPP in the lumen of OMVs (Rapaport *et al.* 1997; Rapaport *et al.* 1998). Tom40, through chemical crosslinking studies, has been shown to be a part of the *trans* binding site (Rapaport *et al.* 1997). Site-specific photocrosslinking has also shown that the Tom22 C-terminal IMS domain and Tom7 are also either part of, or close to, the *trans* site and that both may be involved in the passage of the preproteins to the TIM23 complex (Esaki *et al.* 2004). The *trans* site is known to be important for the translocation process as binding of the presequence to the site stimulated preprotein unfolding. Cleavage of the presequence in the lumen of OMVs caused the rest of the preprotein to slide back out of the TOM complex (Mayer *et al.* 1995).

1.4.4.1. Tom20

The first receptor protein identified was Tom20 when antibodies against it were shown to cause a decrease in import of preproteins *in vitro* using isolated mitochondria (Söllner *et al.* 1989; Schneider *et al.* 1991; Moczko *et al.* 1993; Ramage *et al.* 1993). Tom20 has an N-terminal anchor, a large hydrophilic C-terminal domain in the cytosol and a single tetratricopeptide repeat (TPR) motif, which is thought to be important for

interacting with other Tom proteins (Neupert 1997; Blatch and Lassle 1999). Tom20 in both *N. crassa* and yeast is not essential but lack of the protein causes severe growth rate defects, and yeast cannot grow on non-fermentable carbon sources without the protein. Tom22 levels are lower without Tom20, suggesting that Tom22 depends on Tom20 for its import and/or assembly. Tom70 can compensate for the loss of Tom20 but loss of both receptors is lethal (Ramage *et al.* 1993; Lithgow *et al.* 1994). Tom20 was found to be the receptor for most mitochondrial preproteins because the import of almost all preproteins tested, except for AAC and cytochrome c_1, decreased with a Tom20 deficiency (Ramage *et al.* 1993; Harkness *et al.* 1994). Other evidence that Tom20 is a receptor comes from its role in the *cis* binding site for the presequence (see sec 1.4.4) and the finding that it directly interacts with several preproteins (Söllner *et al.* 1992; Bolliger *et al.* 1995; Brix *et al.* 1999). The NMR structure of the aldehyde dehydrogenase (ALDH) presequence bound to Tom20 showed that the receptor binding domain formed an α-helical groove containing leucine residues, which interact with the hydrophobic face of the amphipathic helix (Abe *et al.* 2000). A further study showed that possibly only very short sections of presequences may actually bind to Tom20 (Muto *et al.* 2001).

1.4.4.2. **Tom70**

The other receptor, Tom70, also has a large cytosolic domain and contains seven TPR motifs (Sollner *et al.* 1990; Brix *et al.* 1997). The main role of Tom70 is to recognize the preprotein version of the hydrophobic inner membrane carrier proteins, which have internal targeting signals (Hines *et al.* 1990; Sollner *et al.* 1990; Ryan *et al.* 1999). Tom70 is not essential in either yeast or *N. crassa.* Strains lacking the protein exhibit only mild phenotypes, showing that Tom20 and Tom22 can probably compensate for Tom70 functions (Steger *et al.* 1990; Grad *et al.* 1999). The double loss in yeast of Tom70 and its homologue Tom71 does not intensify the phenotype (Schlossmann *et al.* 1996). A core region containing four of the TPR motifs in the purified Tom70 cytosolic domain can bind to internal targeting sequences, such as those found in PiC, but does not bind classic N-terminal targeting presequences (Brix *et al.* 1997; Brix *et al.* 1999).

Preproteins of the carrier family have to be bound to cytosolic chaperones to achieve productive recognition of targeting sequences by Tom70 (Young *et al.* 2003).

The four TPR motifs that are found in the binding region of Tom70 resemble a structure in cofactors of Hsp70 and Hsp90 such as the cyclophilin Cyp40 and the immunophilins FKBP52 (Young *et al.* 2001). It has been proposed that Tom70 acts as a cofactor with these chaperones and the interaction between them and Tom70 is necessary for efficient import of the preproteins (Young *et al.* 2003).

1.4.4.3. Tom22

Tom22 is part of the GIP but also works with Tom20 and Tom70 as a receptor and is thought to pass preproteins from the receptor stage to the pore (Kiebler *et al.* 1993). Tom22 is involved in the import of most preproteins except some of those destined to the outer membrane (Kiebler *et al.* 1993; Lithgow *et al.* 1994; Nargang *et al.* 1995). It was originally thought that Tom22 was essential in yeast (Lithgow *et al.* 1994; Hönlinger *et al.* 1995) but it was later shown that yeast could still grow very slowly on fermentable media in the absence of the protein (van Wilpe *et al.* 1999). Conversely, in *N. crassa*, a sheltered heterokaryon containing a *tom22* disruption could not produce viable homokaryotic colonies containing the disruption, demonstrating that Tom22 is essential in this organism (Nargang *et al.* 1995). This may be due to the fact that unlike yeast, *N. crassa* cannot grow by fermentation alone.

Both the cytosolic and an intermembrane space domain on Tom22 have many negative charges (Kiebler *et al.* 1993). The roles of these in the function of Tom22 were analyzed through deletion mutants (Nakai *et al.* 1995; Court *et al.* 1996). It was found that the IMS domain was not essential for viability and there were only minor import defects of matrix proteins and some inner membrane proteins. It was also shown that binding of inner membrane proteins to the *trans* site was unaffected by the loss of the intermembrane space domain. But, when the cytosolic domains of the receptors and the Tom22 IMS domain were removed, N-terminal presequence binding at the *trans* site was affected (Moczko *et al.* 1997). Newer evidence showing site-specific photocrosslinking between the Tom22 IMS domain and the presequence suggests a role of this domain in the *trans* binding site (see section 1.4.4). The purified cytosolic domain preferentially binds the N-terminal presequences and this binding was inhibited by salt (Brix *et al.* 1997). It was also determined that most of the negative charges thought to be important

for electrostatic binding could be removed from the cytosolic domain without affecting function (Nargang *et al.* 1998). Tom22 also has a role in organizing the core complex and acts as a docking point for Tom20 and Tom70 as TOM complexes completely lacking Tom22 are also devoid of Tom20 and Tom70 (van Wilpe *et al.* 1999).

1.4.4.4. Tom40

Tom40 is essential in yeast and *N. crassa* (Baker *et al.* 1990; Taylor *et al.* 2003). It is the major component of the translocation pore and was first identified because of its ability to cross-link with mitochondrial preproteins as they cross the membrane (Vestweber *et al.* 1989; Kiebler *et al.* 1990; Hill *et al.* 1998; Künkele *et al.* 1998; Künkele *et al.* 1998). Tom40 is part of both the *cis* and *trans* binding sites as it can cross-link to presequences at the cytosolic and intermembrane space side of the outer membrane (Rapaport *et al.* 1997; Rapaport *et al.* 1998). Conflicting evidence exists concerning the topology of the protein. In *N. crassa*, both the N and C-termini have been shown by protease accessibility studies on intact mitochondria to be in the IMS (Künkele *et al.* 1998). However, in yeast, the N-terminus can be cleaved by externally added trypsin (Hill *et al.* 1998). Tom40 exists in the TOM complex as oligomers with a basic structure of dimers (Dekker *et al.* 1998; Hill *et al.* 1998; Rapaport *et al.* 1998; Ahting *et al.* 1999; Model *et al.* 2001). During preprotein translocation there are changes to the interactions between Tom40 molecules and between Tom40 and other Tom proteins (Rapaport *et al.* 1998). Based on computer predictions, Tom40 is expected to exist as a β-barrel that spans the outer membrane with 14 β-strands (Fig. 3) (Court *et al.* 1995; Mannella *et al.* 1996) (T. Schirmer, via D. Rappaport, personal communication). Circular dichroism studies of yeast Tom40 expressed in bacteria, refolded from exclusion bodies and inserted into liposomes, revealed a structure containing more than 60% β-sheet (Hill *et al.* 1998). However, *N. crassa* Tom40 purified directly from mitochondria gave only about 30% β-sheet when examined by FTIR (Fourier transform infrared) spectroscopy (Ahting *et al.* 2001).

1.4.4.5.Tom5

Tom5 has mainly been studied in yeast as it was only recently identified in *N. crassa* (W. Neupert, personal communication). It is a tail-anchored protein that spans the outer membrane as an α-helix and has a cytosolic helical N-terminus with alternating positive and negative charges (Dietmeier *et al.* 1997; Hammen and Weiner 2000). It is not essential as strains can grow without it at 30°C, on fermentable or non-fermentable carbon sources, although there is a growth defect, but cannot grow at 37°C on both types of carbon source. The deletion of Tom5 does not affect the levels of other TOM complex proteins or the stability of the complex but it does cause synthetic lethality when deleted in combination with any of the other TOM complex proteins that are viable as single deletions (Tom6, Tom7, Tom20, Tom70) (Dietmeier *et al.* 1997).

Tom5 is proposed to have a role in the passage of preproteins to the GIP from the receptor proteins (Dietmeier *et al.* 1997). Tom5 is in close contact with the preprotein as it translocates across the membrane at a post receptor stage, and import independent of the complex receptors is strongly dependent on Tom5 (Dietmeier *et al.* 1997). Consistent with this, import of all classes of preproteins was strongly reduced into mitochondria without Tom5 (Dietmeier *et al.* 1997). Specifically, the import of the small Tim proteins (Tim9 and Tim13), which do not require the cytosolic domains of the receptor proteins, was strongly inhibited without Tom5 (Kurz *et al.* 1999). This shows that Tom5 is involved in many different import pathways since all classes of preprotein import are affected without Tom5, and precursors with divergent pathways such as the small Tim proteins, Tim22 and Tim54, are affected as well (Kurz *et al.* 1999) (Hammen and Weiner 2000). It was originally proposed that the negative charges in the cytosolic domain were needed for the receptor-like function (Dietmeier *et al.* 1997), but deletion of the first 22 residues, which contain these charges, did not alter the growth phenotype (Horie *et al.* 2003). In addition, structural studies of the cytosolic domain suggested that it would not make a good binding site for presequence signals because the alternating pattern of negative and positive charges is not complementary to the N-terminal presequence and there is no hydrophobic surface present that would complement the hydrophobic face of the amphipathic presequence helix.

1.4.4.6. Tom6

Tom6 is a non-essential tail anchored protein with its N-terminus in the cytosol. It is needed to stabilize the TOM complex (Alconada *et al.* 1995; Cao and Douglas 1995). The amounts of Tom40, Tom20 and Tom70 are the same when Tom6 is absent but the complex is destabilized. A decrease in import of MPP, F_1B and AAC was seen in mitochondria without Tom6 (Alconada *et al.* 1995). Tom6 also appears to have a role in modulating the interactions of the preprotein with the receptors and Tom40, probably in an indirect way, as it does not interact with the preprotein itself (Alconada *et al.* 1995). When the preprotein is at the *cis* site, the interactions between Tom22 and Tom6 are decreased and the interactions between Tom40 and Tom6 are stabilized. When the preprotein is at the *trans* site there is no change in the amount of crosslinking between Tom6 and Tom40 (Rapaport *et al.* 1998; Dembowski *et al.* 2001).

1.4.4.7. Tom7

Tom7 is also a non-essential, tail anchored protein (Hönlinger *et al.* 1996). It is thought to have an opposing role to Tom6, as the removal of Tom7 causes the associations between Tom40, Tom20 and Tom22 to be more stable (Honlinger *et al.* 1996). Tom7 is also proposed to have a role in the productive accumulation of precursors at the outer membrane. It appears to be involved in preprotein sorting as import rates of preproteins such as F_1B, AAC and cytochrome c_1 into ΔTom7 mitochondria are only slightly affected while the import rate of porin is strongly affected (Honlinger *et al.* 1996). An interaction of Tom7 with the receptors is also suggested by the observation of synthetic lethality in a Tom7/Tom20 double deletion strain. Yeast Tom7 deletion strains are very slow growing on non-fermentable carbon sources at 30°C and the strains do not grow on any carbon source at 37°C. The combination of ΔTom7ΔTom5 is lethal in yeast (Honlinger *et al.* 1996; Dietmeier *et al.* 1997). Tom7 has also been found to have a role in the *trans* binding site and preprotein transfer to the TIM23 complex, and may have overlapping functions with the C-terminal IMS domain of Tom22, which is involved in these activities (Esaki *et al.* 2004).

1.4.4.8. Assembly of the TOM complex

The TOM complex components are unique mitochondrial proteins as they are an integral part of the complex that imports them. The subunit composition of the complex seems to be dynamic, with a constant exchange of new and old subunits (Dembowski *et al.* 2001; Rapaport *et al.* 2001). Tom20 and Tom70, the two amino-terminally anchored proteins of the TOM complex, do not require their own cytosolic domains to be imported but use either Tom40 directly or have prior interactions with Tom5 (Schneider *et al.* 1991; Schlossmann and Neupert 1995; Suzuki *et al.* 2000). Tom40, Tom6 and Tom7 all require Tom20 and Tom22. Tom40 and Tom22 require Tom70 in addition to Tom20 and Tom22 (Keil and Pfanner 1993; Dembowski *et al.* 2001; Model *et al.* 2001). Tom5 appears to use Tom40 directly for import (Horie *et al.* 2003).

Assembly of most of the components occurs directly into the TOM complex with the exception of Tom6 and Tom40 (Rapaport and Neupert 1999; Model *et al.* 2001), although one report suggested that human Tom7 assembles via a 120 kDa complex (Johnston *et al.* 2002). Tom6 assembles into the 400 kDa TOM core complex via a 100 kDa intermediate (Model *et al.* 2001). Tom40 follows a more complex pattern of assembly (Fig. 4a) (Model *et al.* 2001). First, the incoming monomer binds to the cytosolic side of the MOM at the TOM complex receptors. It is then translocated to the IMS side of the outer membrane and is associated with the Tim8/Tim13 and/or the Tim9/Tim10 complex which may act as chaperones to bring Tom40 to the SAM complex (Hoppins and Nargang 2004; Wiedemann *et al.* 2004). At this point Tom40 is partially inserted into the membrane and is associated with the SAM complex to form a 250 kDa intermediate (Wiedemann *et al.* 2003). Upon leaving the SAM complex, Tom40 is found in a 100 kDa intermediate, which is fully integrated into the membrane and likely represents a Tom40 dimer consisting of one newly imported Tom40 and one pre-existing Tom40 subunit, along with Tom5 and Tom6. Authentic TOM complex of 400 kDa is formed when the 100 kDa intermediate assembles with Tom22, Tom7, and more Tom40 molecules with the assistance of Mdm10 (Meisinger *et al.* 2004) and Tom13 (Ishikawa *et al.* 2004).

The requirements for Tom40 assembly are not fully known. So far, experiments have shown that Tom20, Tom22 and Tom5 are needed for Tom40 import into the 250

kDa intermediate along with the SAM complex and small Tim complexes, and that Tom6 is required for the formation of the fully assembled 400 kDa form (Model *et al.* 2001; Wiedemann *et al.* 2003; Hoppins and Nargang 2004; Wiedemann *et al.* 2004). It has been shown that conserved residues near the N-terminus (residues 41-60) of Tom40 and three near the C-terminus are required for Tom40 assembly, but not for receptor recognition (Rapaport *et al.* 2001; Taylor *et al.* 2003). Unpublished studies in the Nargang lab (R. Taylor and E. L. Sherman) have investigated the signals required for the Tom40 protein to interact with the TOM complex and to form each of the intermediates. Several mutants affecting highly conserved regions of Tom40 were made (Fig. 5) and *in vitro* import of the radiolabeled mutant Tom40 molecules into isolated mitochondria of *N. crassa* was performed (Fig. 4b, Table 1, Appendix 1). Four different assembly phenotypes were seen in the Tom40 assembly pathway. Some Tom40 variants arrested at the 250 kDa intermediate, and formed no 400 kDa complex even after extended incubation at 25°C. Other mutant proteins showed an accumulation of the 250 kDa intermediate with some assembly to the 400 kDa complex. The individual mutants in this class of mutations showed variation in the amount of 400 kDa complex formed. The third phenotype showed an accumulation of the 100 kDa intermediate with less of the 400 kDa and 250 kDa intermediates formed. Finally, some of the Tom40 variants showed a wild type assembly pattern. Some of these finding are discussed in more detail in the Results section of this thesis.

1.4.4.8.1. SAM complex

The SAM complex (Fig. 1) was first discovered when Mas37, which was originally misclassified as a TOM complex receptor protein (Gratzer *et al.* 1995; Ryan *et al.* 1999), was found to be part of the 250 kDa intermediate in Tom40 assembly (Wiedemann *et al.* 2003). Deletion of Mas37 not only affected Tom40 assembly but also porin import showing that this complex is used generally by β-barrel proteins. Sam50 (also called Tob55 or Omp85) was also identified as part of this complex and is another essential β-barrel protein of the MOM (Kozjak *et al.* 2003; Paschen *et al.* 2003; Gentle *et al.* 2004). This protein is conserved in bacteria, has channel activity, and could possibly

release the β-barrel proteins into the membrane (Paschen *et al.* 2003). Another essential subunit, Sam35 (also called Tob38 or Tom38) is peripherally associated with the outer membrane and is needed for efficient β-barrel insertion into the membrane (Ishikawa *et al.* 2004; Milenkovic *et al.* 2004; Waizenegger *et al.* 2004). Mdm10, a protein originally defined as being essential for proper mitochondrial morphology, has also been shown to have a role in Tom40 assembly. Mdm10 is a component of the SAM complex but appears to affect the import of Tom40 and not other β-barrel proteins (Meisinger *et al.* 2004). It is needed at a later stage of Tom40 assembly, when the protein assembles with Tom6 and Tom7. Another protein, Tom13 was identified as necessary for Tom40 assembly but does not affect the 250 kDa or 100 kDa intermediates (Ishikawa *et al.* 2004). It also does not seem to be a part of the SAM complex but is found in a separate 180 kDa complex.

1.4.5. Translocases of the mitochondrial inner membrane

There are two TIM complexes in the mitochondrial inner membrane. The import of a subset of mitochondrial preproteins such as the metabolite carriers (eg, the ADP/ATP carrier, AAC) into the inner membrane requires the chaperone function of the small Tim protein complexes in the intermembrane space before they are inserted via the TIM22 twin pore complex. Insertion by the TIM22 complex requires a membrane potential across the inner membrane (Gasser *et al.* 1982; Schleyer and Neupert 1982; Sirrenberg *et al.* 1996; Endres *et al.* 1999). Import of matrix proteins by the TIM23 complex (Dekker *et al.* 1997) not only requires a membrane potential, but also the associated import motor that uses ATP. (Gasser *et al.* 1982; Schleyer and Neupert 1982; Eilers *et al.* 1987; Truscott *et al.* 2003). The motor has recently been referred to as the PAM complex (Truscott *et al.* 2003). Membrane potential also tightly regulates the TIM23 and TIM22 pores so ions and membrane potential are not lost (Truscott *et al.* 2001). The OXA complex also exists in the MIM. This complex inserts proteins from the matrix into the inner membrane (Hell *et al.* 2001).

1.4.5.1. The TIM 23 complex and the import motor

The TIM23 complex takes over the import of N-terminal presequence preproteins after *trans* site binding at the TOM complex. Transfer from the TOM complex to the TIM complexes occurs at contact sites between the outer and inner membrane. It was found that the IMS domain of Tom22 and the IMS domain of Tim50 of the TIM23 complex are needed for this interaction but the IMS N-terminal region of Tim23, contrary to a previous suggestion, is not (Schleyer and Neupert 1985; Rassow *et al.* 1989; Donzeau *et al.* 2000; Chacinska *et al.* 2003). There are three core proteins in the TIM23 complex, Tim23, Tim17 and Tim50 (Fig. 1) (Rehling *et al.* 2004). Tim23 is an essential protein that requires a membrane potential to form dimers and its N-terminus is a receptor for the presequences in the IMS (Dekker *et al.* 1993; Bauer *et al.* 1996; Komiya *et al.* 1998). Tim17 is also essential and is homologous to Tim23. Tim23 and Tim17 exist in a 90 kDa complex and these two proteins form a pore of 13 Å called the MCC (mitochondrial inner membrane conductance channel) (Ryan and Jensen 1993; Kübrich *et al.* 1994; Haucke and Schatz 1997). However, purified Tim23 can form a pore with similar characteristics to the MCC and there is also evidence from studies using mutants and antibodies against the two proteins that Tim23 alone forms the pore (Lohret *et al.* 1997; Truscott *et al.* 2001). The role of Tim17 remains elusive, as Tim17 cannot substitute for Tim23, supporting a unique role for Tim17 in import (Maarse *et al.* 1994; Ryan *et al.* 1994; Blom *et al.* 1995). Tim50 has one predicted membrane spanning domain and a large IMS domain. It plays a role in linking the TIM23 complex to the TOM complex and binds with the IMS domain of Tim23 to help guide preproteins through the IMS (Geissler *et al.* 2002; Yamamoto *et al.* 2002; Mokranjac *et al.* 2003).

Tim44, an essential protein in the matrix that is peripherally associated with the TIM23 complex and inner membrane, connects the TIM23 complex to mitochondrial heat shock protein 70 (mtHsp70). mtHsp70 is the central component of the presequence import motor that completes translocation of the preprotein into the matrix after the force from the membrane potential has pulled the positively charged presequence into the matrix (Maarse *et al.* 1992; Blom *et al.* 1993; Berthold *et al.* 1995; Blom *et al.* 1995). This motor, also termed PAM (presequence-translocase associated-import-motor) is comprised of mtHsp70 along with Tim14/Pam18, Tim16/Pam16 and Mge1 (Mokranjac

et al. 2003; Truscott *et al.* 2003; Frazier *et al.* 2004; Kozany *et al.* 2004; Rehling *et al.* 2004). Two major hypotheses exist for the mechanism of the import motor. The Brownian ratchet model suggests that Hsp70 would trap segments of the preprotein as they enter the matrix and prevent backsliding. As more of the protein is unfolded outside the mitochondria, it could enter and be trapped by another mtHsp70 molecule (Schneider *et al.* 1994; Voos *et al.* 1996; Gaume *et al.* 1998; Voisine *et al.* 1999). The pulling model suggests that mtHsp70 is simultaneously bound to the TIM23 complex through Tim44 and the translocating preprotein. ATP-induced conformational changes to mtHsp70 are thought to generate a force capable of pulling the preprotein into the matrix and stimulate unfolding of the portions of the preprotein still remaining on the outer surface of the MOM (Kang *et al.* 1990; Schneider *et al.* 1994; Voos *et al.* 1996; Matouschek *et al.* 1997; Voisine *et al.* 1999).

With the recent discovery of Tim14 and Tim16, a more detailed picture of the import motor has emerged (Mokranjac *et al.* 2003; Truscott *et al.* 2003; Frazier *et al.* 2004; Kozany *et al.* 2004). Both Tim14 and Tim16 are essential components of the motor and are related to the J-domain proteins, which act as cochaperones with Hsp70 and are members of the DnaJ protein family. Tim14 is an integral inner membrane protein with its J-domain containing the conserved His-Pro-Asp (HPD) motif exposed to the matrix. Tim14 interacts with Tim44 and mtHsp70 and stimulates the ATPase activity in mtHsp70. Tim16, however, does not contain the HPD motif found in J-domains and does not stimulate the ATPase activity of mtHsp70. It interacts with mtHsp70 and Tim14 and is crucial for the association of Tim14 with the TIM23 complex. It is also proposed that these interactions between Tim16 and Tim14 and mtHsp70 are regulatory and Tim16 may prevent Tim14 from constantly activating mtHsp70.

Based on this new information, the function of the motor can be better understood. When the preprotein emerges from the TIM23 complex, it associates with Tim44, which recruits mtHsp70 where the peptide can sit loosely in the peptide-binding pocket of mtHsp70. The J-domain of Tim14 then stimulates the ATPase activity of mtHsp70 causing it to close tightly on the peptide, efficiently trapping the precursor in the matrix. This ADP-bound mtHsp70 is then released from the import motor allowing the peptide to move further into the matrix but not to slide back out (Liu *et al.* 2003;

Mokranjac *et al.* 2003). Mge1 then associates with mtHsp70 and the ADP-bound mtHsp70 is regenerated to the ATP form so it can start another cycle. Tim16 would stabilize the motor and regulate the cycling, preventing the Tim14 stimulatory effect on mtHsp70 (Mokranjac *et al.* 2003; Truscott *et al.* 2003; Frazier *et al.* 2004; Kozany *et al.* 2004; Li *et al.* 2004). Although this theory is appealing, more work is required to determine the sequence of events that occur as the motor cycles. This may clear up the debate as to how the inward movement of the translocating peptide is acheived.

1.4.5.1.1. Precursor processing and folding

The final steps in the import of matrix preproteins are cleavage of the presequence and folding into the mature form of the protein (Gakh *et al.* 2002). The mitochondrial processing peptidase (MPP) is a complex that recognizes the presequence and cleaves it when it is in an extended conformation, not in an amphipathic helix (Hawlitschek *et al.* 1988; Witte *et al.* 1988; Yang *et al.* 1988; Taylor *et al.* 2001). Another protease in the matrix, MIP (mitochondrial intermediate peptidase), is required for removing an octapeptide which is found after the presequence in a subset of matrix proteins (Isaya *et al.* 1994). Protein folding must also occur properly, and is accomplished by several chaperone proteins found in the matrix, including the essential protein mtHsp60 (Cheng *et al.* 1989; Ostermann *et al.* 1989; Rowley *et al.* 1994; Rassow *et al.* 1995).

1.4.5.2. Small Tim proteins of the intermembrane space

There are two 70 kDa complexes made of the small Tim proteins in the intermembrane space, the essential Tim9/Tim10 complex that can also contain Tim12 (Bauer *et al.* 1999) and the homologous non-essential Tim8/Tim13 complex (Koehler *et al.* 1999). Each of these contains three molecules of each protein found in the respective complex. In the Tim9/Tim10/Tim12 complex, one Tim12 protein substitutes for one Tim10 protein. All these proteins contain highly conserved cysteine residues that can form a zinc finger motif (Koehler *et al.* 1998; Sirrenberg *et al.* 1998; Koehler *et al.* 1999). While the cysteines are important for the function and assembly of the complex, there is controversy over their role. One hypothesis is that they coordinate zinc ions, while another suggests they form disulfide bonds. There is evidence for both of these views

depending on the conditions of the experiments (Curran *et al.* 2002; Allen *et al.* 2003; Lutz *et al.* 2003; Koehler 2004). Recently, another protein has been found in the IMS, Mia40/Tim40, which is required for the import of small IMS proteins and also for assembly of the small Tim complexes (Chacinska *et al.* 2004; Naoe *et al.* 2004). Based on results from these studies and others (Chacinska *et al.* 2004; Lu *et al.* 2004; Lu *et al.* 2004), it seems that both disulfide bonds and zinc binding are necessary for the biogenesis of the small Tims. The addition of zinc ions to the import reaction increased the amount of the Mia40-Tim10 assembly intermediate while addition of β-mercaptoethanol after the import reaction caused the Mia intermediate to dissociate but did not affect the final Tim9/Tim10 complex.

The small Tim complexes are involved in chaperoning a subset of inner membrane proteins from the TOM complex to the TIM22 complex, which inserts them into the membrane. The carrier proteins, such as AAC, are thought to translocate through the TOM complex in a slightly folded conformation as two membrane spanning helices connected by a loop, with the loop moving through the pore first (Endres *et al.* 1999; Wiedemann *et al.* 2001). The Tim9/Tim10 complex associates with the preprotein as it emerges in the IMS and then transfers it to the Tim9/Tim10/Tim12, complex which is associated with the TIM22 complex at the inner membrane (Endres *et al.* 1999; Ryan *et al.* 1999; Wiedemann *et al.* 2001; Curran *et al.* 2002). The TOM complex and the Tim9/Tim10 complex were found to be sufficient for transporting AAC across the outer membrane of OMVs (Vasiljev *et al.* 2004).

The Tim8/Tim13 complex has a role in the import of Tim23 (Davis *et al.* 2000; Paschen *et al.* 2000). Tim8/Tim13 binds the N-terminus of Tim23 and traps it in the intermembrane space. Although this complex is not essential, it is needed for efficient import of Tim23 when there is a low membrane potential (Kaldi *et al.* 1998; Davis *et al.* 2000; Paschen *et al.* 2000; Hoppins and Nargang 2004). The human homologue of Tim8 is the DDP1 protein (deafness dystonia protein). Lack of the protein results in Mohr-Tranebjaerg syndrome. It is thought that this disease occurs because the human Tim8/Tim13 complex cannot import TIM23 properly, leading to mitochondrial dysfunction because of reduced import of matrix proteins. However, no defects in mitochondrial function have been observed in the tissues of Mohr-Tranebjaerg patients

(Koehler *et al.* 1999; Paschen *et al.* 2000; Tranebjaerg *et al.* 2000; Bauer and Neupert 2001; Rothbauer *et al.* 2001; Roesch *et al.* 2002). The Tim8/Tim13 complex is also involved in the import of β-barrel proteins (Hoppins and Nargang 2004; Wiedemann *et al.* 2004).

1.4.5.3. TIM22 complex

The 300 kDa TIM22 complex of the mitochondrial inner membrane is responsible for inserting proteins into the inner membrane (Fig. 1)(reviewed in Rassow *et al.* 1999; Tokatlidis and Schatz 1999; Pfanner and Wiedemann 2002; Rehling *et al.* 2004). This complex contains three membrane embedded components, Tim22, Tim54 and Tim18 (Sirrenberg *et al.* 1996; Kerscher *et al.* 1997; Kerscher *et al.* 2000; Koehler *et al.* 2000). Tim22 is essential but Tim18 and Tim54 are not. The roles of Tim54 and Tim18 are not understood. Tim22 forms the voltage activated twin pore in the complex and responds to proteins with internal targeting signals (Kovermann *et al.* 2002).

The TIM22 complex was purified and the steps of import were studied *in vitro*. It was proposed that preprotein insertion into the inner membrane occurs in three steps (Rehling *et al.* 2003; Rehling *et al.* 2004). The first step is 'tethering' the preprotein to the complex via the Tim9/Tim10 complex through Tim12 and requires no membrane potential. Then the complex is partially activated and one loop is inserted into the pore by electrophoretic force of the positive charges on the matrix-exposed loop even if there is a low membrane potential. Next, the TIM22 complex recognizes the internal signal and in the presence of a full membrane potential is fully activated. The next set of transmembrane domains is then inserted into the other pore. The actual insertion into the membrane is thought to occur through lateral openings in the complex.

1.4.5.4. Export of matrix proteins to the inner membrane

There are a few proteins that are nuclear-encoded that enter the matrix and require cleavage of their presequence before they are inserted into the inner membrane by the OXA export complex. This complex also exports most, if not all, of the mtDNA-encoded proteins that need to be inserted into the inner membrane (Bauer *et al.* 1994; Bonnefoy *et al.* 1994; He and Fox 1997; Hell *et al.* 1997; Hell *et al.* 1998; Hell *et al.* 2001). The

OXA complex is a homooligomeric complex made up of the Oxa1 protein, which has five transmembrane domains with its N-terminus in the IMS and C-terminus in the matrix (Herrmann *et al.* 1997; Nargang *et al.* 2002). Oxa1 is essential in *N. crassa* and for growth of yeast on non-fermentable carbon sources (Bonnefoy *et al.* 1994; Nargang *et al.* 2002).

Additional export proteins, may form other complexes in the inner membrane. Mba1 has overlapping but also independent functions from Oxa1 in exporting proteins (Preuss *et al.* 2001; Baummann *et al.* 2002). Cox18, Pnt1 and Mss2 in yeast and Oxa2 in *N. crassa*, along with Oxa1 are required for the biogenesis of cytochrome oxidase (He and Fox 1999; Broadley *et al.* 2001; Saracco and Fox 2002; Funes *et al.* 2004).

1.5. Objectives of this study

One of the goals of this study was to elucidate residues in Tom40 important for both the function of the TOM complex and for Tom40 assembly. This was examined by creating strains with mutations in conserved regions in Tom40. Using this approach, I have identified regions that are involved in the sorting of different precursors to their subcompartments, in general import function, and in the stability of the TOM complex.

As most of the research to date on the small Tom proteins has been performed in yeast, another goal was to study the roles of small Tom proteins, Tom5, Tom6 and Tom7 in *N. crassa*. This was accomplished by creating strains with single and double absences of the proteins. These strains were used to analyze the growth of *N. crassa* without the small Tom proteins, as well as the stability and function of the TOM complex. This revealed that the combined absence of Tom6 and Tom7 caused severe growth defects and destabilized the TOM complex. The loss of all three of the small Tom proteins appears to be lethal. Tom6 and Tom7 are needed for the import of specific preproteins into the mitochondria, while Tom5 is not.

Figure 1. **Import complexes and pathways of mitochondrial preprotein import.** The complexes are shown with each of their components indicated (see sections 1.4.4 to 1.4.5.4 for details). Each protein in a given complex is named for that complex and they are distinguished from each other by their weight in kDa (eg, Tom70, the 70 kDa component of the TOM complex). Preproteins are initially recognized by the receptors of the TOM complex, which have specificity for different classes of preproteins. Preproteins are then passed from the receptors to the GIP portion of the TOM complex. Pathways then diverge depending on the final destination of the preprotein. The TOM complex directly inserts some outer membrane proteins into the outer membrane and also delivers some proteins directly to the IMS. Other IMS proteins, including the small Tims, interact with Mia40 in the IMS, which assembles them into their functional states. β-barrel proteins and inner membrane proteins with internal targeting signals of the carrier class interact with the small Tim protein complexes in the IMS which chaperone them to the SAM complex for insertion into the outer membrane. Many inner membrane proteins, such as those in the carrier class, are passed from the Tim9/Tim10 complex to the TIM22 complex of the inner membrane, which inserts them into the inner membrane. Matrix preproteins are passed from the TOM complex to the TIM23 complex that translocates them across the inner membrane to the import motor, which completes translocation across the inner membrane. In the matrix, the N-terminal presequence is cleaved by MPP and folding is assisted by chaperones. The TIM23 complex also inserts some preproteins into the inner membrane by a stop-transfer mechanism. Finally, some nuclear-encoded proteins that enter the matrix via the TIM23 complex and some mitochondrial-encoded proteins are 'exported' into the inner membrane by the OXA complex.

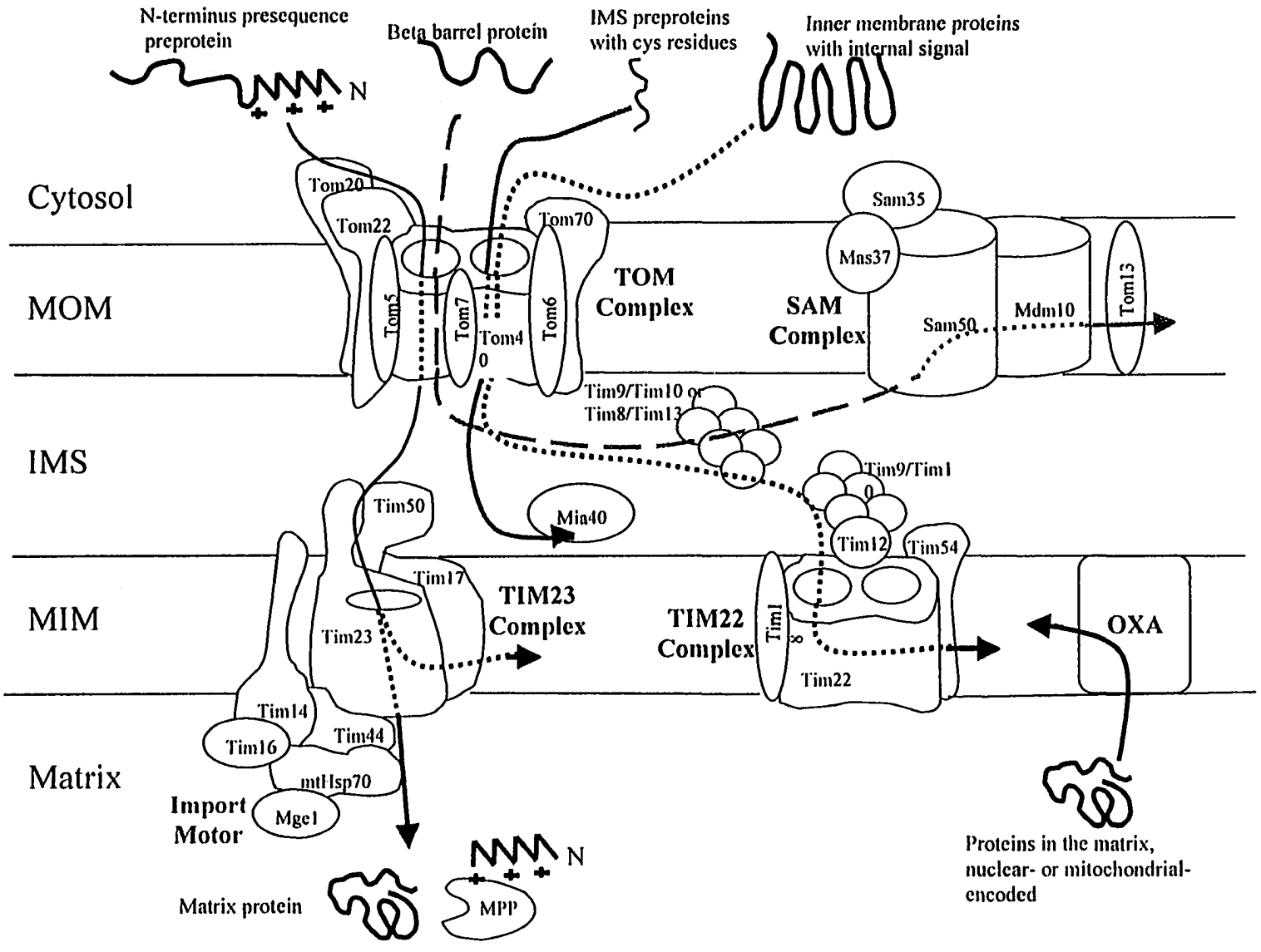


Figure 2. **The TOM complex of *N. crassa*.** Preproteins are initially recognized by the receptors Tom20 or Tom70. Tom20 is the main receptor and recognizes proteins with N-terminal presequence signals and other types of preproteins. Tom70 mainly recognizes preproteins with internal signals like those found in the carrier class of inner membrane proteins. There is functional overlap of the two receptors. Tom5 is proposed to have some receptor like functions in *S. cerevisiae*. Tom22, Tom20 and Tom40 form the *cis* binding site in the cytosol. Tom22 cooperates with the receptors and passes the preprotein from the receptors to the GIP. The GIP consists of Tom40, which is the main component, along with Tom22, Tom5, Tom6 and Tom7. Once the preprotein starts to emerge through the translocation pore into the IMS, it can bind the *trans* site, which is composed of Tom40, Tom22 and Tom7. From here, preproteins are sorted via different routes based on the class of preprotein and their final destination.

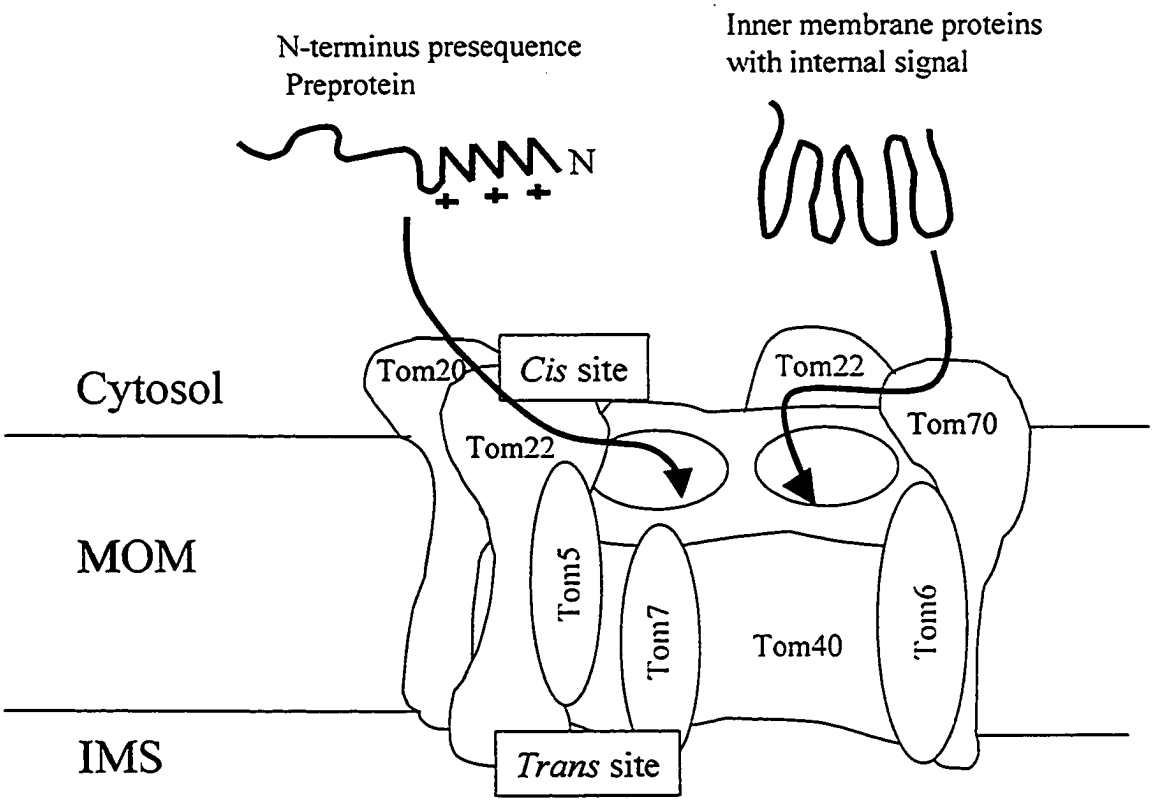


Figure 3. **Computer predicted model of Tom40.** The predicted structure of Tom40 (T. Schirmer, via D. Rapaport, personal communication). The bold letters are predicted to form the β-strands that span the outer membrane. The N- and C-termini are predicted to be in the IMS. The boxes indicate the regions mutated in this study. Solid bold boxes indicate mutations that cannot rescue the *tom40*^{RIP} nucleus. Dotted boxes show mutations that result in import defects.

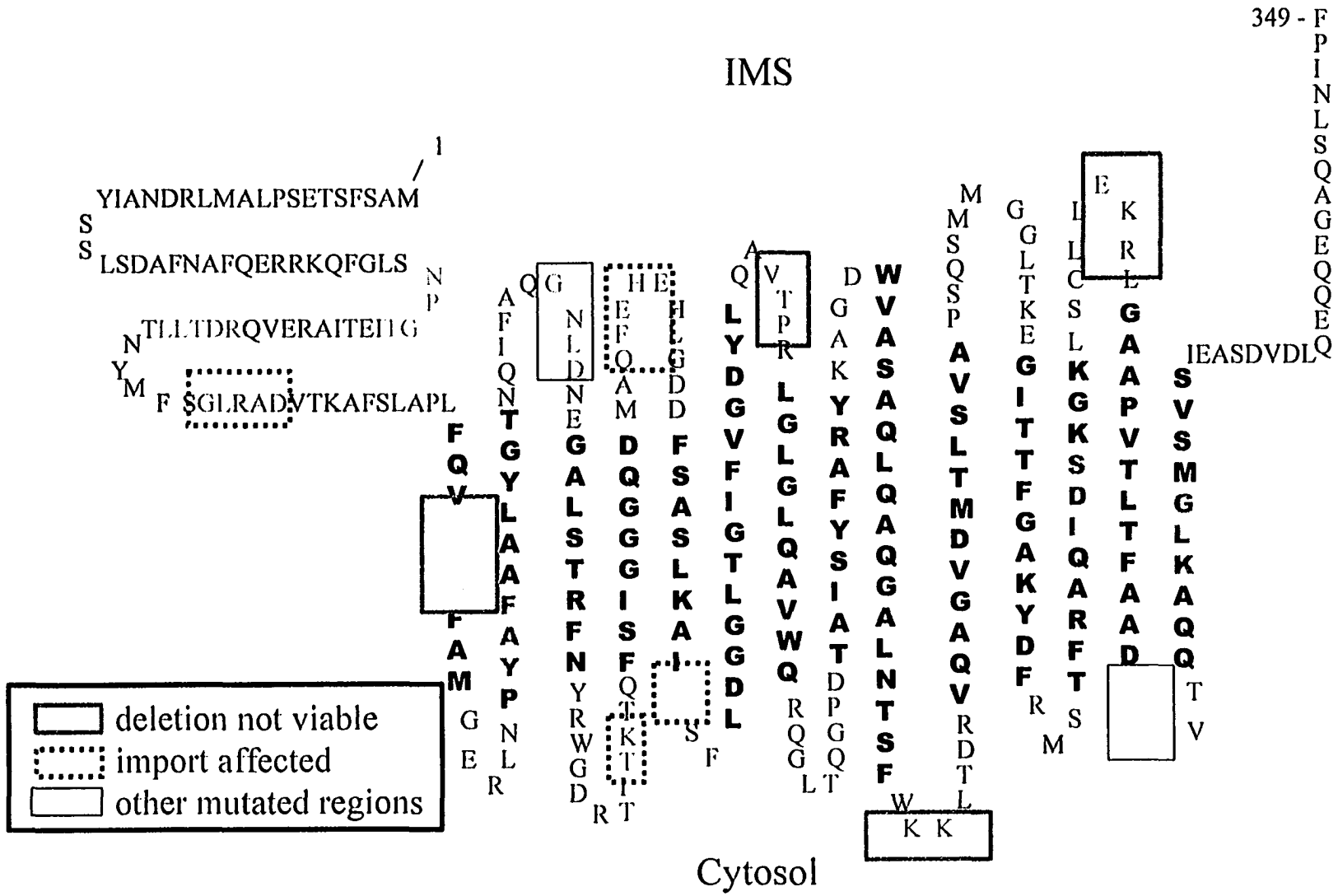
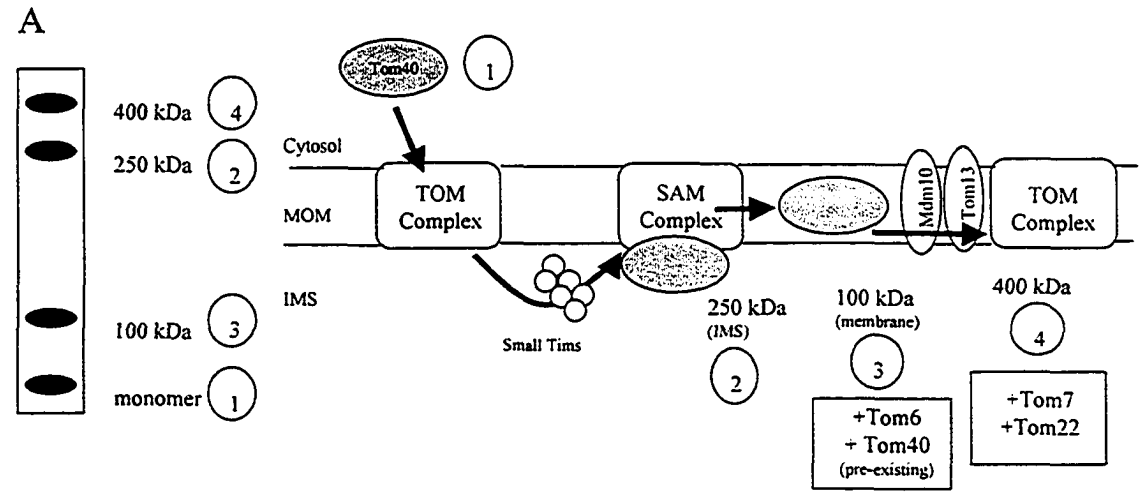


Figure 4. **Assembly of Tom40 into the TOM complex. A.** Schematic drawing of Tom40 assembly showing the intermediates seen during blue native gel electrophoresis (BNGE) and the proposed pathway of insertion. First, the incoming Tom40 monomer binds to mitochondria at the TOM complex. It is then translocated across the outer membrane to the IMS where it is chaperoned by the small Tim protein complexes to the SAM complex. The second identifiable intermediate is the 250 kDa complex consisting of the imported Tom40 subunit associated with the SAM complex. The SAM complex releases Tom40 into the outer membrane where it is now part of a third intermediate of 100 kDa, which also contains Tom5, Tom6, and another pre-existing molecule of Tom40. Finally, it is assembled with the rest of the Tom proteins including more Tom40 subunits and forms the authentic TOM complex of 400 kDa. The latter steps of the TOM complex assembly are assisted by the Mdm10 protein and Tom13. **B.** Examples of BNGE assays of *in vitro* Tom40 assembly (for a figure of each mutant see Appendix 1). 50μg of mitochondria were used in the import assays. Imports were performed at 0°C and 25°C for 20 min using ^{35}S-Met labeled Tom40 precursor variants. Following import, mitochondria were solubilized in 1% DIG, and electrophoresed on blue native gels. Proteins were transferred to PVDF and exposed to X-ray film. Representatives of the different classes of phenotypes seen are shown. (1) Wild type Tom40 assembly pathway. (2) Assembly phenotype where Tom40 stops at the 250 kDa intermediate. Example shown is for the ΔGLRAD variant. (3) Two examples of Tom40 variants with an accumulation of the 250 kDa intermediate but with varying amounts of the 400 kDa complex formed. Examples are for the ΔNP and NP-AA precursors. (4) Example of a Tom40 variant (EKR-AAA) that causes an accumulation of the 100 kDa intermediate. (5) A Tom40 variant (ΔGNLD) with assembly indistinguishable from wild type.



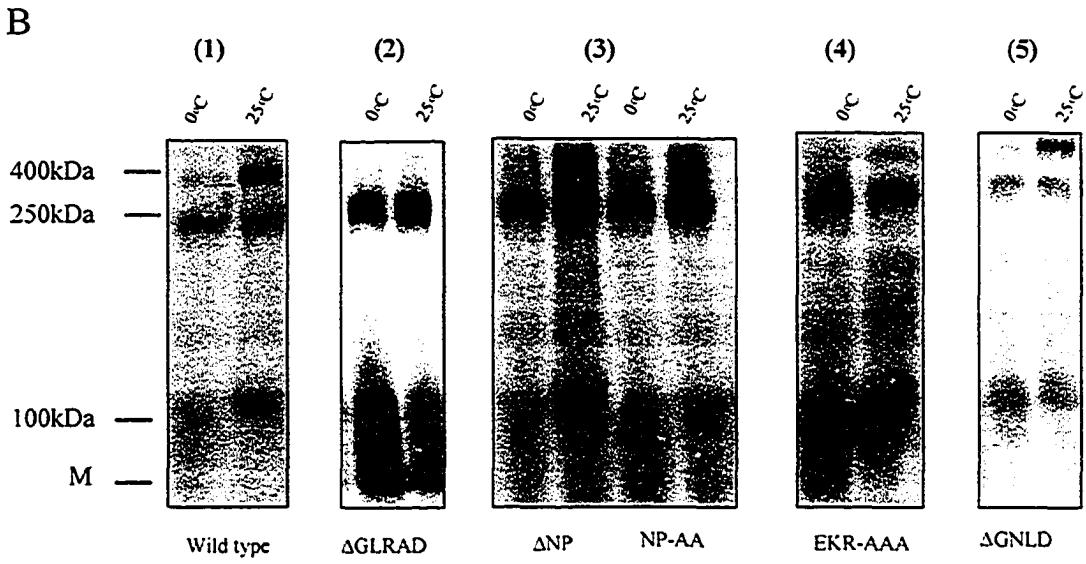


Figure 5. **Alignment of Tom40 protein sequences.** The *Neurospora crassa* (Nc) Tom40 sequence was aligned with Tom40 proteins from *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Mus musculus* (Mm), *Caenorhabditis elegans* (Ce), and *Drosophila melanogaster* (Dm) as shown previously (Taylor *et al* 2003). Residues conserved in at least four of the species are shown in black and similar residues are shown in grey. The residues targeted for site-directed mutagenesis for *in vivo* studies are denoted with # or *. These residues were either deleted and substituted with alanine residues. # indicates regions previously studied in the lab (Rebecca Taylor thesis, 2003) and * indicates regions that are further discussed in the present work. Both # and * were also mutated in a cloned cDNA of Tom40 for *in vitro* assembly assays of Tom40 variants. The line over certain stretches of residues indicates those that have been changed to cysteine for future use in substituted cysteine accessibility method (SCAM) studies.

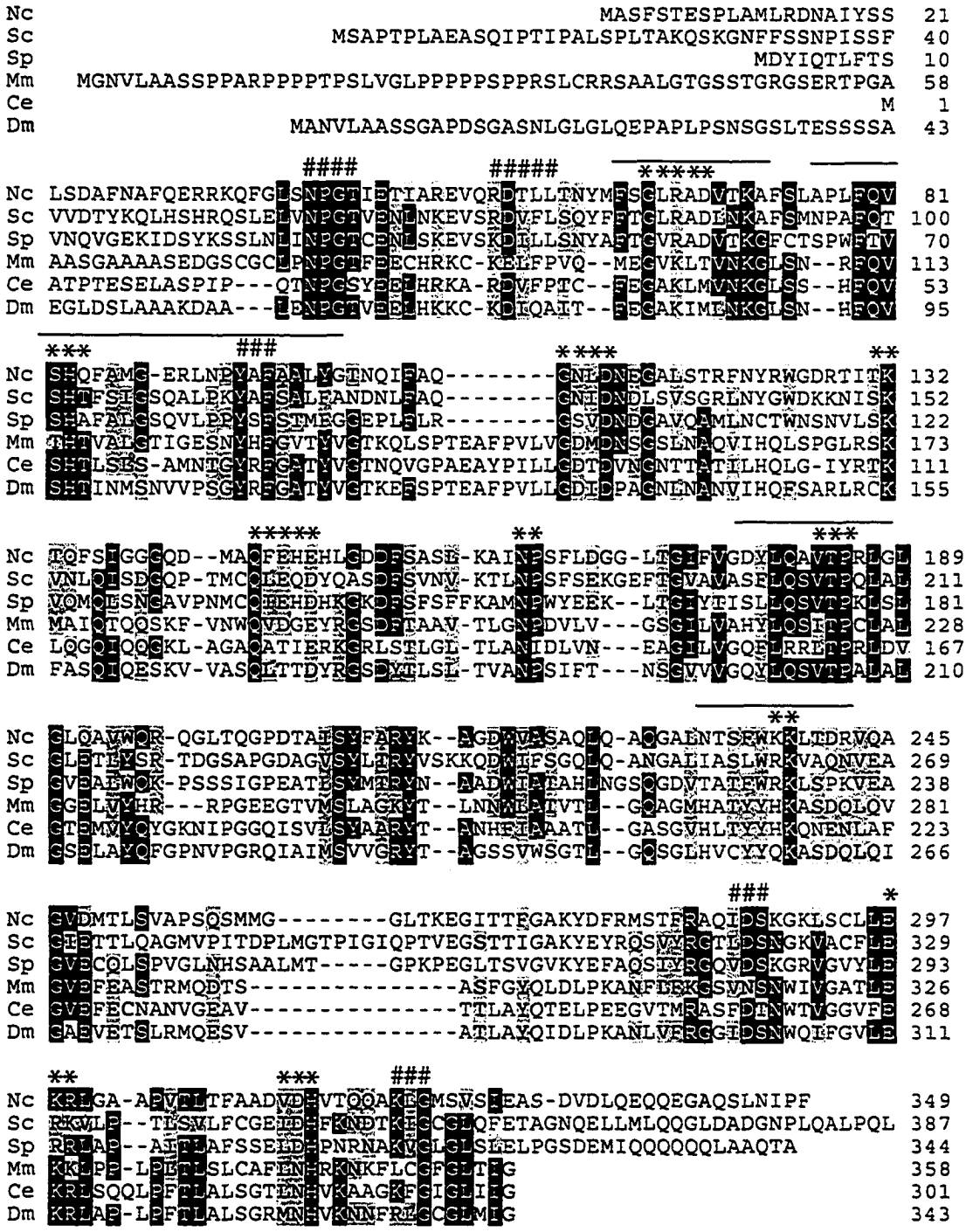


Table 1.

Classification of Tom40 variants by *in vitro* assembly phenotypes.

Computer predicted location of domain	Stops at 250 kDa	Assembles to 400 kDa but accumulates at 250 kDa	Assembles to 400 kDa but accumulates at 100 kDa	Assembly like wild type
IMS	ΔGLRAD GLRAD-AAAAA ΔEKR ΔQFEHE QFEHE-AAAAA ΔVTP VTP-AAA	ΔNPGT NPGT-AAAA ΔRD ΔRDTLL GNLD-AAAA VTPR-VTR	EKR-AAA	ΔGNLD
Membrane	ΔYAF YAF-AAA ΔIDS IDS-AAA ΔKLG	ΔSHQ SHQ-AAA KLG-AAA		
Cytosol	ΔKK	ΔTK TK-AA KK-AA ΔNP NP-AA ΔVDH	VDH-AAA	

The strains characterized can be classified into four different assembly phenotypes. (1) Tom40 stops at the 250 kDa intermediate. (2) Varying amounts of the 400 kDa form but with significant accumulation of the 250 kDa intermediate. (3) Accumulation of the 100 kDa intermediate. (4) Assembly resembles wild type. Strains are also classified according to their computer predicted location (T. Schirmer, via D. Rapaport, personal communication) in the IMS, in a predicted membrane spanning β-strand or in a cytosolic loop (see also Fig. 3 and Appendix 1).

2. MATERIALS AND METHODS

2.1. Growth of *N. crassa*

Growth and handling of *N. crassa* followed standard practices as previously described (Davis and De Serres 1970). The strains used are listed in Table 2. Measurement of growth rate in race tubes was done in 25 ml pipettes as described (Davis and De Serres 1970; White and Woodward 1995). Strains were grown at 30°C or at room temperature and mycelial elongation was recorded every 24 hrs. Liquid growth rates were measured by growing the appropriate number of 250 ml *N. crassa* cultures in 1 L flasks (Vogel's media plus supplements, inoculated with 2 x 10^6 conidia/ml) for the desired number of time points. The mycelium was harvested at the desired time points, dried and weighed. Measurement of growth rates by colony size was done by spotting 10 µl of a suspension containing 2 x 10^7, 2 x 10^6, 2 x 10^5, and 2 x 10^4 conidia/ml onto plates containing standard sorbose media with the appropriate supplements. The plates were incubated at 30°C until growth was seen in the lowest dilution spot of the control strain.

2.2. Crosses involving the small Tom null strains

The mutant *tom5*^{RIP}and *tom6*^{RIP} strains were previously made in the lab by Nancy Go using the process of repeat induced point mutation (RIP) (Selker 1990). The Δ*tom7* strain (Tom7KO-35) was also made by Nancy Go, but in this case the *tom7* gene was replaced with a hygromycin resistance gene via homologous recombination (Colot 2004). The *tom5*^{RIP} strain (Tom5Rip-7) was crossed to the *tom6*^{RIP} strain (Tom6Rip-12) and mitochondria from the strains generated from this cross were screened for the presence of Tom5 and Tom6 protein by western analysis. Two strains lacking both Tom5 and Tom6 were identified. These strains were shown to be sensitive to hygromycin and were crossed to the Δ*tom7* strain. Ascospores from the cross were plated onto media containing hygromycin to select for strains carrying hygromycin resistance as a marker for the deletion of *tom7*. Strains able to grow on hygromycin were then screened for the presence of the Tom5 and Tom6 proteins by western blot analysis. Strains were identified that did not contain either Tom5 or Tom6. The absence of Tom7, which was indicated by the hygromycin resistance, was confirmed by the absence of a PCR product

using primers in the *tom7* gene (see Results section 3.2.1) and by southern blot analysis for the appropriate restriction pattern (Fig. 6).

2.3. Transformation of *N. crassa*

DNA was transformed into *N. crassa* by electroporation of conidia as previously described with slight modifications (Margolin *et al.* 1997; Margolin *et al.* 2000). Conidia that were approximately one week old were harvested in sterile water and washed three times with 50 ml of cold, sterile 1M sorbitol. Conidia were then resuspended to a concentration of 2.0 to 2.5 x 10^9 conidia/ml. 5 μg of linearized plasmid DNA in a volume of 5 μl was mixed with 40 μl of the conidia and set on ice in the electroporation cuvettes for 5 min. Electroporation was performed at 2.1kV, 475 Ω, and 25 microfarads on a Gene Pulser (BioRad, Hercules, CA) with a pulse time constant of 11 to 12 ms. Immediately following the pulse, 1 ml of ice-cold sterile 1M sorbitol was added and the conidia were incubated at 30°C for 45 to 60 min to recover. Aliquots of between 10 and 100 μl of the conidia suspension were added to 50 ml of top agar at 45°C (standard sorbose media with 1M sorbitol (Davis and De Serres 1970)) containing the appropriate antibiotics for selection of the transformants. After gentle but thorough mixing, this was spread onto 5 plates of the same media as the top agar, but lacking the 1M sorbitol, and incubated at 30°C for 3 to 7 days or until transformed colonies were formed. Single transformant colonies were then picked using sterile glass pasteur pipettes and transferred to slants with Vogel's medium containing the appropriate nutritional requirements. The slants also contained the selective antibiotic(s) at 0.5X the normal concentration to allow better conidiation, which is often inhibited by the drugs. The slants were incubated at 30°C until the surface of the agar was covered by the mycelium and then were removed to room temperature to conidiate. Conidia were streaked onto plates identical to those used for the electroporation and incubated at 30°C until colonies formed. These were picked to slants without the antibiotic selection and allowed to grow and conidiate. This was done to ensure that only pure homokaryotic single colonies were chosen. When heterokaryotic strains were transformed directly, the transformed strains were tested for their nutritional requirements to ensure they were the desired homokaryon, following the purification process.

2.4. Creation of strains expressing mutant Tom40 proteins

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 RIP40het, a *tom40*^{RIP} heterokaryotic strain (Fig. 7). Mutant *tom40* alleles that maintained sufficient Tom40 function should rescue the nucleus containing the non-functional *tom40*^{RIP} allele. Selection for transformation into the *tom40*^{RIP}-containing nucleus was achieved by plating on media containing leucine, lysine, and cycloheximide (50 μg/ml), and also bleomycin (1.5 μg/ml) for the presence of the transforming plasmid. Transformation and purification were performed as described in section 2.3. Nutritional testing for the lysine and leucine requirements of the original *tom40*^{RIP}-containing nucleus was done to confirm the rescue of the RIPed nucleus with the mutant *tom40* gene. Conformation that the new mutant allele was present in the rescued homokaryon was obtained by sequencing the PCR amplified ectopic *tom40* gene from isolated genomic DNA (section 2.8). Ectopic *tom40* gene was selectively amplified over the RIP copies of the genes by performing PCR with primers upstream and downstream of the gene. That PCR product was used for subsequent PCR with primers with ends that match the ectopic sequence of the *tom40* gene but that are mismatched at the 3' ends of the RIP alleles of the *tom40* gene (see Table 3, WTPCR Nter and WTPCR Cter primers). Each of the Tom40 variant strains are named for the mutation to the Tom40 protein (eg, ΔGLRAD Tom40 variant strain contains Tom40 protein with the GLRAD residues deleted).

2.5. *In vitro* import of radiolabeled proteins into isolated mitochondria

For *in vitro* import studies, mitochondria were isolated as described (Mayer *et al.* 1993) and import of mitochondrial preproteins was basically 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-polyacrylamide gel electrophoresis and viewed by autoradiography or a phosphorimager system. Quantification of the image

from the latter was done using the Image Quant program (version 5.2, Molecular Dynamics). *In vitro* import of mutant Tom40 proteins was as described (Rapaport *et al.* 2001).

2.6. Pulse import of Tom40 variant precursors

Pulse import studies were modified from Rapaport *et al.* 2001. 50 μg of isolated mitochondria, 2 μl of a lysate containing the desired form of Tom40 labeled with ^{35}S methionine, and 100 μl of the import buffer (0.5% BSA [wt/vol], 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 10 mM MOPS-KOH, pH 7.2) multiplied by the number of time points to be analyzed, were incubated at 25°C for 2 min. Mitochondria were then reisolated by centrifugation at 2°C for 15 min at 15,000 rpm. The mitochondria were then resuspended in 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 isolation buffer (0.25 M sucrose, 10mM MOPS-KOH). Samples were then 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 blue native gel electrophoresis (Sec 2.7).

2.7. Blue native gel electrophoresis (BNGE)

Mitochondria (50μg) were solubilized in 50 μl of buffer containing detergent (either 1% DIG or 1% DDM in 20 mM Tris-Cl, pH 7.4; 0.1mM EDTA; 50mM NaCl; 1% glycerol [vol/vol]; 1mM 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 a 6-13% gradient blue native gel as previously described (Schägger and von Jagow 1991; Schägger *et al.* 1994) except that electrophoresis was performed overnight (about 16 to 20 hrs) at 4°C between 40 and 60 volts before the excess Coomassie Blue was electrophoresed out for 1-1 ½ hr at 500 volts.

2.8. Conidial genomic DNA isolation for PCR

To isolate small quantities of DNA for PCR, a small amount of conidia was added to 100 µl of lysing solution (1 M sorbitol, 20 mM EDTA, 3 mg/ml lysing enzyme (Sigma)) and incubated for 10 min at 37°C. Conidia were washed with 500 µl of washing solution (1 M sorbitol, 20 mM EDTA), pelleted by centrifugation, resuspended in 100 µl of sterile dH_2O, and incubated for 10 min at 100°C. This solution was then subjected to a standard DNA isolation procedure using glass milk (Geneclean II, Q Biogene, Carlsbad, CA). DNA was eluted with 20 µl sterile dH_2O and 10 µl of this was used in a standard PCR reaction.

2.9. 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 Russell 2001).

The following techniques were performed as previously described: separation of mitochondrial proteins by polyacrylamide gel electrophoresis (Laemmli 1970), Western blotting (Good and Crosby 1989), genomic DNA isolation (Wendland *et al.* 1996) and the isolation of mitochondria (Mayer *et al.* 1993).

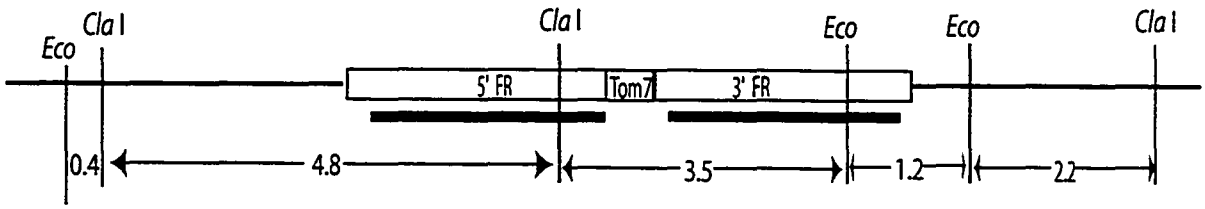
The following procedures were performed as recommended by the supplier: Western blot detection using LumiGLO chemiluminescent substrate (KPL, Mandel), protein determination by the Coomassie dye binding assay (Bio-Rad, Hercules, CA), automated sequencing using a BigDye Terminator Cycle sequencing kit (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 Inc., Santa Clarita, CA) and site-directed mutagenesis based on the Muta-Gene system (Bio-Rad) made from individual reagents (Hahn 2000).

2.10. Oligonucleotides and plasmids

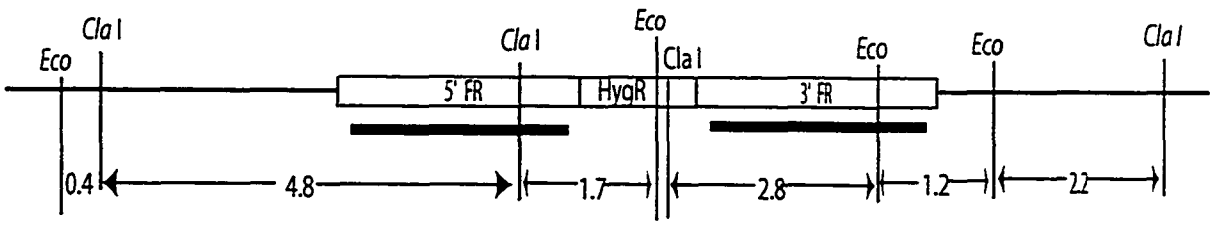
Oligonucleotides and plasmids used in this study are listed in Table 3 and Table 4.

Figure 6. **Schematic of the *tom7* gene and Δ*tom7*.** Schematic drawing of the *tom7* gene with surrounding flanking regions (5'FR and 3'FR). Restriction enzyme sites and the size of the fragments produced for Southern blot analysis are shown. **A.** Wild type *tom7* gene. **B.** Δ*tom7*, where the *tom7* gene has been replaced with the hygromycin resistance gene. Two new restriction sites are present, which change the size of the fragments and make it possible to distinguish between Δ*tom7* and wild type *tom7* by southern blot analysis. The black bar in both panels indicates the region covered by the probe in Southern blots. **C.** Southern blot of *ClaI*/*Eco*RI digested genomic DNAs showing patterns expected for wild type and Δ*tom7* loci (data supplied by N. Go).

A. Wild type *tom7*



B. Tom7 knock-out mutant Δ*tom7*



C. Southern of Tom7 knock-out mutants

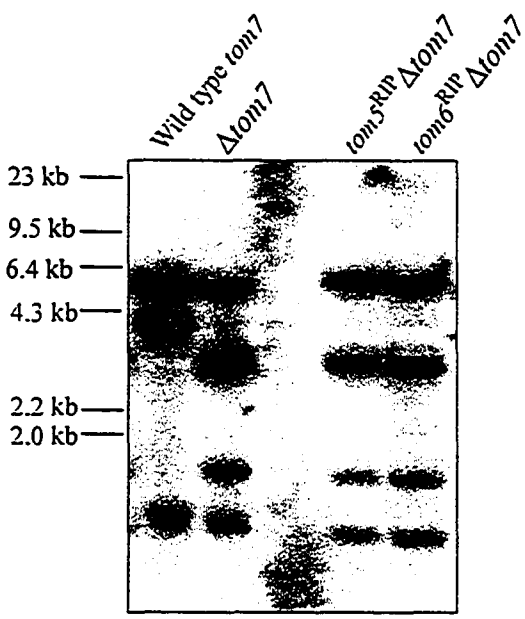
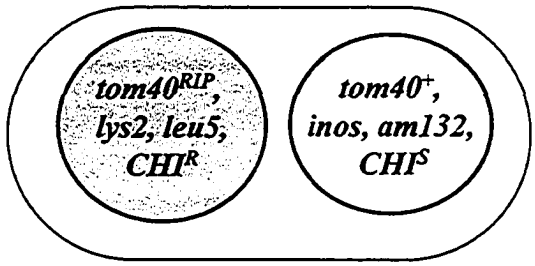


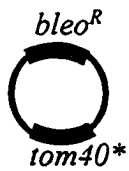
Figure 7. **The *tom40*^{RIP} sheltered heterokaryotic strain.** Strain (RIP40het) contains two nuclei with different genetic markers. One nucleus contains *tom40*^{RIP}, *lys2, leu5* and cycloheximide resistance (CHI^{R}). The other nucleus contains wild type *tom40, inos, am132,* and is cycloheximide sensitive (CHI^{S}). This strain was transformed with plasmids containing altered versions of *tom40* (*tom40**) and the bleomycin resistance gene (bleo^{R}). Growth on media containing lysine, leucine, cycloheximide and bleomycin, allowed selection for the nucleus containing the inserted altered version of *tom40* in the *tom40*^{RIP} nucleus.

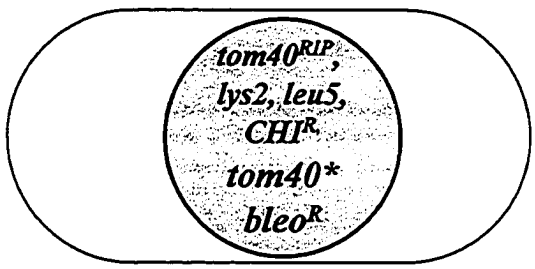


***tom40* sheltered heterokaryon strain (RIP40het)**

Select on lysine, leucine, bleomycin, and cycloheximide

Transform





Transformed Tom40 variant strains

Table 2.

Strains used in this study.

STOCK STRAINS Strain	Genotype	Origin or Source
H V	*a cyh-2 lys-2 leu-5 mei-2*	Fungal Genetics Stock Center #7255
7626	*his*$^-$	R. L. Metzenberg
H I	*nic-2 cyh-1: inl inv mei-2*	Fungal Genetics Stock Center #7251
M I	*ad-3A; am*$_{132}$ *inl inv mei-2*	Fungal Genetics Stock Center #7261
NCN251 (also called 74A)	*A*	Fungal Genetics Stock Center #2489 (74-OR23-1VA)
Dupl40	As HV, but carries an ectopic copy of *tom40*. *hyg*^{r}	Transformation of Host V with the *tom40* plasmid pRIP-4
RIP40het (F40-6)	Sheltered heterokaryon: (*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} + *am132 inl inv mei-2*), mating type unknown. Both nuclei also contain an ectopic RIPed copy of *tom40*.	Cross of MateV (Fungal Genetics Stock Center #7265) x 40Dupl

SMALL TOM MUTANT STRAINS Strain	Genotype	Origin or Source
tom5^{RIP} (strain used Tom5Rip-7)	*nic-2 inl inv tom5*^{RIP}	Sheltered RIP using H I and M I
tom6^{RIP} (strain used Tom6Rip-12)	his$^-$ *tom6*^{RIP}	RIP using 7626 and NCN251
Δ*tom7* (strain used Tom7KO-35)	*Δtom7, hyg*^{r}	Tom7 replacement 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}*Δtom7, hyg*^{r}	Cross between Δ *tom7* and *tom5*^{RIP} *tom6*^{RIP}
tom6^{RIP} Δ *tom7* (strain used LV10-11 and LV10-2)	*nic-2 tom6*^{RIP}*Δtom7, hyg*^{r}	Cross between Δ *tom7* and *tom5*^{RIP} *tom6*^{RIP}

Table 2 continued

Tom40 VARIANT STRAINS		
Strain	**Genotype**	**Origin or Source**
ΔGLRAD (strain used 19-9)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with a deletion of amino acids 64-68.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
GLRAD-AAAAA (strain used 20-2)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues at positions 64-68 changed to alanine residues.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
SHQ-AAA (strain used 22-9)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues at positions 82-84 changed to alanine residues.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
ΔGNLD (strain used 46-4)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with a deletion of amino acids 109-112.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
GNLD-AAAA (strain used 47-4)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues at positions 109-112 changed to alanine. residues.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
ΔTK (strain used 48-8)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with a deletion of amino acids 131-132.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
TK–AA (strain used 49-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues at positions 131-132 changed to alanine.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
ΔQFEHE (strain used 50-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with a deletion of amino acids 145-149.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
QFEHE-AAAAA (strain used 51-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues at positions 145-149 changed to alanine.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
ΔNP (strain used 72-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with a deletion of amino acids 163-164.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
NP–AA (strain used 74-2)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues at positions 163-164 changed to alanine.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)

Table 2 continued

Strain	Genotype	Origin or Source
VTP-AAA (strain used 53-8)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues at positions 183-185 changed to alanine residues.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
VTPR-VTR (strain used 54-9)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residue 185 deleted.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
KK-AA (strain used 56-4)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues 237-238 changed to alanine	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
EKR-AAA (strain used 18-12)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with residues 297-299 changed to alanine residues	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
ΔVDH (strain used 73-8)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with a deletion of amino acids 313-315.	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)
VDH-AAA (strain used 75-3)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} with an ectopic copy of *tom40* with deletion of residues 313-315 changed to alanine	Transformation of RIP40het with specific *tom40* plasmid (see Table 4)

Tom40 SCAM[1] STRAINS

Strain	Genotype	Origin or Source
F62C (strain studied 62-2)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 62 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
S63C (strain studied 63-3)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 63 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
G64C (strain studied 64-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 64 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
L65C (strain studied 65-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 65 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)

[1] SCAM, substituted cysteine accessibility mapping

Table 2 continued

Strain	Genotype	Origin or Source
R66C (strain studied 66-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 66 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
A67C (strain studied 67-2)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 67 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
D68C (Strain studied 68-3)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 68 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
V69C (strain studied 69-1)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and 69 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
T70C (strain studied 70-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 70 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
K71C (strain studied 71-3)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 71 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
A72C (strain studied 72-2)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 72changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
A76C (strain studied 76-10)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 76changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
D77C (strain studied 77-3)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 77 changed to Ala and residue 77 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
F79C (strain studied 79-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 79changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
Q80C (strain studied 80-8)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 80 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)

Table 2 continued

Strain	Genotype	Origin or Source
V81C (strain studied 81-9)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 81 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
S82C (strain studied 82-1)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 82 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
H83C (strain studied 83-7)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 83 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
Q84C (strain studied 84-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 84 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
F85C (strain studied 85-1)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 85 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
A86C (strain studied 86-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 86 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
M87C (strain studied 87-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 87 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
E89C (strain studied 89-1)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 88 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
R90C (strain studied 90-3)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 90 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
L91C (strain studied 91-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 91 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
N92C (strain studied 92-4)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 92 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)

Table 2 continued

Strain	Genotype	Origin or Source
P93C (strain studied 93-11)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 93 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
Y94C (strain studied 94-3)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 94 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
A95C (strain studied 95-4)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 95 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
F96C (strain studied 96-4)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 96 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
A97C (strain studied 97-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 97 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
A98C (strain studied 98-2)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 98 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
L99C (strain studied 99-1)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 99 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 3)
Y100C (strain studied 100-10)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 100 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
G101C (strain studied 101-2)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 101 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
D178C (strain studied 178-3)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 178 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
Y179C (strain studied 179-7)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 179 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)

Table 2 continued

Strain	Genotype	Origin or Source
L180C (strain studied 180-4)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 180 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
Q181C (strain studied 181-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 181 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
A182C (strain studied 182-4)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 182 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
V183C (strain studied 183-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 183 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
T184C (strain studied 184-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 184 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
P185C (strain studied 185-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 185 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
R186C (strain studied 186-8)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 186 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
L187C (strain studied 187-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 187 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
G188C (strain studied 188-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 188 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
L189C (strain studied 189-5)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 189 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
N232C (strain studied 232-11)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 232 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)

Table 2 continued

Strain	Genotype	Origin or Source
T233C (strain studied 233-9)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 233 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
S234C (strain studied 234-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 234 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)
F235C (strain studied 235-6)	*cyh-2 lys-2 leu-5 mei-2 tom40*^{RIP} and an ectopic copy of *tom40* with residue 294 changed to Ala and residue 235 changed to Cys	Transformation of RIP40het with specific cysteine plasmid (see Table 4)

Table 3.

Primers used in this study.

Tom40 sequencing and PCR primers

PRIMER	SEQUENCE (5' - 3')	COMMENTS
FNA 113	CGGTATCTTTGTCGGCGACTAC	Tom40 sequencing primer
FNA 114	TGCCTTTGCTGCTCTCTACG	Tom40 sequencing primer.
FNA 181	GGCTTTCAGCCTCGCCCCTC	Tom40 sequencing primer.
FNA 183	TTAAAAAGGGGATGTTGAGGG	Tom40 sequencing primer
FNA 253	GGCCTCCGCGCCGACGTCAC	Tom40 sequencing primer
RTA78	CGAGCGTTCTGAACAAATCCAG	Sequencing primer - pQE40 - DHFR
RTA85	TTTAAAAGGGGATGTTGAGGGAC	See Wt-TOM40 PCR Cter
RTA86	GAAAAGGTATGACCCACACCCTTTC	Tom40 sequencing and PCR primer
RTA87	CACCATCCGCTGCGTCCTCGATTCCC	Tom40 sequencing and PCR primer
RTA31	GGTGACTGGGTTGCTAGCGCTCAGC	Tom40 sequencing primer
RTA43	GTGCGCGGAAACAACAGAGCG	Tom40 sequencing primer. Same as FNA169.
WTPCR Nter	CCGATGCCTTCAACGCCTTTC	Used to eliminate ectopic RIP in PCR from potential transformants.
WTPCR Cter	TTTAAAAGGGGATGTTGAGGGAC	Used to eliminate endogenous RIP in PCR from potential transformants.
Tom40a	CCAAGTACGACTTCAGAATGTCC	Tom40 sequencing primer

Tom40 variant primers

PRIMER	SEQUENCE (5' - 3')	COMMENTS
RTA10	GAGGCCGAGTCTGGCAGTGACGGC	Mutagenic primer for VTPR- VTAR for genomic construct.
RTA17	GACGGGGGCGGCACCAAGGAGCAAGCA GCTGAGCTTC	Mutagenic primer for ΔEKR for genomic construct.
RTA18	GACGGGGGCGGCACCAAGGGCCGCCGCG AGCAAGCAGCTGAGCTTG	Mutagenic primer for ERK - AAA for genomic construct.

Table 3 continued

PRIMER	SEQUENCE (5' - 3')	COMMENTS
RTA19	GAGGCTGAAAGCCTTGGTGACAGAGAAC ATGTAGTTGGTGAGG	Mutagenic primer for ΔGLRAD for genomic construct.
RTA20	GAGGCTGAAAGCCTTGGTGACGGCTGCG GCTGCGGCAGAGAACATGTAGTTGGTGA GG	Mutagenic primer for GLRAD - AAAAA for genomic construct.
RTA21	CAACTCGCCCATGGCAAAAACTTGGAAG CTTGGGTTATAG	Mutagenic primer for ΔSHQ for genomic construct.
RTA22	CAACCTCTCGCCCATGGCAAACGCGGCG GCAACTTGGAAGCTTGGGTTATAG	Mutagenic primer for SHQ -AAA for genomic construct.
RTA46	GTCGAGAGAGCGCCCTCGTTCTGAGCGA AGATCTGTTGAC	Mutagenic primer for ΔGNLD for genomic construct.
RTA47	GTCGAGAGAGCGCCCTCGTTGGCCGCGG CCGCCTGAGCGAAGATCTGTTGAC	Mutagenic primer for GNLD - AAAA for genomic construct.
RTA48	CCACCAATCGAGAACTGCGTGATGGTCC TGTCACCCCATC	Mutagenic primer for ΔTK for genomic construct.
RTA49	CCACCAATCGAGAACTGCGCGGCGATGG TCCTGTCACCCCATC	Mutagenic primer for TK - AA for genomic construct.
RTA50	GAGGCACTGAAGTCGTCGCCAAGGTGGG CCATATCCTGGCCGCCACCAATCG	Mutagenic primer for ΔQHFEHE for genomic construct.
RTA51	GAGGCACTGAAGTCGTCGCCAAGGTGCG CGGCCGCGGCCGCGGCCATATCCTGGCC GCCACCAATCG	Mutagenic primer for QHEFE - AAAAA for genomic construct.
RTA52	CTGGAGACCGAGGCCGAGTCTGGCCTGG AGGTAGTCGCCGAC	Mutagenic primer for ΔVTP for genomic construct.
RTA53	CTGGAGACCGAGGCCGAGTCTGGCCGCG GCCTGGAGGTAGTCGCCGA	Mutagenic primer for VTP-AAA for genomic construct.
RTA54	CTGGAGACCGAGGCCGAGTCTAGTGACG GCCTGGAGGTAGTC	Mutagenic primer for VTPR - VTR for genomic construct.
RTA55	GCCTGCACCCTATCCGTCAGCCAGAAGG AAGTGTTGAGAG	Mutagenic primer for ΔKK for genomic construct.
RTA56	GCCTGCACCCTATCCGTCAGCGCCGCCCA GAAGGAAGTGTTGAGAG	Mutagenic primer for KK-AA for genomic construct.
RTA72	GACCGCCGTCAAGGAAAGAGATGGCCTT GAGGGAGGCAC	Mutagenic primer for ΔNP for genomic construct.
RTA73	GAGCTTGGCTTGTTGAGTGACATCAGCA GCGAAGGTCAGAG	Mutagenic primer for ΔVDH for genomic construct.
RTA74	GACCGCCGTCAAGGAAAGAGGCGGCGAT GGCCTTGAGGGAGGCAC	Mutagenic primer for NP-AA for genomic construct.
RTA75	GAGCTTGGCTTGTTGAGTGACCGCGGCC GCATCAGCAGCGAAGGTCAGAG	Mutagenic primer for VDH-AAA for genomic construct.

Table 3 continued

SCAM[1] Primers

PRIMER	SEQUENCE (5' - 3')	COMMENTS[2]
Y60C	GAGGCCAGAGAACATACAGTTGGTGAGGAGG G	Mutagenic primer for cysteine mutant Y60C for genomic construct.
M61C	GCGGAGGCCAGAGAAACAGTAGTTGGTGAGG AG	Mutagenic primer for cysteine mutant M61C for genomic construct.
F62C	GGCGCGGAGGCCAGAACACATGTAGTTGGTT GAG	Mutagenic primer for cysteine mutant F62C for genomic construct.
S63C	GTCGGCGCGGAGGCCACAGAACATGTAGTTG GT	Mutagenic primer for cysteine mutant S63C for genomic construct.
G64C	GACGTCGGCGCGGAGACAAGAGAACATTGAG TT	Mutagenic primer for cysteine mutant G64C for genomic construct.
L65C	GGTGACGTCGGCGCGACAGCCAGAGAACATG TA	Mutagenic primer for cysteine mutant L65C for genomic construct.
R66C	CTTGGTGACGTCGGCACAGAGGCCAGAGAAC AT	Mutagenic primer for cysteine mutant R66C for genomic construct.
A67C	AGCCTTGGTGACGTCACAGCGGAGGCCAGAG AA	Mutagenic primer for cysteine mutant A67C for genomic construct.
D68C	GAAAGCCTTGGTGACACAGGCGCGGAGGCCA GA	Mutagenic primer for cysteine mutant D68C for genomic construct.
V69C	GCTGAAAGCCTTGGGTACAGTCGGCGCGGAG GCC	Mutagenic primer for cysteine mutant V69C for genomic construct.
T70C	GAGGCTGAAAGCCTTACAGACGTCGGCGCGG AG	Mutagenic primer for cysteine mutant T70C for genomic construct.
K71C	GGCGAGGCTGAAAGCACAGGTGACGTCGGCG CG	Mutagenic primer for cysteine mutant K71C for genomic construct.
A72C	GGGCGAGGCTGAAACACTTGGTGACGTCGGC G	Mutagenic primer for cysteine mutant A72C for genomic construct.
A76C	GAGTAAAGCAACGTACAGAGGACAGAGGCTG AAAGCCTTGGTGAC	Mutagenic primer for cysteine mutant A76C for genomic construct.
P77C	CAATTAGAATAAAGCAACGTACAGACAGGCG AGGCTGAAAGCCTTGG	Mutagenic primer for cysteine mutant P77C for genomic construct.
F79C	GCAAACTGGTGGGAAACTTGACAGCTTGGGT TATAGGTCAGCG	Mutagenic primer for cysteine mutant F79C for genomic construct.
Q80C	CATGGCAAACTGGTGGGAAACACAGAAGCTT GGTTGATAGGTCAG	Mutagenic primer for cysteine mutant Q80C for genomic construct.
V81C	CCCATGGCAAACTGGTGGGAACATTGGAAGC TTGGGTTATAGG	Mutagenic primer for cysteine mutant V81C for genomic construct.
S82C	CTCGCCCATGGCAAACTGGTGACAAACT TGGAAGCTTGGGTTATAG	Mutagenic primer for cysteine mutant S82C for genomic construct.

[1] SCAM, substituted cysteine accessibility mapping
[2] All primers in this section do not contain a 5' phosphate

Table 3 continued

PRIMER	SEQUENCE (5' - 3')	COMMENTS
H83C	CCTCTCGCCCATGGCAAATCGACAGGAA ACCTGGAAGCTTGGG	Mutagenic primer for cysteine mutant H83C for genomic construct.
Q84C	CAACCTCTCGCCCATGGCAAAACAGTGG GAAACTTGGAAGCTTG	Mutagenic primer for cysteine mutant Q84C for genomic construct.
F85C	GTTCAACCTCTCGCCCATTGCACACTTGT GGGAAACTTGGAAGC	Mutagenic primer for cysteine mutant F85C for genomic construct.
A86C	GGGTTCAACCTCTCGCCCATACAAAACTG GTGGGAAACTTGGAAG	Mutagenic primer for cysteine mutant A86C for genomic construct.
M87C	CATAAAAGTTCAACCTCTCGCCACAGGC AAACTGGTGGGAAACTTG	Mutagenic primer for cysteine mutant M87C for genomic construct.
G88C	GCATAAGGGTTCAACCTCTCACCACATG GCAAACTGGTGGGAAAC	Mutagenic primer for cysteine mutant G88C for genomic construct.
E89C	CAAAGGCATAAGGGTTCAACCTACAGCC CATGGCAAACTGGTGGG	Mutagenic primer for cysteine mutant E89C for genomic construct.
R90C	GCAAAGGCATAAGGGTTCAAACACTCGC CCATGGCAAACTGGTG	Mutagenic primer for cysteine mutant R90C for genomic construct.
L91C	GCAGCAAAGGCATAAGGGTTACACCTCT CGCCCATGGCAAACTG	Mutagenic primer for cysteine mutant L91C for genomic construct.
N92C	GAGAGCAGCAAAGGCATAGGACACAACC TCTCGCCCATGGCAAAC	Mutagenic primer for cysteine mutant N92C for genomic construct.
P93C	GTAGAGAGCAGCAAAGGCATAAACAGTT CAACCTCTCGCCCATGG	Mutagenic primer for cysteine mutant P93C for genomic construct.
Y94C	CCGTAGAGAGCAGCAAAGGCACAAGGGT TCAACCTCTCGCCCATG	Mutagenic primer for cysteine mutant Y94C for genomic construct.
A95C	GTTCCGTAGAGAGCAGCAAACAATAAGG GTTCAACCTCTCGC	Mutagenic primer for cysteine mutant A95C for genomic construct.
F96C	GTTGGTTCCGTAGAGAGCAGCCACAGGC ATAAGGGTTCAACCTCTC	Mutagenic primer for cysteine mutant F96C for genomic construct.
A97C	CTGGTTGGTTCCGTAGAGAGCACAAAAG GCATAAGGGTTCAACC	Mutagenic primer for cysteine mutant A97C for genomic construct.
A98C	CCTGGTTGGTTTTCCGTAGAGACAAGCAA AGGCATAAGGGTTCAAC	Mutagenic primer for cysteine mutant A98C for genomic construct.
L99C	CATACCTGGTTGGTTCCGTAACAAGCAGC AAAGGCATAAGGGTTC	Mutagenic primer for cysteine mutant L99C for genomic construct.
Y100C	GCGCATACCTGGTTGGTTCCACAGAGAG CAGCAAAGGACTAAG	Mutagenic primer for cysteine mutant Y100C for genomic construct.
G101C	CAAGCGCATACCTGGTTGGTACAGTAGA GAGCAGCAAAGGCATAAG	Mutagenic primer for cysteine mutant G101C for genomic construct.
D178C	GGGAGTGACGGCCTGGAGGTAACAGCCG ACAAAGATACCGGTGAG	Mutagenic primer for cysteine mutant D178C for genomic construct.
Y179D	TCTGGGAGTGACGGCCTGGAGACAGTCG CCGACAAAGATACCGGTG	Mutagenic primer for cysteine mutant Y179D for genomic construct.

Table 3 continued

PRIMER	SEQUENCE (5' - 3')	COMMENTS
L180C	GGAGTCTGGGAGTGACCGGCCTGACAGT AGTCGCCGACAAAGATACCG	Mutagenic primer for cysteine mutant L180C for genomic construct.
Q181C	GCCGAGTCTGGGAAGTGACGGCACAGGA GGTAGTCGCCGACAAGAAT	Mutagenic primer for cysteine mutant Q181C for genomic construct.
A182C	GAGGCCGAGTCTGGGAGTGACACACTGG AGGTAGTCGCCGCACAAAG	Mutagenic primer for cysteine mutant A182C for genomic construct.
V183C	ACCGAGGCCGAGTCTGGGAGTACAGGCC TGGAGGTAGTCGCCGAC	Mutagenic primer for cysteine mutant V183C for genomic construct.
T184C	GAGACCGAGGCCGAGTCTGGGACAGACG GCCTGGAGGTAGTCGCC	Mutagenic primer for cysteine mutant T184C for genomic construct.
P185C	CTGGAGACCGAGGCCGAGTCTACAAGTG ACGGCCTAAAGGTAGTC	Mutagenic primer for cysteine mutant P185C for genomic construct.
R186C	GGCCTGGAGACCGAGGCCGAGACAGGGA GTGACGGCCTGGACCTAG	Mutagenic primer for cysteine mutant R186C for genomic construct.
L187C	GACGGCCTGGAGACCGAGGCCACATCTG GGAGTGACGGCCTGGAG	Mutagenic primer for cysteine mutant L187C for genomic construct.
G188C	CCAGACGGCCTGGAGACCGAGACAGAGT CTGGGAGTGACGGCCTG	Mutagenic primer for cysteine mutant G188C for genomic construct.
L189C	TTGCCAGACGGCCTGGAGACCACAGCCG AGTCTGGGAGTGACGGC	Mutagenic primer for cysteine mutant L189C for genomic construct.
G190C	ACGTTGCCAGACGGCCTGGAGACAGAGG CCGAGTCTGGGAGTGAC	Mutagenic primer for cysteine mutant G190C for genomic construct.
N232C	CAGCTTCTTCCAGAAGGAAGTACAGAGA GCACCCTGAGCCTGGAG	Mutagenic primer for cysteine mutant N232C for genomic construct.
T233C	CGTCAGCTTCTTCCAGAAGGAACACTTGA GAGCACCCTGAGCCTG	Mutagenic primer for cysteine mutant T233C for genomic construct.
S234C	ATCCGTCAGCTTCTTCCAGAAACAAGTGT TGAGAGCACCCTGAGC	Mutagenic primer for cysteine mutant S234C for genomic construct.
F235C	CCTATCCGTCAGCTTCTTCCAACAGGAAG TGTTGAGAGCACCCTG	Mutagenic primer for cysteine mutant F235C for genomic construct.

Table 4.

Plasmids used in this study.

Plasmid name	Made from	Comments
Tom6 rescue	Nancy's Tom6 Rip Hyg Plasmid	Contains Bleo resistance. Tom6 cloned using NotI from Tom6 Rip Hyg Plasmid into pBR322
Tom7 rescue	Nancy's Tom7 Rip Hyg plasmid	Contains Bleo resistance. Tom7 cloned using NotI from Tom7 Rip Hyg plasmid into pBS522
pB3	Tom40 genomic construct	R. Taylor subclone of Tom40. Used for site directed mutagenesis. Contains Bleo520.
pC8	pB3	As plasmid pB3 with residue 293 (cysteine) changed to alanine. Used for SCAM site directed mutagenesis.

Tom40 variant plasmids used during this study.

Plasmid name	Made from	Comments
p17-5	pB3	Tom40 variant plasmid containing residues EKR deleted
p18-3	pB3	Tom40 variant plasmid containing residues EKR changed to alanine
p19-8	pB3	Tom40 variant plasmid containing residues GLRAD deleted
p20-19	pB3	Tom40 variant plasmid containing residues GLRAD changed to alanine
p21-3	pB3	Tom40 variant plasmid containing residues SHQ deleted
p22-10	pB3	Tom40 variant plasmid containing residues SHQ changed to alanine
p46-7	pB3	Tom40 variant plasmid containing residues GNLD deleted
p47-5	pB3	Tom40 variant plasmid containing residues GNLD changed to alanine
p48-7	pB3	Tom40 variant plasmid containing residues TK deleted
p49-1	pB3	Tom40 variant plasmid containing residues TK changed to alanine
p50-3	pB3	Tom40 variant plasmid containing residues QFEHE deleted
p51-6	pB3	Tom40 variant plasmid containing residues QFEHE changed to alanine
p52-6	pB3	Tom40 variant plasmid containing residues VTP deleted
p53-1	pB3	Tom40 variant plasmid containing residues VTP changed to alanine
p54-3	pB3	Tom40 variant plasmid containing residue P of VTP deleted
p55-2	pB3	Tom40 variant plasmid containing residues KK deleted
p56-1	pB3	Tom40 variant plasmid containing residues KK changed to alanine
p72-2	pB3	Tom40 variant plasmid containing residues NP deleted
p74-1	pB3	Tom40 variant plasmid containing residues NP changed to alanine
p73-3	pB3	Tom40 variant plasmid containing residues VDH deleted
p75-6	pB3	Tom40 variant plasmid containing residues VDH changed to alanine

SCAM plasmids used during this study.

Plasmid name	Made from	Comments
p62-4	pC8	SCAM plasmid containing Tom40 residue F62 changed to cysteine by site-directed mutagenesis
p63-3	pC8	SCAM plasmid containing Tom40 residue S63 changed to cysteine by site-directed mutagenesis

Table 4 continued

Plasmid name	Made from	Comments
p64	pC8	SCAM plasmid containing Tom40 residue G64 changed to cysteine by site-directed mutagenesis
p65	pC8	SCAM plasmid containing Tom40 residue L65 changed to cysteine by site-directed mutagenesis
p66	pC8	SCAM plasmid containing Tom40 residue R66 changed to cysteine by site-directed mutagenesis
p67	pC8	SCAM plasmid containing Tom40 residue A67 changed to cysteine by site-directed mutagenesis
p68	pC8	SCAM plasmid containing Tom40 residue D68 changed to cysteine by site-directed mutagenesis
p69-3	pC8	SCAM plasmid containing Tom40 residue V69 changed to cysteine by site-directed mutagenesis
p70-7	pC8	SCAM plasmid containing Tom40 residue T70 changed to cysteine by site-directed mutagenesis
p71-1	pC8	SCAM plasmid containing Tom40 residue K71 changed to cysteine by site-directed mutagenesis
p72-2	pC8	SCAM plasmid containing Tom40 residue A72 changed to cysteine by site-directed mutagenesis
p76-2	pC8	SCAM plasmid containing Tom40 residue A76 changed to cysteine by site-directed mutagenesis
p77-1	pC8	SCAM plasmid containing Tom40 residue P77 changed to cysteine by site-directed mutagenesis
p79-1	pC8	SCAM plasmid containing Tom40 residue F79 changed to cysteine by site-directed mutagenesis
p80-3	pC8	SCAM plasmid containing Tom40 residue Q80 changed to cysteine by site-directed mutagenesis
p81-1	pC8	SCAM plasmid containing Tom40 residue V81 changed to cysteine by site-directed mutagenesis
p82-1	pC8	SCAM plasmid containing Tom40 residue S82 changed to cysteine by site-directed mutagenesis
p83-1	pC8	SCAM plasmid containing Tom40 residue H83 changed to cysteine by site-directed mutagenesis
p84-1	pC8	SCAM plasmid containing Tom40 residue Q84 changed to cysteine by site-directed mutagenesis
p85-3	pC8	SCAM plasmid containing Tom40 residue F85 changed to cysteine by site-directed mutagenesis
p86-1	pC8	SCAM plasmid containing Tom40 residue A86 changed to cysteine by site-directed mutagenesis
p87-33	pC8	SCAM plasmid containing Tom40 residue M87 changed to cysteine by site-directed mutagenesis
p88-2	pC8	SCAM plasmid containing Tom40 residue G88 changed to cysteine by site-directed mutagenesis
p89-3	pC8	SCAM plasmid containing Tom40 residue E89 changed to cysteine by site-directed mutagenesis
p90-3	pC8	SCAM plasmid containing Tom40 residue R90 changed to cysteine by site-directed mutagenesis
p91-2	pC8	SCAM plasmid containing Tom40 residue L91 changed to cysteine by site-directed mutagenesis
p92-14	pC8	SCAM plasmid containing Tom40 residue N92 changed to cysteine by site-directed mutagenesis

Table 4 coninued

Plasmid name	Made from	Comments
p93-3	pC8	SCAM plasmid containing Tom40 P93 changed to cysteine by site-directed mutagenesis
p94-35	pC8	SCAM plasmid containing Tom40 residue Y94 changed to cysteine by site-directed mutagenesis
p95-1	pC8	SCAM plasmid containing Tom40 residue A95 changed to cysteine by site-directed mutagenesis
p96-3	pC8	SCAM plasmid containing Tom40 F96 changed to cysteine by site-directed mutagenesis
p97-20	pC8	SCAM plasmid containing Tom40 A97 changed to cysteine by site-directed mutagenesis
p98-20	pC8	SCAM plasmid containing Tom40 residue A98 changed to cysteine by site-directed mutagenesis
p99-20	pC8	SCAM plasmid containing Tom40 residue L99 changed to cysteine by site-directed mutagenesis
p100-20	pC8	SCAM plasmid containing Tom40 Y100 changed to cysteine by site-directed mutagenesis
p101-21	pC8	SCAM plasmid containing Tom40 residue G101 changed to cysteine by site-directed mutagenesis
p178-1	pC8	SCAM plasmid containing Tom40 residue D178 changed to cysteine by site-directed mutagenesis
p179-5	pC8	SCAM plasmid containing Tom40 residue Y179 changed to cysteine by site-directed mutagenesis
p180-1	pC8	SCAM plasmid containing Tom40 residue L180 changed to cysteine by site-directed mutagenesis
p181-3	pC8	SCAM plasmid containing Tom40 residue Q181 changed to cysteine by site-directed mutagenesis
p182-1	pC8	SCAM plasmid containing Tom40 residue A182 changed to cysteine by site-directed mutagenesis
p183-1	pC8	SCAM plasmid containing Tom40 residue V183 changed to cysteine by site-directed mutagenesis
p184-8	pC8	SCAM plasmid containing Tom40 residue T184 changed to cysteine by site-directed mutagenesis
p185-1	pC8	SCAM plasmid containing Tom40 residue P185 changed to cysteine by site-directed mutagenesis
p186-3	pC8	SCAM plasmid containing Tom40 residue R186 changed to cysteine by site-directed mutagenesis
p187-1	pC8	SCAM plasmid containing Tom40 residue L187 changed to cysteine by site-directed mutagenesis
p188-4	pC8	SCAM plasmid containing Tom40 residue G188 changed to cysteine by site-directed mutagenesis
p189-1	pC8	SCAM plasmid containing Tom40 residue L189 changed to cysteine by site-directed mutagenesis
p190-2	pC8	SCAM plasmid containing Tom40 residue G190 changed to cysteine by site-directed mutagenesis
p232-1	pC8	SCAM plasmid containing Tom40 residue N232 changed to cysteine by site-directed mutagenesis
p233-3	pC8	SCAM plasmid containing Tom40 residue T233 changed to cysteine by site-directed mutagenesis
p234-1	pC8	SCAM plasmid containing Tom40 residue S234 changed to cysteine by site-directed mutagenesis
p235-2	pC8	SCAM plasmid containing Tom40 residue F235 changed to cysteine by site-directed mutagenesis

3. RESULTS

3.1 ANALYSIS OF THE TOM40 PROTEIN OF THE TOM COMPLEX

3.1.1 Further characterization of *tom40* variants with the *in vitro* 100 kDa assembly intermediate phenotype

Although the main function of Tom40 as the pore forming component of the TOM complex is known, the specific regions in the protein that are important for its targeting, assembly into the TOM complex, and function as a pore are poorly understood. To identify regions that might play a role in these functions, an alignment of Tom40 protein sequences from several species was made prior to my arrival in the lab (Taylor *et al.* 2003) (Fig. 5). Sequences that showed high conservation were targeted for site-directed mutagenesis in both cloned genomic and cDNA versions of *tom40*. As a summer student, I assisted in the creation of many of the mutants. The resulting mutant versions of Tom40 cDNA were then radiolabeled and imported into isolated wild type mitochondria by Rebecca Taylor and me. Assembly of the mutant forms into the TOM complex was monitored using BNGE. We found five classes of assembly phenotypes as discussed in section 1.4.4.8 (Fig. 4). Rebecca and I were individually involved in characterizing the different classes of mutants.

Two mutants made up the class which accumulated Tom40 at the 100 kDa intermediate (EKR-AAA and VDH-AAA, Fig. 8a, Table 1). Models of Tom40 assembly predict that Tom40 has integrated into the membrane at the 100 kDa stage and is in a complex with a pre-existing molecule of Tom40 along with Tom5 and Tom6 (Model *et al.* 2001; Wiedemann 2003). I chose to examine these two mutants further because they may be unable to undergo further assembly with additional Tom subunits. To see if there was very slow assembly into the 400 kDa core complex, a pulse version of the assembly assay was done. Labeled Tom40 variants were added to isolated 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 hrs at 25°C (Fig. 8b). No further assembly of the VDH-AAA mutant occurred after 1 hr and both the 250 kDa and 100 kDa intermediates were present after 6 hrs. The EKR-AAA mutant did assemble further

after longer times, with a steady increase in the amount of the 400 kDa complex and a slight decrease of the 100 kDa intermediate. Thus, it appears that both of these Tom40 variants have reduced ability to progress along the Tom40 assembly pathway. This appears to be due to a defect in the conversion of the 100 kDa intermediate to the 400 kDa fully assembled form. These Tom40 variants were further examined with respect to their effect on TOM complex stability (section 3.1.3.2).

3.1.2 Creation of *tom40* mutant strains

I continued my investigation of Tom40 assembly and function by *in vivo* examination of the effect of other mutations in the conserved regions of the protein. Rebecca Taylor and I constructed plasmids with the same mutations as those used in the *in vitro* assembly assay (Fig. 5), but in a genomic version of *tom40*. These were used to transform the RIP40het strain (Fig. 7). Since Tom40 is essential, this strain is a heterokaryon containing a wild type copy of *tom40* in one nucleus with inositol-leucine-requirements while the other nucleus contained a null allele of *tom40* as well as leucine-lysine-requirements and cycloheximide resistance. Transformations that gave rise to homokaryotic strains that were lysine-leucine requiring and cycloheximide resistant, contained viable mutations in the *tom40* gene and could rescue the lethal phenotype of the Tom40 deficiency (Fig. 7). Table 2 lists the strains that I created and investigated further. For each strain the presence of the introduced mutant gene was confirmed by conidial PCR with primers selecting for the ectopic copy of *tom40* over the $tom40^{RIP}$ alleles (section 2.4 and 2.8) and sequencing of the resulting product. It is of interest to note that four of the 21 *tom40* variants that were transformed into RIP40het were unable to rescue the $tom40^{RIP}$ nucleus. All four of these were deletions (ΔEKR, ΔSHQ, ΔVTP and ΔKK).

3.1.3 Characterization of *tom40* variant strains

Each of the viable Tom40 mutant strains was examined with respect to growth rate, the stability of the TOM complex containing the mutant Tom40, and the ability of mitochondria from these strains to import mitochondrial precursor proteins.

3.1.3.1 Growth phenotypes

Of the 17 strains tested in race tubes or liquid medium, only 4 showed a growth defect (Table 5). Strains containing the SHQ-AAA and ΔVDH Tom40 variants grew slightly slower on solid media in race tubes but did not show slower growth in liquid media (Fig. 9). On the other hand, the ΔGLRAD strain grew more slowly than the control in liquid media but a growth defect was not observed in race tubes (Fig. 10). The strain containing the ΔQFEHE version of Tom40 was the only one to show a substantial reduction in growth in both liquid culture and race tubes (Fig. 9, Fig. 10). Once my study had progressed to the analysis of mitochondrial protein import, six strains were found to be defective in importing at least some precursors (section 3.1.3.3). These strains were re-tested for growth defects by spotting different concentrations of conidia onto sorbose-containing[1] plates and comparing the amount of growth to the control strain. This showed that the mutants expressing the ΔGLRAD and the ΔQFEHE Tom40 variants had obvious growth defects, while the other strains had only minor growth defects (Fig. 11). Although many strains generated in this study did not show growth defects, all of the mutant strains had a reduced ability to climb the walls of the conidia flasks compared to the control strain.

3.1.3.2 TOM complex stability

The stability of the TOM complex containing the mutant Tom40 proteins was examined by BNGE following solubilization of isolated mitochondria with either 1% DIG, a mild detergent, which leaves the wild type complex intact (Künkele *et al.* 1998; Künkele *et al.* 1998) or with a stronger detergent, 1% DDM, which leaves only the wild type core complex intact (Dekker *et al.* 1998; Ahting *et al.* 1999; Meisinger *et al.* 2001). After samples were subjected to BNGE, they were transferred to PVDF membrane, and decorated with antibodies against Tom40.

Three classes of stability were found: the first had a slight destabilization of the TOM complex in DDM, the second had severe destabilization in DDM and the third had

[1] The presence of L-sorbose in growth medium causes *Neurospora* to form colonies (Davis and De Serres 1970).

severe destabilization in both DIG and DDM. Each of the mutants is shown in Fig. 12 and the results from each strain are summarized in Table 5.

The EKR-AAA and VDH-AAA containing TOM complexes showed a severely destabilized complex in the presence of DDM (Fig. 12, Table 6). This observation supports the notion that interactions of these variants with additional Tom components may be sub optimal. This could lead to accumulation of the 100 kDa intermediate during assembly.

3.1.3.3 Ability to import mitochondrial precursor proteins

The effect of each of the mutations on the function of the TOM complex was studied using *in vitro* import assays. Isolated mitochondria from the mutant strains were incubated with labeled preproteins containing S^{35}-methionine that are targeted to three of the four mitochondrial subcompartments. By treating the mitochondria with proteinase K following import, the amount of the protein that was imported into a protease protected location in the mitochondria could be determined. Import to the inner membrane was examined using the precursor of AAC, a protein of the carrier family with an internal targeting sequence, which is inserted into the inner membrane via the TIM22 complex. $F_1\beta$, a preprotein with an N-terminal targeting presequence, was used to test the import of matrix targeted preproteins via the TIM23 complex. The porin precursor, a β-barrel protein, was used to test import to the outer membrane. In some cases when import defects were seen with these mitochondrial preproteins, import was examined further using other preproteins as import substrates. Each import experiment was repeated at least two times and a representative result is shown in the figures.

Of the 17 strains tested, only six were shown to have import defects: ΔQFEHE, ΔTK, ΔGLRAD, GLRAD-AAAAA, ΔNP and NP-AA (Table 5). ΔQFEHE (Fig. 13) and ΔTK (Fig. 14) have general import defects for preproteins destined to each of the subcompartments tested. Interestingly, the other strains have more specific defects, with only preproteins destined to certain subcompartments having decreased import compared to controls.

Mitochondria from the strain containing the ΔGLRAD Tom40 variant were not deficient in the import of AAC and PiC, another inner membrane protein. However,

import of preproteins with N-terminal targeting signals to the matrix was strongly affected, as shown by the decreased import of $F_1\beta$, and Su9-DHFR, a fusion of the matrix targeting signal of subunit 9 of the ATPase to dihydrofolate reductase (Fig. 15). The import of the MOM β-barrel protein porin was also reduced in the ΔGLRAD strain. Therefore, other β-barrel proteins of the MOM were tested. Interestingly, like porin, Mdm10 and Sam50 showed decreased import, but Tom40 import was virtually unaffected (Fig. 15). This is in contrast to mitochondria from GLRAD-AAAAA, the partner strain of ΔGLRAD, where the residues were changed to alanine instead of being deleted. Mitochondria containing this Tom40 variant showed no severe import defects for preproteins destined to the inner membrane or the matrix, but were still defective in their ability to import β-barrel proteins to the outer membrane, including a decreased rate of Tom40 import (Fig. 16).

Mitochondria from the Tom40 ΔNP strain have a specific defect, where the preproteins destined to the inner membrane or the matrix are imported in comparable amounts to control, but β-barrel proteins targeted to the outer membrane have significantly reduced import (Fig. 17). Strangely, the strain NP-AA, in which the NP residues in Tom40 are changed to alanine rather than being deleted, has a more severe phenotype. Import of both β-barrel and inner membrane proteins import is reduced in this strain (Fig. 18).

3.2 ANALYSIS OF THE SMALL TOM PROTEINS OF THE TOM COMPLEX

3.2.1 Creation of Strains lacking the small Tom proteins

As discussed in the introduction (section 1.4.4.5 to 1.4.4.8), the GIP of the TOM complex contains three small Tom proteins: Tom5, Tom6 and Tom7, which are not essential in yeast. However, deletion of any of the small Tom proteins in yeast results in mitochondria with decreased rates of mitochondrial preprotein import (Alconada *et al.* 1995; Hönlinger *et al.* 1996; Dietmeier *et al.* 1997). Tom5, is thought to have a function as a receptor in yeast, either acting directly as a receptor for the small Tim preproteins, or as a step between the TOM complex receptors and the GIP for other preproteins. (Dietmeier *et al.* 1997; Kurz *et al.* 1999). Tom6 and Tom7 are thought to have opposing

roles in the stability of the TOM complex, with Tom6 stabilizing the interaction between the Tom proteins and Tom7 destabilizing the interactions (Alconada *et al.* 1995; Hönlinger *et al.* 1996). Tom6 is also thought to modulate the interactions between Tom proteins in the complex during preprotein translocation (Rapaport *et al.* 1998; Dembowski *et al.* 2001). Tom7 might also have a role in protein sorting, as the import of porin is decreased more than the import of other classes of preproteins in Tom7 deletion mutants (Hönlinger *et al.* 1996). As part of my project, I wished to characterize mutant strains in *N. crassa* lacking the small Tom proteins. In addition, I wanted to construct and characterize strains with all possible combinations of the mutant small *tom* genes.

In *N. crassa*, a process termed Repeated Induced Point mutation (RIP) occurs if there is a duplication of a DNA sequence of a certain minimum length (~ 2.0 kb) present in the genome of one nucleus during a sexual cross (Selker 1990). RIP results in GC to AT transitions within both copies of the duplicated sequence. Thus, RIP is a useful tool for targeted mutagenesis. Strains containing RIPed alleles of the *tom5* and *tom6* genes were created in our lab by Nancy Go. She also created a Δ*tom7* strain using an homologous recombination technique (section 2.2). I used the $tom5^{RIP}$ (Tom5Rip-7) and $tom6^{RIP}$ (Tom6Rip-12) strains in a cross to produce $tom5^{RIP}tom6^{RIP}$ double mutants. The lack of both proteins in these strains was confirmed by Western blot analysis and is shown for one of these strains (Tom(5/6)Rip-9) (Fig. 19). These strains (Tom(5/6)Rip-9 or Tom(5/6)Rip-10) were then crossed to a Δ*tom7* strain (tom7KO-35) to produce $tom5^{RIP}$Δ*tom7* and $tom6^{RIP}$Δ*tom7* double mutant strains. The ascospores were plated on hygromycin containing medium since this served as a marker for the Δ*tom7* gene and the $tom5^{RIP}tom6^{RIP}$ strains were both hygromycin sensitive. A total of 412 spores were picked from this cross and the resulting strains either started growing immediately after being transferred to slants or started growing a few days after being transferred (Table 6). The strains that started growing later had slower growth rates. The $tom5^{RIP}$Δ*tom7* double mutant strains were found in the group which started growing immediately and had a normal growth rate. On the other hand, the $tom6^{RIP}$Δ*tom7* double mutant strains were found in the group that started growing later and had a slow growth rate. The presence or absence of Tom5 and Tom6 proteins was confirmed by Western blot analysis. Due to the fact that we have no antibody to *N. crassa* Tom7, the absence of the Δ*tom7* allele was

confirmed by PCR (Fig. 19) and by Southern analysis (Fig. 6, Nancy Go, personal communication). The combination of *tom6*^{RIP}, *tom5*^{RIP} and Δ*tom7* was not found after screening 19 potential strains which had the slowest growth rates (Table 6) supporting the notion that loss of all the small Tom proteins is lethal.

3.2.2 Characterization of strains without the small Tom proteins

All the strains missing different combinations of small Tom proteins, including the single mutations and each of the double mutations were analyzed to determine their growth rates, levels of other mitochondrial proteins, the stability of the TOM complex, and the ability of mitochondria isolated from the strains to import mitochondrial preproteins.

3.2.2.1 Growth phenotypes

The growth of these strains were tested by spotting varying amounts of conidia onto sorbose containing plates with appropriate supplements (Fig. 20). The *tom5*^{RIP}, *tom6*^{RIP}, Δ*tom7*, *tom5*^{RIP}*tom6*^{RIP} and *tom5*^{RIP}Δ*tom7* strains showed no growth defects compared to the parental strains. The *tom6*^{RIP}Δ*tom7* strain showed a strong growth defect. In addition, the *tom6*^{RIP}Δ*tom7* strain had severely reduced conidiation (Fig. 20).

3.2.2.2 Levels of other mitochondrial proteins

The levels of mitochondrial proteins were examined in each of the mutant strains by western blot analysis. The levels of the Tom components are unaffected by the absence of each small Tom protein individually. Similarly, the lack of both Tom5 and Tom6 or Tom5 and Tom7 had no effect on other Tom components (Fig. 19). However, the loss of Tom6 and Tom7 reduced the levels of Tom5, Tom20 and Tom22 (Fig. 19). The levels of other mitochondrial proteins, Tim23 of the inner membrane, CCHL of the IMS and Hsp60 of the matrix were not affected by the absence of any of the small Tom proteins (Fig. 19).

3.2.2.3 TOM complex stability

The stability of the TOM complex in the different small Tom mutant strains was examined by solubilizing mitochondria in either DIG or DDM and analyzing the complex by BNGE as described for the Tom40 mutant strains in section 3.1.3.2. The absence of Tom5 had no effect on the stability of the Tom complex (Fig. 21). The absence of Tom6 caused some loss of Tom40 from the complex in 1% DIG but caused the complex to completely dissociate in 1% DDM. This extended to any of the strains without the Tom6 protein. The absence of Tom7 caused slight loss of Tom40 from the complex in both 1% DIG and 1% DDM. The combined loss of Tom6 and Tom7 resulted in severe destabilization of the TOM complex even in the presence of DIG (Fig. 21). In the mitochondria solubilized in DIG, the dissociated Tom40 in the double mutants lacking Tom6 migrate at a lower molecular weight from those in the single *tom6* or *tom7* mutants. This may indicate that Tom40 is completely dissociated from other subunits in the double mutants but retains association with certain subunits in the single mutants. Similarly, in DDM, any strain carrying a *tom6* mutation gives rise to a more fully dissociated Tom40.

3.2.2.4 Ability to import mitochondrial precursor proteins

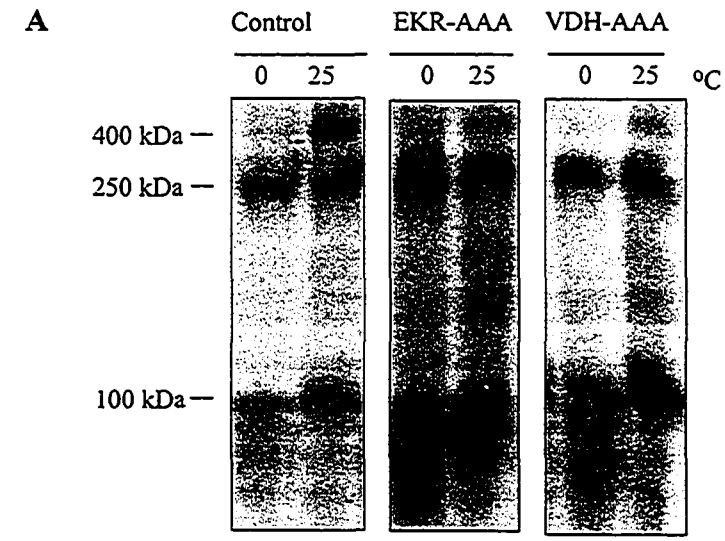
The ability of the small Tom mutant strains to import mitochondrial proteins was tested in the same way as for the Tom40 mutant strains (section 3.1.3.3), by *in vitro* import of radiolabeled preproteins into isolated mitochondria from each of the strains. The ability of the *tom5*^{RIP} strain to import mitochondrial preproteins into the matrix, to the outer membrane or to the inner membrane was not distinguishable from the control strain (Fig.22). In yeast, Tom5 was found to have a role in importing the small Tim proteins to the IMS (Kurz *et al.* 1999). Therefore, mitochondria from the *tom5*^{RIP} strain were also examined for their ability to import Tim10 into the IMS. Again, no difference from the control was observed. The strain lacking Tom6 had a slight defect in importing $F_1\beta$ into the matrix but had comparable import to the control for AAC and porin, to the inner and the outer membranes, respectively (Fig.23). Mitochondria without Tom5 or Tom6 had a defect in importing matrix proteins as well, as shown by the reduced import of $F_1\beta$, but import of AAC and PiC to the inner membrane, and porin to the outer

membrane was not affected (Fig. 24). The strains lacking Tom7 or Tom7 and Tom5 had a defect in importing porin to the outer membrane and $F_1\beta$ to the matrix, but import to the inner membrane was unaffected (Fig. 25 and Fig. 26). I was unable to obtain enough conidia from the *tom6*^{RIP}Δ*tom7* strain to grow cultures for analysis of mitochondrial protein import (see Fig. 20b).

3.2.2.5 Effects of the small Tom proteins on Tom40 assembly into the TOM complex

Mitochondria from each of the small Tom mutant strains were tested for effects on Tom40 assembly. Again, the *tom6*^{RIP}Δ*tom7*strain was not evaluated because of the severe growth phenotype. Each strain was able to fully assemble Tom40 into the 400 kDa complex at 25°C (Fig. 27). However, it appears that the Δ*tom7* and *tom5*^{RIP} mitochondria can assemble Tom40 more efficiently than the control, as 400 kDa complex could be seen after 20 min at 0°C, which is not seen in the control mitochondria. In mitochondria lacking both Tom5 and Tom7, no 250 kDa intermediate could be seen at 0°C or 25°C and the amount of fully assembled Tom40 following import at 0°C was more pronounced than in either single mutant. The lack of the 250 kDa intermediate in the double mutant suggests an altered assembly pathway for Tom40.

Figure 8. **Import of Tom40 variants into mitochondrial TOM complexes *in vitro*.** Radiolabeled Tom40 variant preproteins with the EKR-AAA or VDH-AAA changes were imported into isolated mitochondria and compared to the assembly of wild type Tom40. Mitochondria were isolated from the import reactions, lysed with 1% DIG and electrophoresed on blue native gels. **A.** Import was done for 20 min at 0°C and 25°C. **B.** Mitochondria were incubated with radiolabeled Tom40 protein for 2 min, washed by re-isolation, and suspended in import mix without Tom40 protein. Mitochondria were then incubated at 25°C and aliquots were taken immediately after the pulse (0 hrs) and at 1, 2, 4, and 6 hrs.



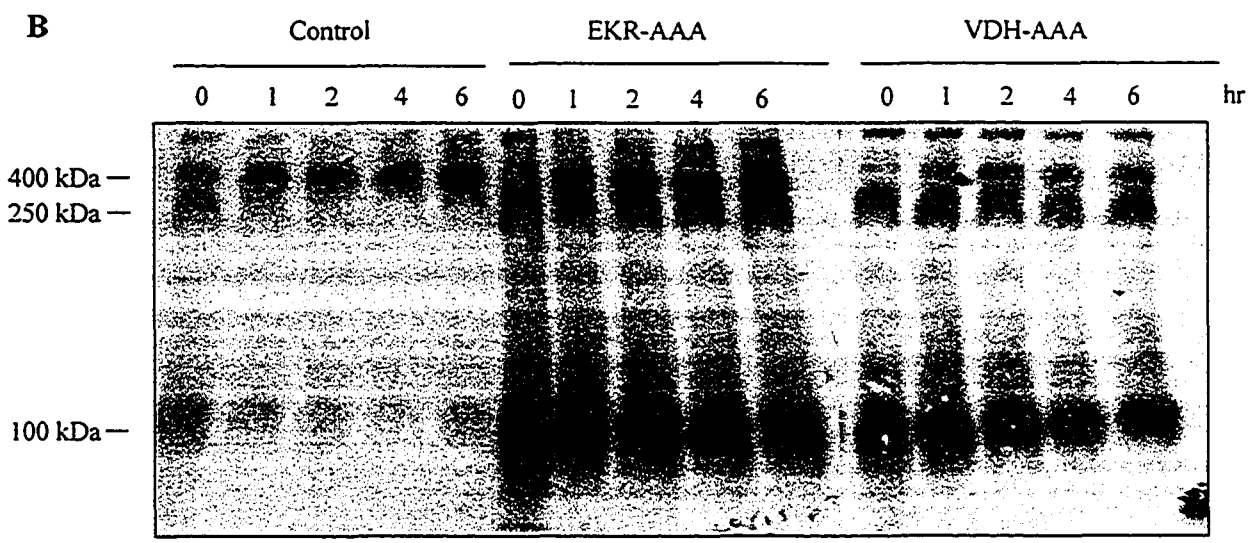


Figure 9. **Growth of strains containing Tom40 variants in race tubes.** Growth was tested at 22°C (room temperature) and 30°C on solid media in 25 ml tubes. Extension of mycelial growth down the tube was measured every 24 hr. Several isolates of each strain with slower growth than the control strain are shown. A. SHQ-AAA. B. ΔVDH. C. ΔQFEHE.

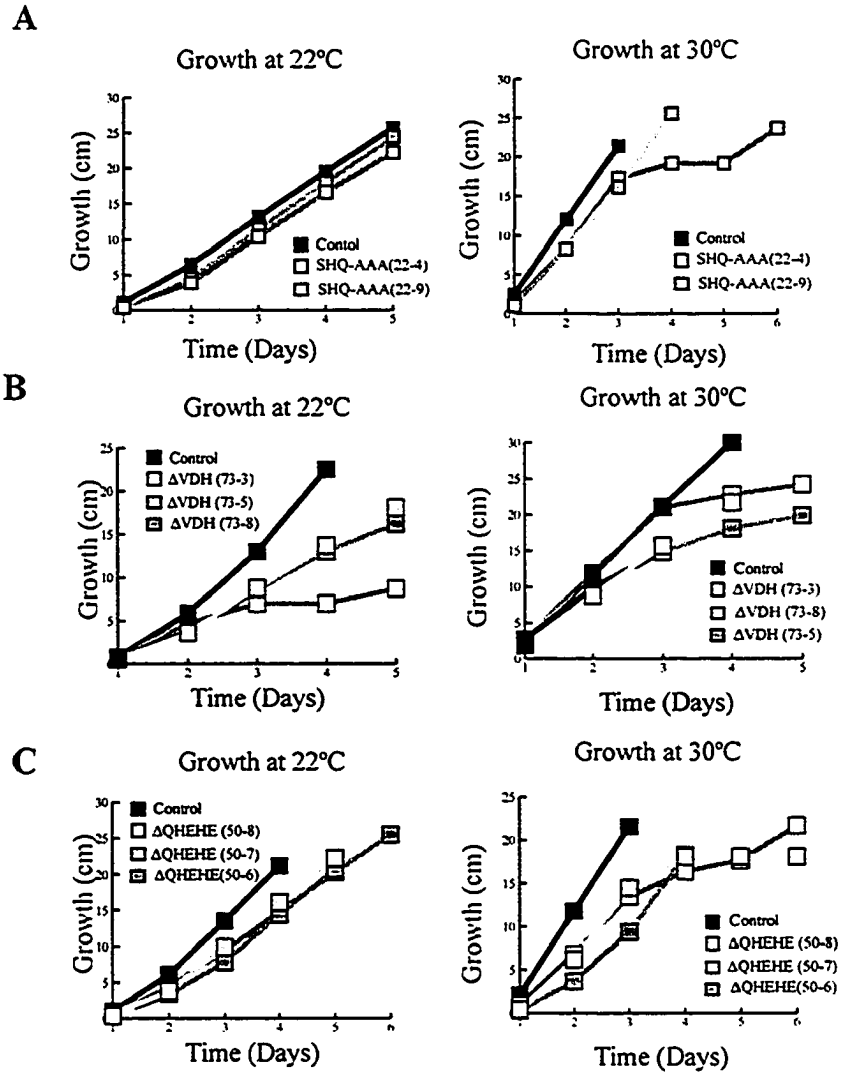


Figure 10. **Growth of strains containing Tom40 variants in liquid media.** For each 12 hr time point strains were grown in 1 L flasks containing 250 ml liquid cultures at 30°C, harvested at the times indicated, dried, and weighed. Only the strains that had slower growth, ΔQFEHE (A) and ΔGLRAD (B) are shown.

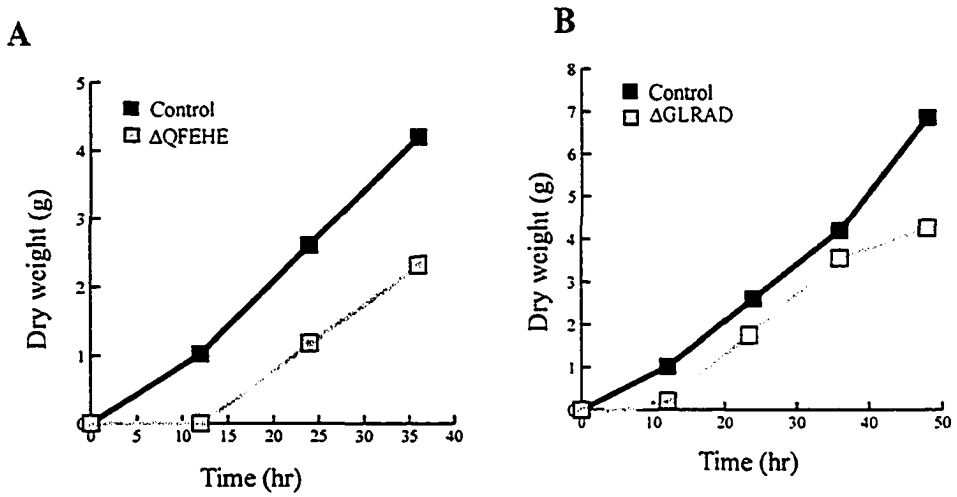


Figure 11. **Growth rates of import defective Tom40 variant strains on sorbose-containing medium.** Conidia from each strain were diluted to 2 x 10^7, 2 x 10^6, 2 x 10^5 and 2 x 10^4 conidia/ml and 10µl of each dilution was spotted onto a plate containing Vogel's sorbose medium with appropriate supplements. Plates were incubated at 30°C for approximately 2 days until the control strain showed growth for all the dilutions.

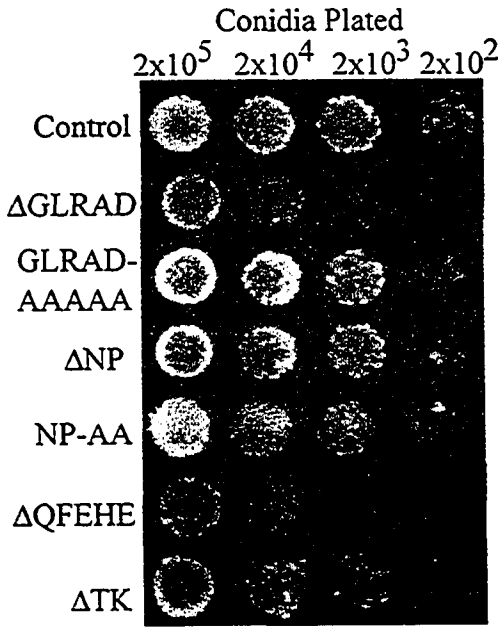


Figure 12. **Stability of the TOM complex containing mutant Tom40 proteins.** Isolated mitochondria containing the Tom40 variants were dissolved in 1% DIG or 1% DDM, subjected to BNGE and blotted to PVDF. Membranes were decorated with αTom40 antiserum. Each Tom40 variant is shown, according to its class of stability. **A.** Wild type Tom40. **B.** Variants with slight destabilization seen in DDM: ΔTK, GNLD-AAAA, VTP-AAA, QFEHEH-AAAAA, TK-AA, VTPR-VTP, ΔNP and NP-AA. **C.** Variants with severe destabilization in DDM: ΔQFEHE, GLRAD-AAAAA, EKR-AAA, ΔGNLD, KK-AA, ΔVDH and VDH-AAA. **D.** Variants that show severe destabilization in both DIG and DDM: ΔGLRAD and SHQ-AAA.

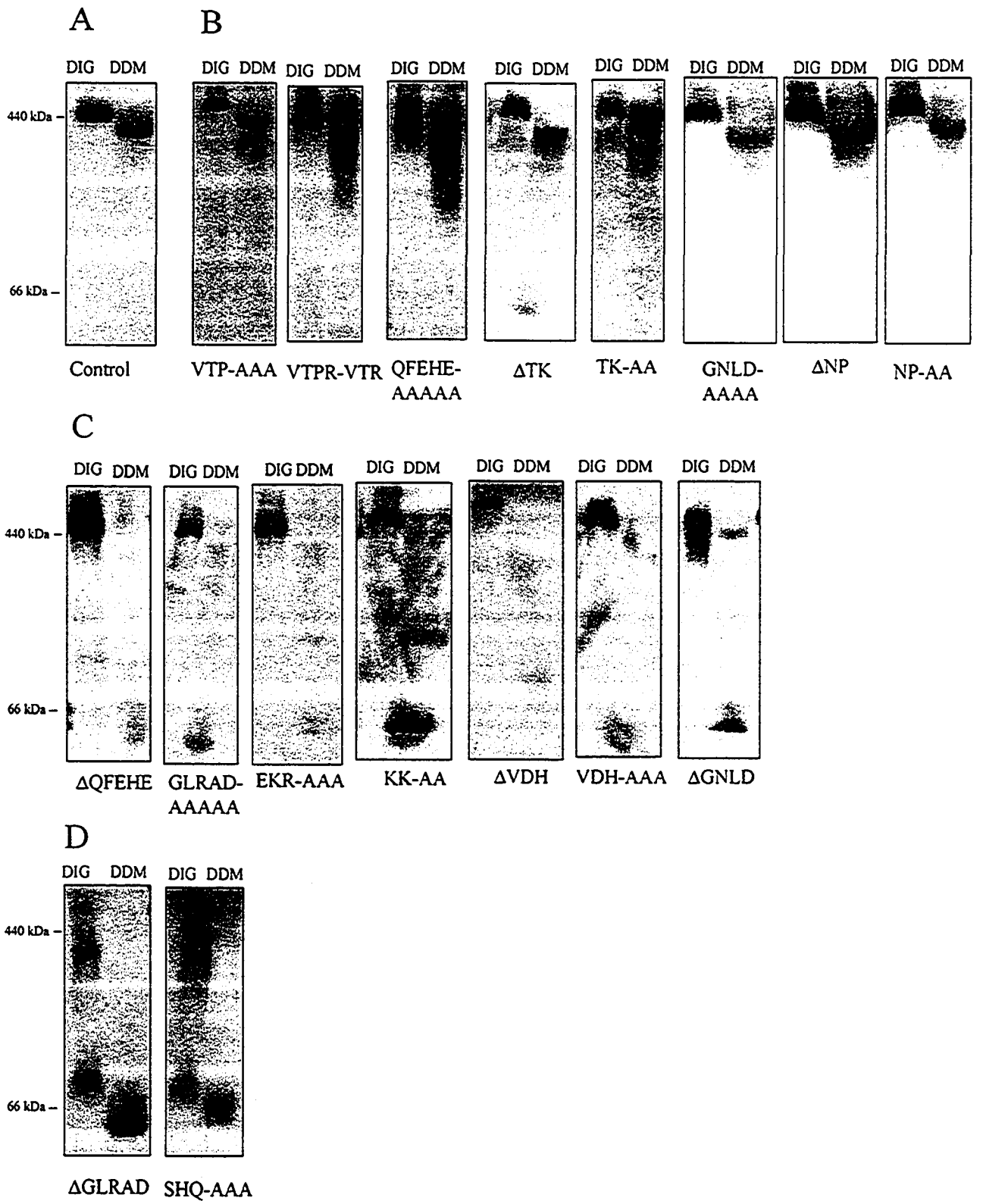


Figure 13. ***In vitro*** **import of preproteins into mitochondria containing mutant Tom40 ΔQFEHE.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains containing wild type Tom40 or ΔQFEHE Tom40 for 1, 3 and 5 min at 25°C (AAC and $F_1\beta$) or at 15°C (porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A.** Import of $F_1\beta$ to the matrix. "p" and "m" indicate the precursor and mature forms of the protein. **B.** Import of AAC to the inner membrane. **C.** Import of porin to the outer membrane.

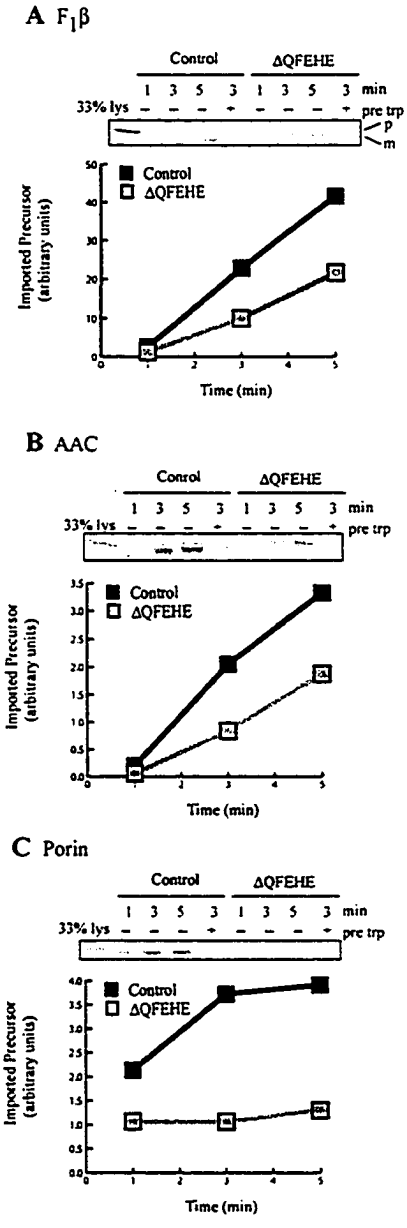


Figure 14. ***In vitro*** **import of preproteins into mitochondria containing mutant Tom40 ΔTK.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains containing wild type Tom40 or ΔTK Tom40 for 1, 3 and 5 min at 25°C (AAC and $F_1\beta$) or at 15°C (porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A.** Import of $F_1\beta$ to the matrix. "p" and "m" indicate the precursor and mature forms of the protein. **B.** Import of AAC to the inner membrane. **C.** Import of porin to the outer membrane.

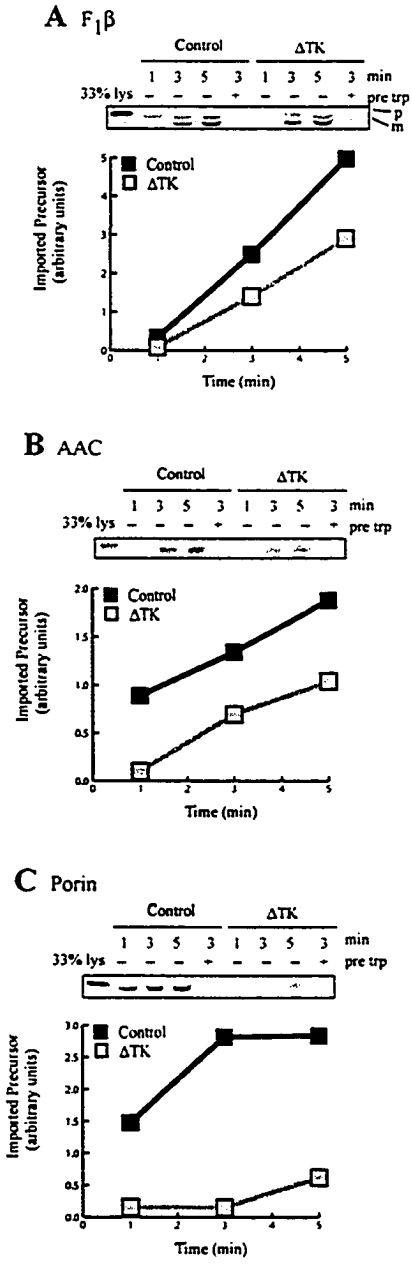


Figure 15. ***In vitro*** **import of preproteins into mitochondria containing mutant Tom40 ΔGLRAD.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains containing wild type Tom40 or ΔGLRAD Tom40 for 1, 3 and 5 min at 25°C (AAC, Sam50, Mdm10, Su9DHFR, PiC and $F_1\beta$) or at 15°C (Tom40 and porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A.** Import of $F_1\beta$ and Su9DHFR to the matrix. "p" and "m" indicate the precursor and mature forms of the proteins. **B.** Import of AAC and PiC to the inner membrane. **C.** Import of porin, Sam50, Tom40 and Mdm10 to the outer membrane. When Tom40 is properly assembled in the membrane it is cut once by the proteinase K treatment and forms a 26 kDa and 12 kDa fragment. The 12 kDa fragment is not shown on the gel insets, the 40 kDa and 26 kDa proteins are indicated. The 26 kDa fragment was used for quantification. The Sam50 protein is also cleaved to produce a 45 kDa fragment as indicated. The 45 kDa fragment was used for quantification.

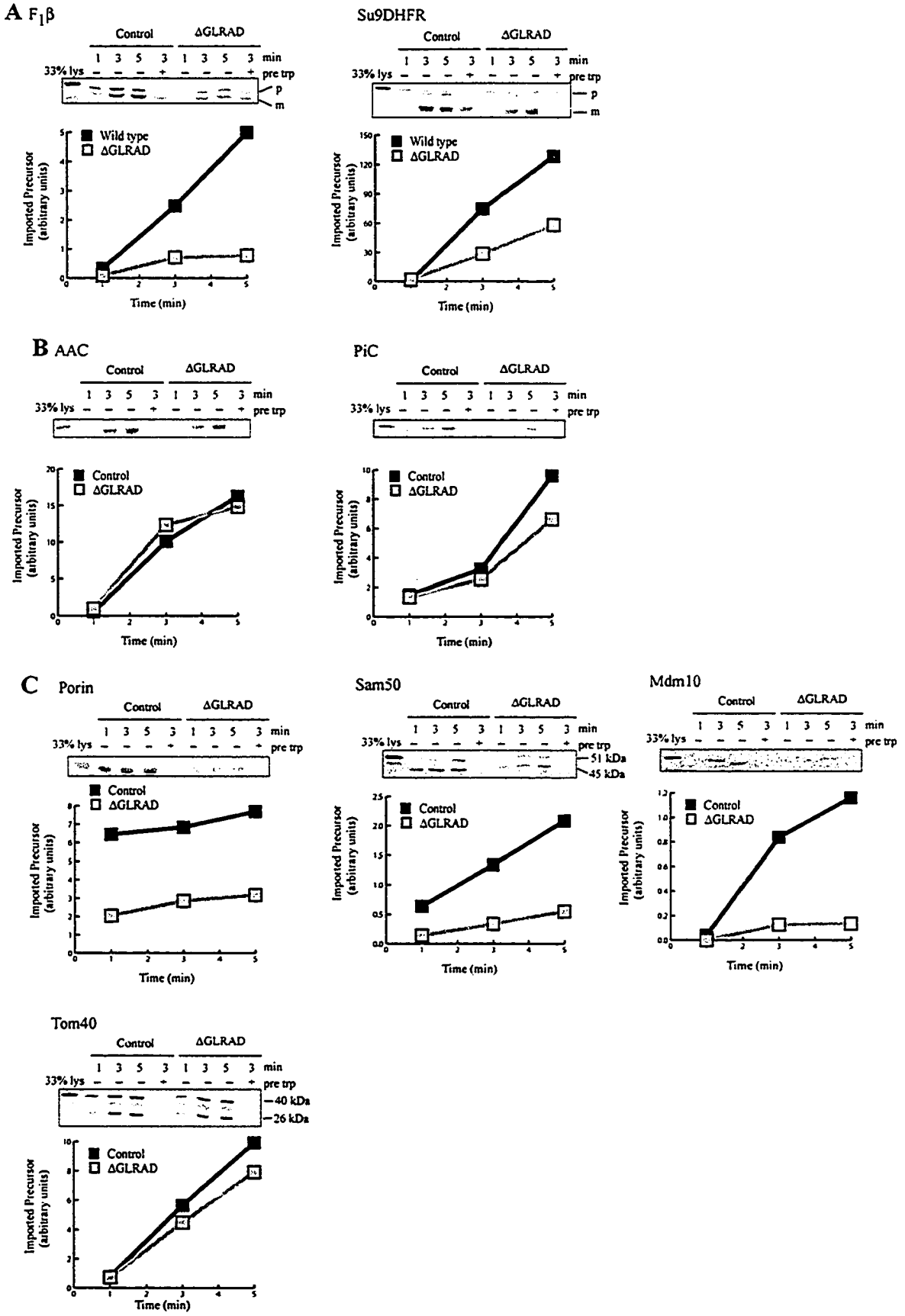


Figure 16. ***In vitro*** **import of preproteins into mitochondria containing mutant Tom40 GLRAD-AAAAA.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains containing wild type Tom40 or GLRAD-AAAAA Tom40 for 1, 3 and 5 min at 25°C (AAC, Sam50, Mdm10, Su9DHFR, PiC and $F_1\beta$) or at 15°C (Tom40 and porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A.** Import of $F_1\beta$ and Su9DHFR to the matrix. "p" and "m" indicate the precursor and mature forms of the proteins. **B.** Import of AAC and PiC to the inner membrane. **C.** Import of porin, Sam50, Tom40 and Mdm10 to the outer membrane. When Tom40 is properly assembled in the membrane it is cut once by the proteinase K treatment and forms a 26 kDa and 12 kDa fragment. The 12 kDa fragment is not shown on the gel insets, the 40 kDa and 26 kDa proteins are indicated. The 26 kDa fragment was used for quantification. The Sam50 protein is also cleaved to produce a 45 kDa fragment as indicated. The 45 kDa fragment was used for quantification.

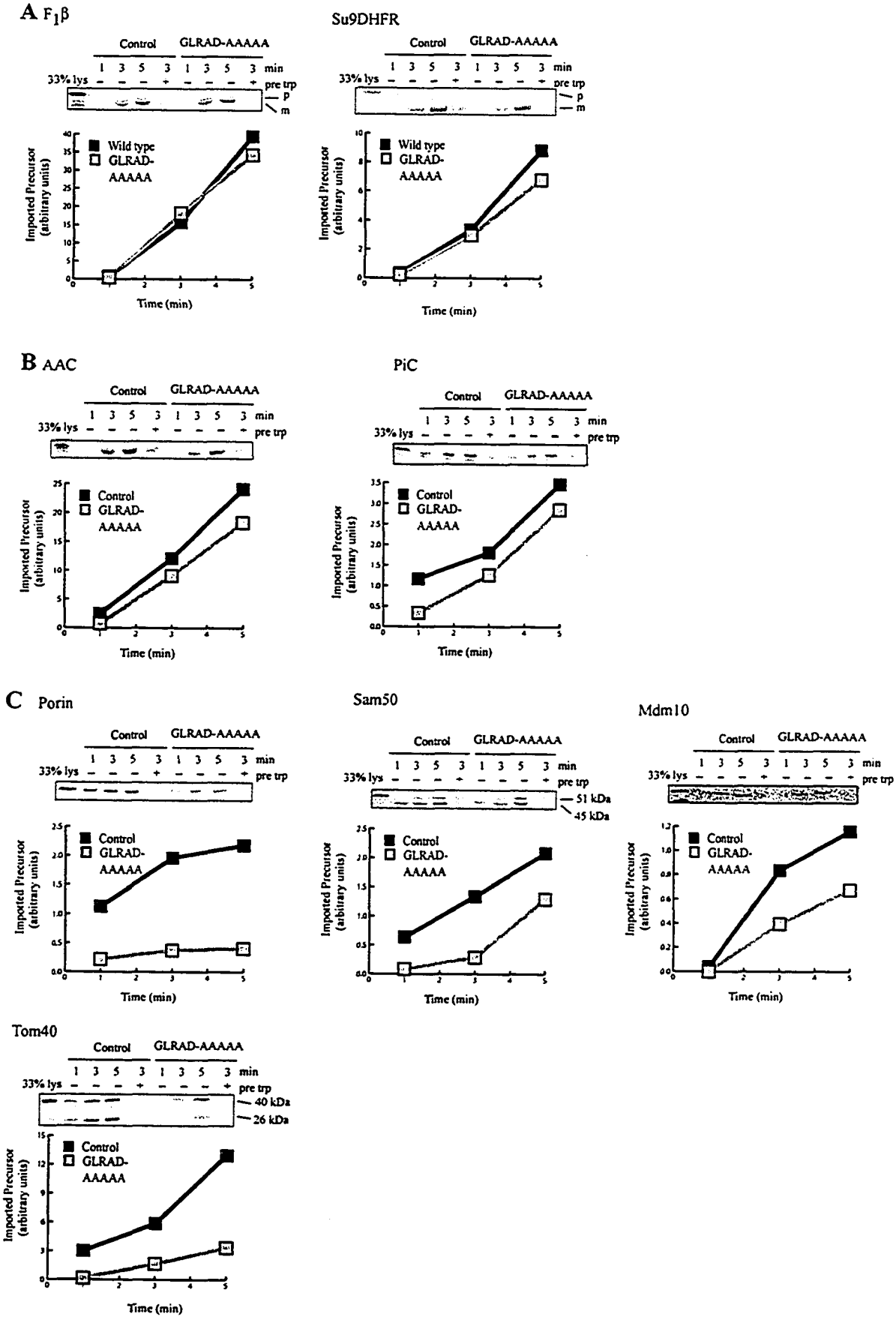


Figure 17. ***In vitro* import of preproteins into mitochondria containing mutant Tom40 ΔNP.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains containing wild type Tom40 or ΔNP Tom40 for 1, 3 and 5 min at 25°C (AAC, Sam50, Su9DHFR, PiC and $F_1\beta$) or at 15°C (Tom40 and porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. Import of CCHL to the intermembrane space. **A.** Import of $F_1\beta$ and Su9DHFR to the matrix. "p" and "m" indicate the precursor and mature forms of the proteins. **B.** Import of AAC and PiC to the inner membrane. **C.** Import of porin, Sam50, Tom40 and Mdm10 to the outer membrane. When Tom40 is properly assembled in the membrane it is cut once by the proteinase K treatment and forms a 26 kDa and 12 kDa fragment. The 12 kDa fragment is not shown on the gel insets, the 40 kDa and 26 kDa proteins are indicated. The 26 kDa fragment was used for quantification. The Sam50 protein is also cleaved to produce a 45 kDa fragment as indicated. The 45 kDa fragment was used for quantification.

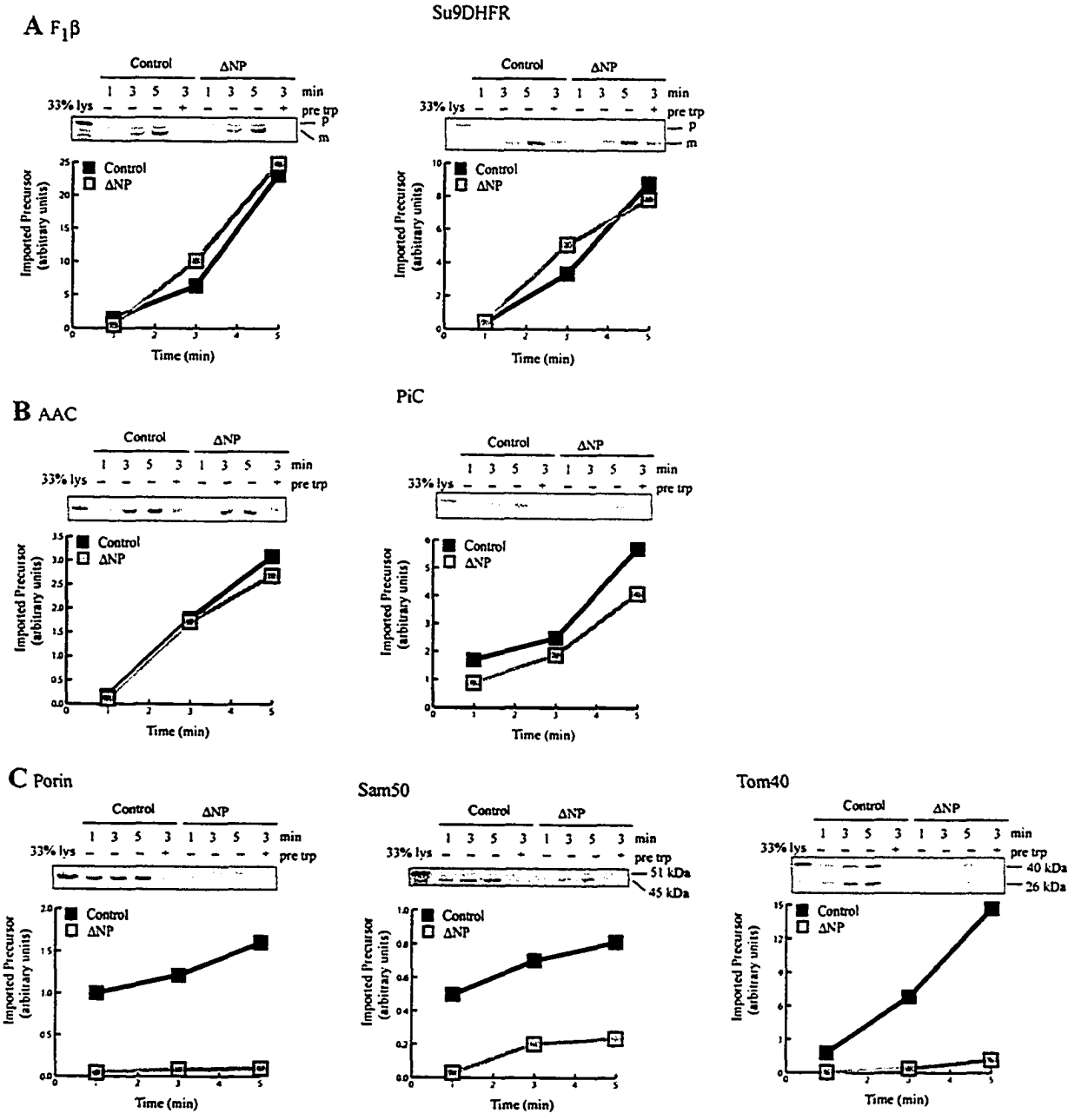


Figure 18. ***In vitro* import of preproteins into mitochondria containing mutant Tom40 NP-AA.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains containing wild type Tom40 or NP-AA Tom40 for 1, 3 and 5 min at 25°C (AAC, Sam50, Su9DHFR, PiC and $F_1\beta$) or at 15°C (Tom40 and porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. Import of CCHL to the intermembrane space. **A.** Import of $F_1\beta$ and Su9DHFR to the matrix. "p" and "m" indicate the precursor and mature forms of the proteins. **B.** Import of AAC and PiC to the inner membrane. **C.** Import of porin, Sam50, Tom40 and Mdm10 to the outer membrane. When Tom40 is properly assembled in the membrane it is cut once by the proteinase K treatment and forms a 26 kDa and 12 kDa fragment. The 12 kDa fragment is not shown on the gel insets, the 40 kDa and 26 kDa proteins are indicated. The 26 kDa fragment was used for quantification. The Sam50 proteins is also cleaved to produce a 45 kDa fragment as indicated. The 45 kDa fragment was used for quantification.

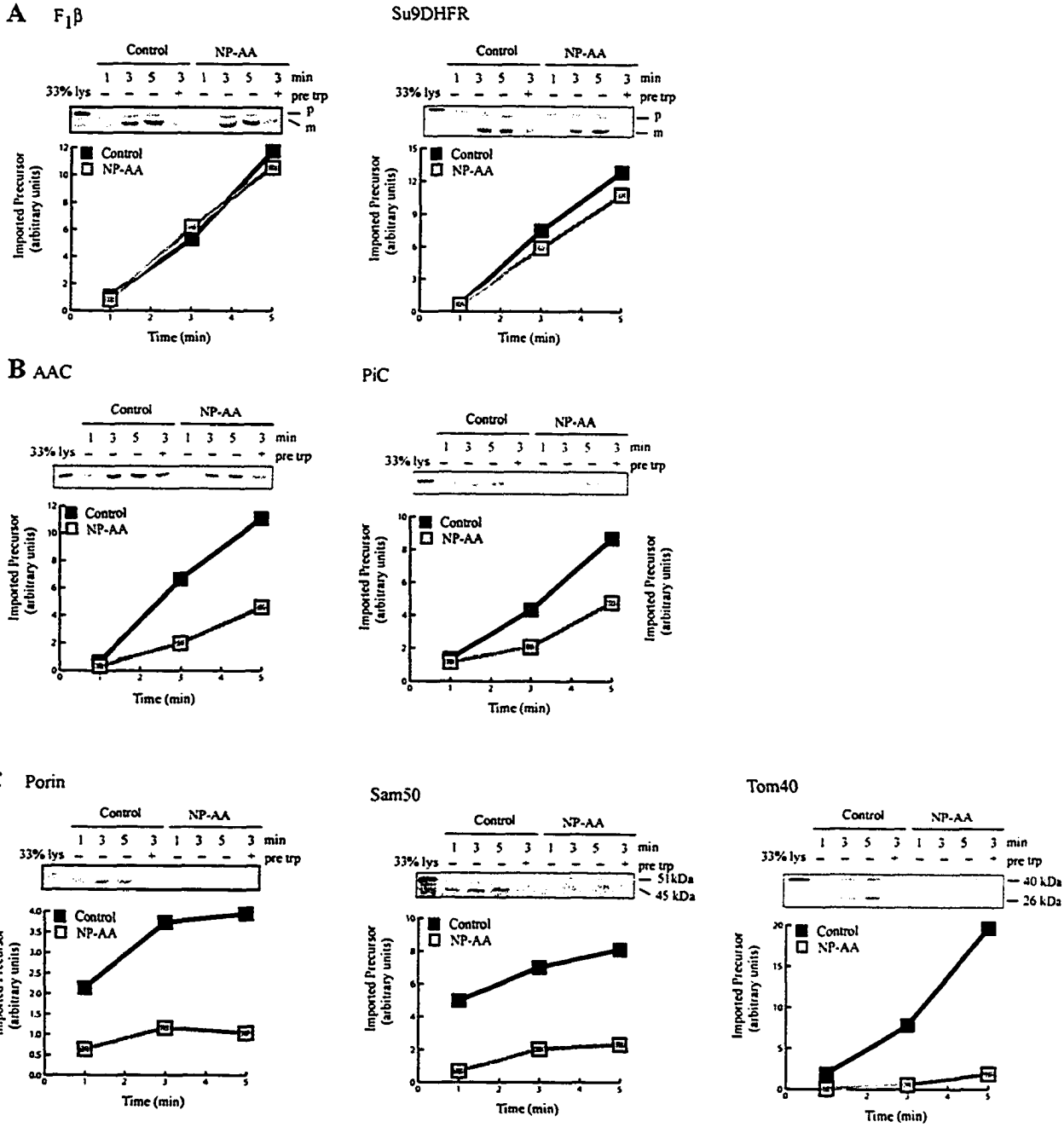


Figure 19. **Levels of TOM complex and other mitochondrial proteins in strains lacking the small Tom proteins.** 30 μg of mitochondria isolated from each strain was subjected to SDS-PAGE and transferred to nitrocellulose. Western blot analysis was done for each of the TOM complex proteins and for mtHsp60 in the matrix, Tim23 in the inner membrane and CCHL in the intermembrane space. The presence/absence of Tom7 was determined by PCR because there is no αTom7 antibody. DNA was isolated from conidia of each strain and PCR was performed using primers outside the *tom7* gene. Since the hygromycin resistance gene is larger than the *tom7* gene, the resulting PCR fragments from the Δ*tom7* strains will be larger than the wild type *tom7* strains.

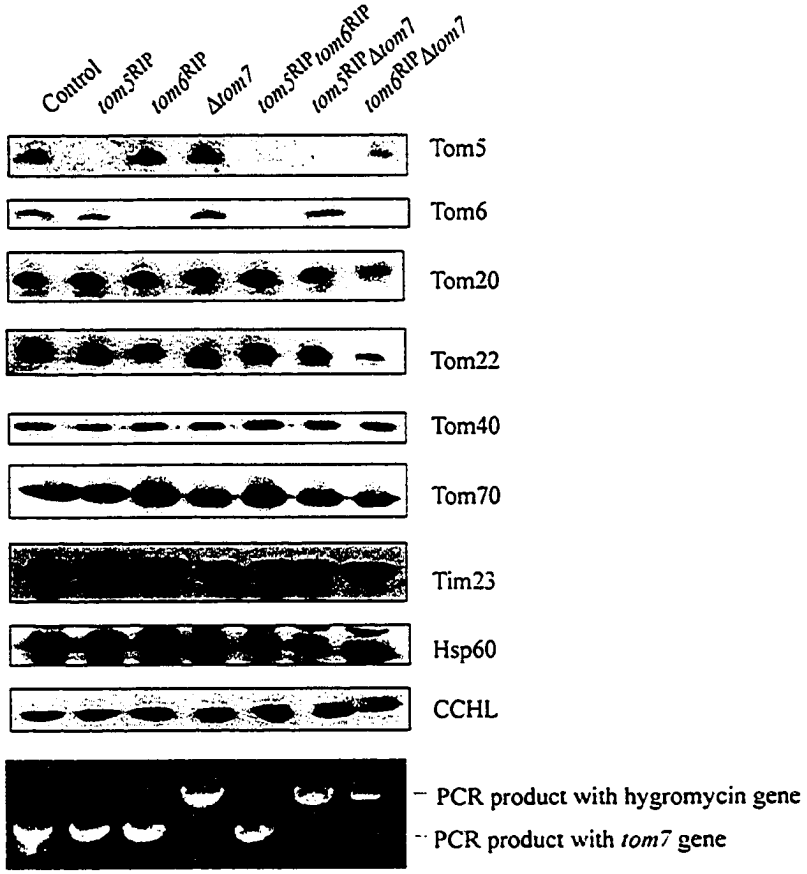


Figure 20. **Growth of strains lacking the small Tom proteins. A.** Conidia from each strain were diluted to 2 x 10^7, 2 x 10^6, 2 x 10^5 and 2 x 10^4 conidia/ml and 10 µl of each dilution was plated on sorbose-containing medium. Plates were incubated at 30°C for approximately 2 days. Control strains 7251 and 7261, which were the parent strains for the *tom5*^{RIP} strain do not grow well on this media but can grow when supplemented with 1% yeast extract. This was not included in the medium because it causes a larger increase in growth rate than for other strains. **B.** Conidia flasks containing the Δ*tom7* strain, which climbs the walls of the conidia flask and conidiates like wild type strains, and the *tom6*^{RIP}Δ*tom7* strain, which does not climb and conidiates poorly.

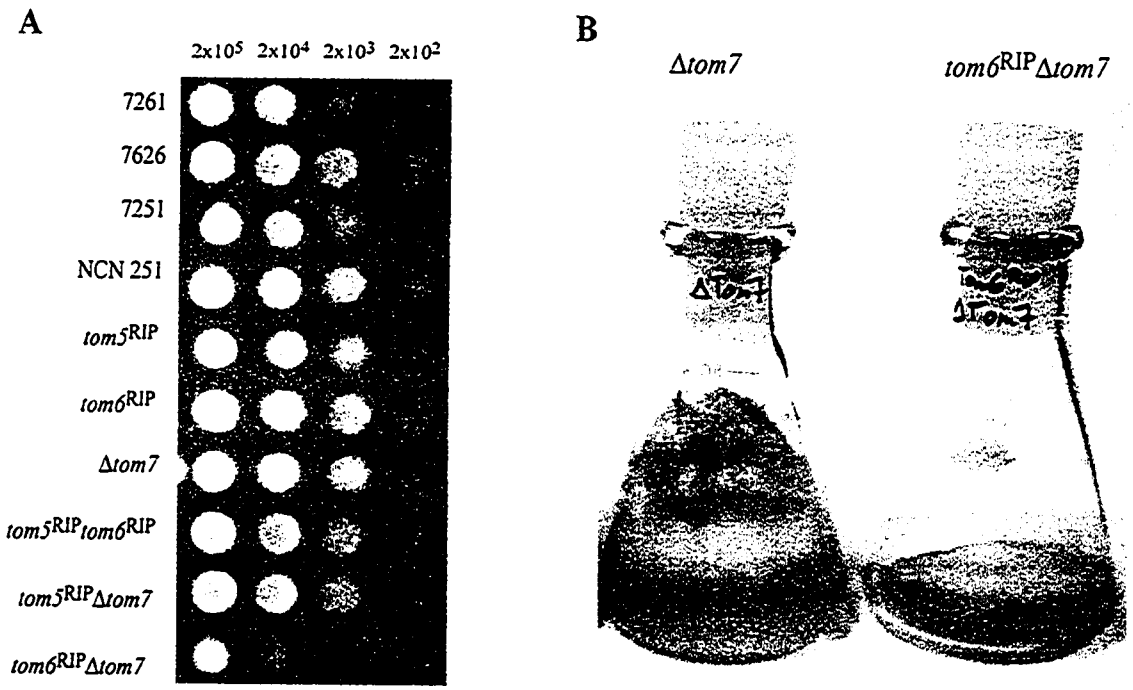
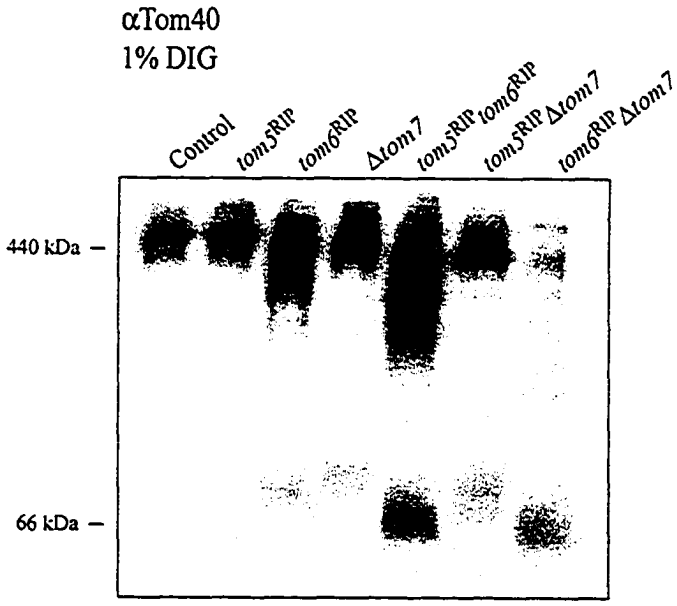


Figure 21. **Stability of the TOM complex in strains lacking the small Tom proteins.** Mitochondria (50μg) lacking different small Tom proteins were dissolved in 1% DIG or 1% DDM, subjected to BNGE and blotted to PVDF. Membranes were decorated with αTom40 antiserum.



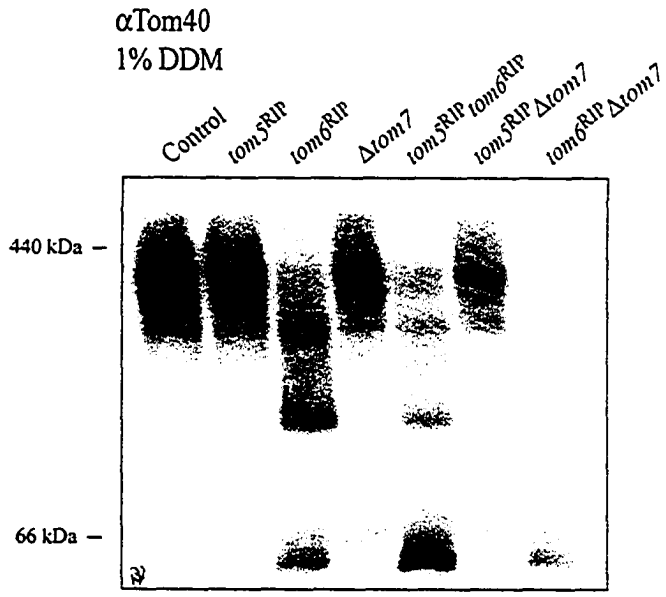
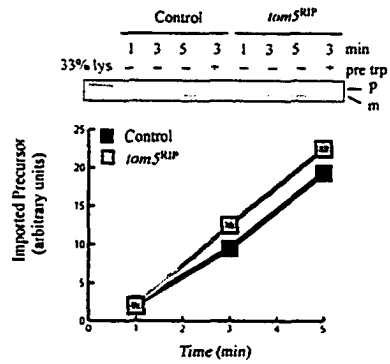
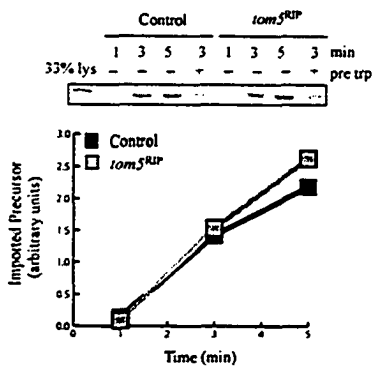


Figure 22. ***In vitro* import of preproteins into mitochondria lacking Tom5.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains with or without Tom5 for 1, 3 and 5 min at 25°C ($F_1\beta$, Tim10 and AAC) or at 15°C (porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A.** Import of $F_1\beta$ to the matrix. "p" and "m" indicate the precursor and mature forms of the protein. **B.** Import of AAC and PiC to the inner membrane. **C.** Import of Tim10 to the intermembrane space. **D.** Import of porin to the outer membrane.

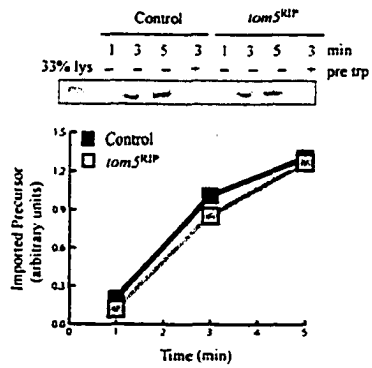
A $F_1\beta$



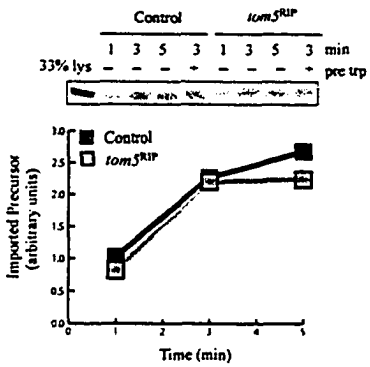
B AAC



PiC



C Tim10



D Porin

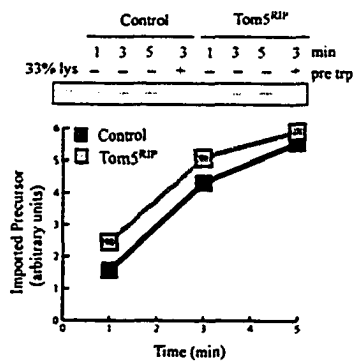
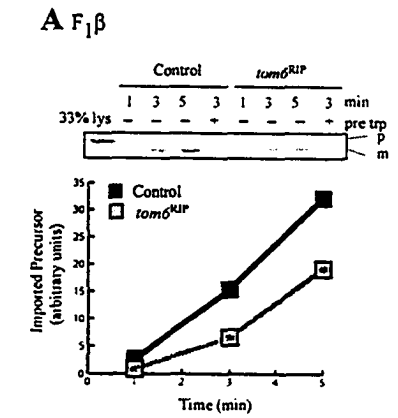
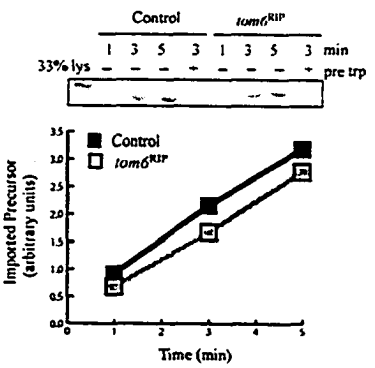


Figure 23. ***In vitro*** **import of preproteins into mitochondria lacking Tom6.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains with or without Tom6 for 1, 3 and 5 min at 25° ($F_1\beta$ and AAC) or at 15°C (porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A.** Import of $F_1\beta$ to the matrix. "p" and "m" indicate the precursor and mature forms of the protein. **B.** Import of AAC to the inner membrane. **C.** Import of porin to the outer membrane.





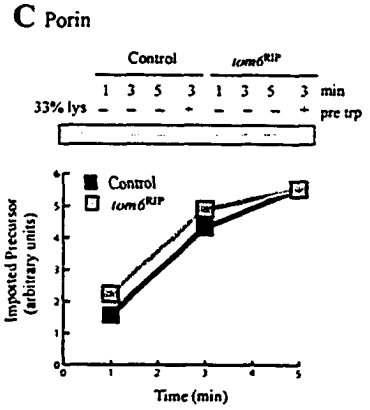


Figure 24. ***In vitro*** **import of preproteins into mitochondria lacking Tom5 and Tom6.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains with or without Tom5 and Tom6 for 1, 3 and 5 min at 25°C (AAC, PiC, and $F_1\beta$) or at 15°C (porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A** Import of $F_1\beta$ and MPP to the matrix. "p" and "m" indicate the precursor and mature forms of the protein. **B.** Import of AAC and PiC to the inner membrane **C.** Import of porin to the outer membrane.

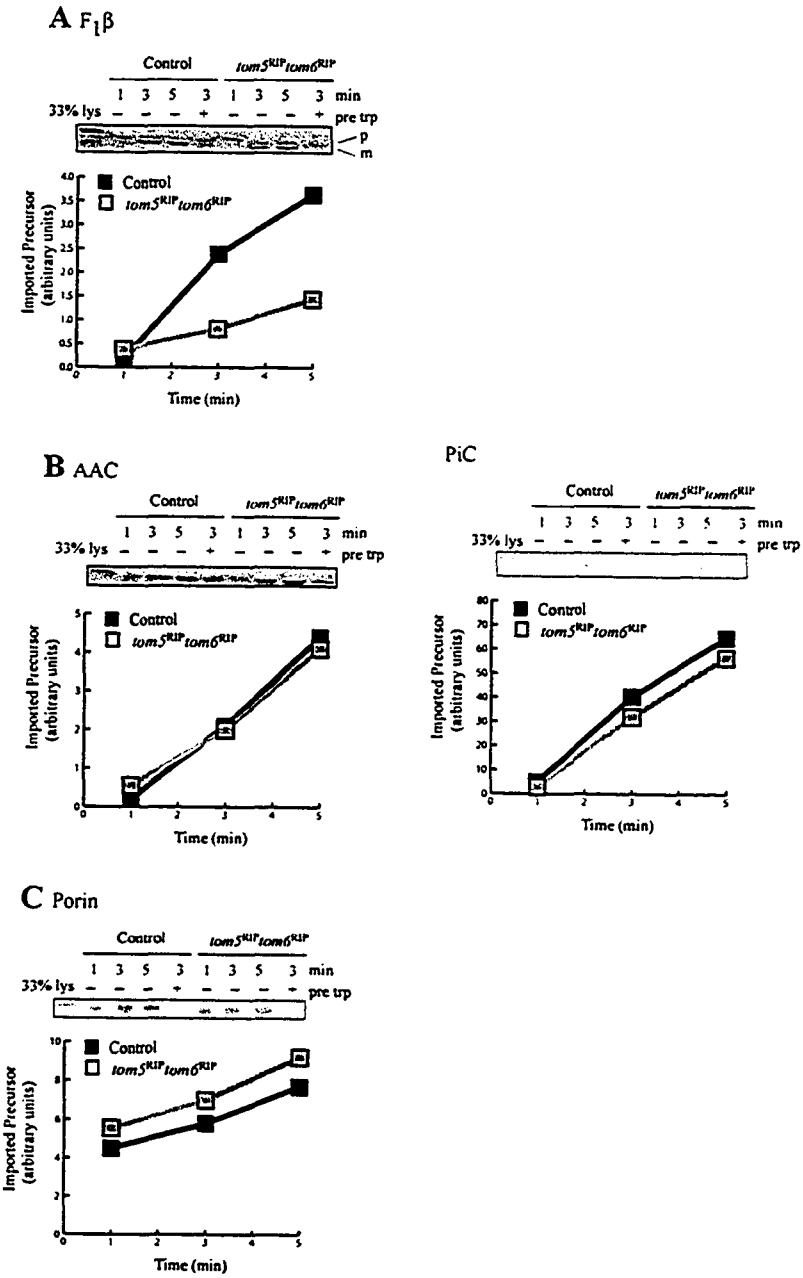
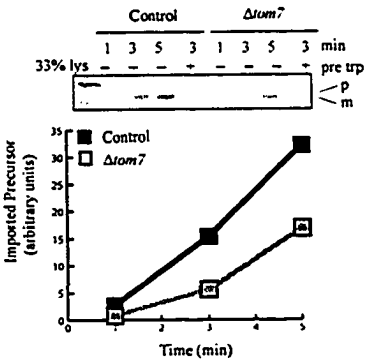
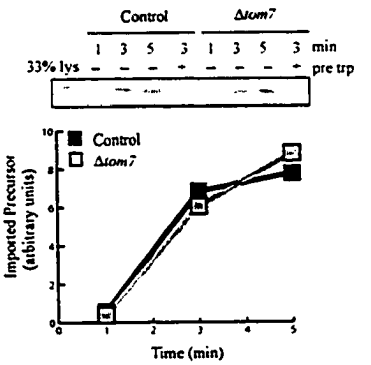


Figure 25. ***In vitro*** **import of preproteins into mitochondria lacking Tom7.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains with or without Tom7 for 1, 3 and 5 min at 25° ($F_1\beta$ and AAC) or at 15°C (porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A.** Import of $F_1\beta$ to the matrix. "p" and "m" indicate the precursor and mature forms of the protein. **B.** Import of AAC to the inner membrane. **C.** Import of porin to the outer membrane.

A $F_1\beta$



B AAC



C Porin

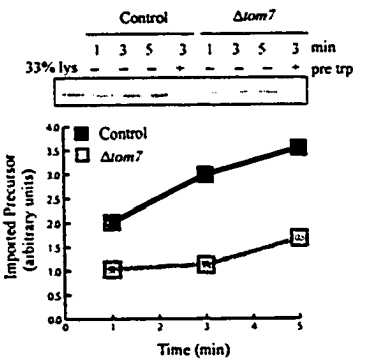
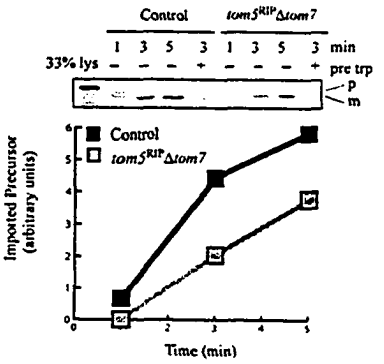
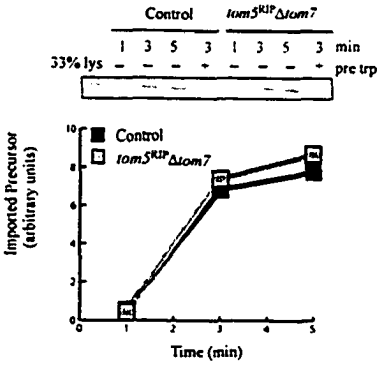


Figure 26. ***In vitro* import of preproteins into mitochondria lacking Tom5 and Tom7.** Radiolabeled mitochondrial preproteins were incubated with mitochondria isolated from strains with or without Tom5 and Tom7 for 1, 3 and 5 min at 25° ($F_1\beta$ and AAC) or at 15°C (porin). A sample of mitochondria from each strain was pretreated with trypsin (pre trp), to show the amount of receptor independent import. After incubation, mitochondria were treated with proteinase K to remove excess radiolabeled protein. Samples were then re-isolated and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and either exposed to film or a Phosphorimager screen for visualization and quantification. **A.** Import of $F_1\beta$ to the matrix. "p" and "m" indicate the precursor and mature forms of the protein. **B.** Import of AAC to the inner membrane. **C.** Import of porin to the outer membrane.

A $F_1\beta$



B AAC



C Porin

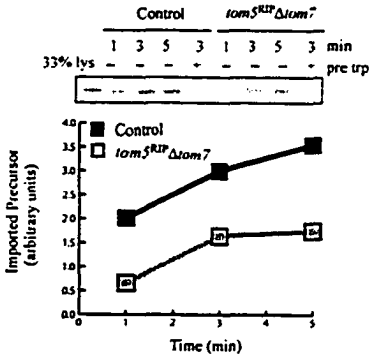


Figure 27. **Tom40 assembly in strains lacking the small Tom proteins.** Radiolabeled Tom40 protein was incubated with isolated mitochondria from each of the small Tom mutant strains for 20 min at 0°C or 25°C. After the incubation, mitochondria were pelleted, dissolved in 1% DIG, subjected to BNGE, transferred to PVDF membrane and exposed to film.

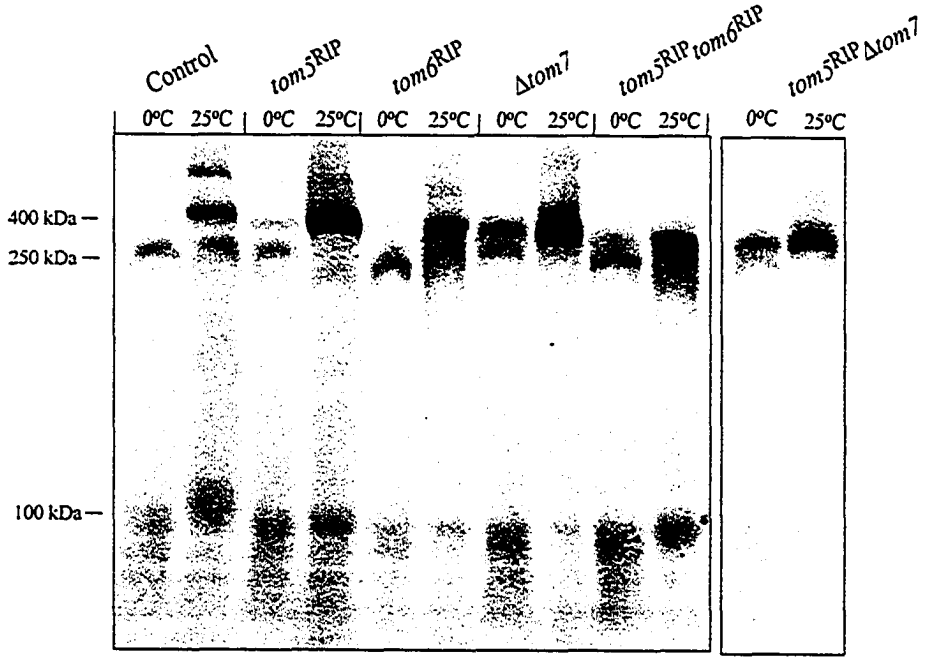


Table 5. **Summary of data on Tom40 variants.** Column one describes the mutation in the conserved region of Tom40 and the starting residue number (see Fig. 6). Column two shows the computer predicted location of the mutated region. "Assembly phenotype" describes how the mutant Tom40s assemble in vitro as assayed by BNGE (see methods section 2.5 and 2.7). "Rescue?" shows if the mutant Tom40 containing the indicated changes can rescue the tom40RIP nucleus of RIP40het. The grey rows indicate the four mutant tom40 variants that were incapable of rescuing the tom40RIP nucleus. The Stability section summarizes if Tom40 is released from mitochondria of each of the strains following solubilization in 1% DIG or 1% DDM. The "Precursor Import" column shows if preproteins imported into mitochondria containing the mutant Tom40 were affected in the import of any mitochondrial preprotein.

Mutation	Predicted Region	Assembly Phenotype	Rescue?	Growth Rate[1] Race Tubes	Growth Rate[1] Liquid Media	Growth Rate[1] Sorbose Plate	Complex Stability[2] DIG	Complex Stability[2] DDM	Precursor import
ΔGLRAD (64)	IMS	Stops at 250 kDa	Yes	N	S	S	N	S	Affected
GLRAD-AAAA	IMS	Stops at 250 kDa	Yes	N	n.d.	S	Y	S	Affected
ΔSHQ (82)	MEMB	Assembles but accumulates at 250 kDa	No						
SHQ-AAA	MEMB	Assembles but accumulates at 250 kDa	Yes	S	N	n.d.	N	S	Not affected
ΔGNLD (109)	IMS	Like WT	Yes	N	n.d.	n.d.	Y	S	Not affected
GNLD-AAAA	IMS	Assembles but accumulates at 250 kDa	Yes	N	n.d.	n.d.	Y	N	Not affected
ΔTK (131)	CYT	Stops at 250KDa	Yes	N	N	S	Y	N	Affected
TK-AA	CYT	Assembles but accumulates at 250 kDa	Yes	N	n.d.	n.d.	Y	N	Not affected
ΔQFEHE (145)	IMS	Stops at 250 kDa	Yes	S	S	S	Y	S	Affected
QFEHE-AAAAA	IMS	Stops at 250 kDa	Yes	N	n.d.	n.d.	Y	N	Not affected
ΔNP (163)	CYT	Assembles but accumulates at 250 kDa	Yes	N	n.d.	S	Y	N	Affected
NP-AA	CYT	Assembles but accumulates at 250 kDa	Yes	N	n.d.	S	Y	N	Affected
ΔVTP (183)	IMS	Stops at 250 kDa	No						
VTP-AAA	IMS	Stops at 250 kDa	Yes	N	N	n.d.	Y	N	Not affected
VTPR-VTR	IMS	Assembles but accumulates at 250 kDa	Yes	N	n.d.	n.d.	Y	N	Not affected
ΔKK (237)	CYT	Stops at 250 kDa	No						
KK-AA	CYT	Assembles but accumulates at 250 kDa	Yes	N	N	n.d.	Y	S	Not affected
ΔEKR (297)	IMS	Stops at 250 kDa	No						
EKR-AAA	IMS	Accumulates at 100 kDa	Yes	N	N	n.d.	Y	S	Not affected
ΔVDH (313)	CYT	Assembles but accumulates at 250 kDa	Yes	S	n.d.	n.d.	Y	S	Not affected
VDH-AAA	CYT	Accumulates at 100 kDa	Yes	N	n.d.	n.d.	Y	N	Not affected

[1] N = normal growth, S = slow growth, n.d. = not determined

[2] Y= Yes stable, N = Not stable, S = severely destabilized

Table 6.

Summary of strains produced from the cross of Δ*tom7* x *tom5*^{RIP}*tom6*^{RIP}.

	Spores picked from medium with hygromycin	Number Screened	Double Mutants Found
Immediate growth start – normal growth rate	262	30	12 *tom5*^{RIP}Δ*tom7*
Later growth start – slow growth rate	72	19	19 *tom6*^{RIP}Δ*tom7*
Did not grow	78	-	
Total	412	49	31

Ascospores from the cross between Δ*tom7* (Tom7KO-35) x *tom5*^{RIP}*tom6*^{RIP} (Tom5/6Rip–9 and Tom5/6Rip–10) were plated onto Vogel's sorbose-containing medium with appropriate supplements. The media also contained hygromycin to select for the Δ*tom7*allele. Spores that grew on the plates were picked to slants containing supplemented Vogel's sucrose medium with hygromycin and incubated at 30°C. Strains that showed 'immediate growth start' showed growth in the slant within 24 hours and continues growing at a normal rate. Strains with 'later growth start' showed growth in the slant 2-7 days later and had slow growth rates. The presence or absence of Tom5 and Tom6 was then tested by western analysis in the 49 strains that were screened and Tom7 was presumed absent through selection on hygromycin (eight random isolates were tested by PCR to confirm strains with hygromycin resistance were missing *tom7*. This was found to be the case in each isolate). 12 of the 30 'immediate growth' strains tested were missing Tom5 and Tom7 and 18 of 30 were missing Tom7 only based on the progenies ability to grow in the presence of hygromycin. All 19 of the slow growth strains screened were missing Tom6 and Tom7. No isolates were found to be missing all three small Tom proteins.

4. DISCUSSION

4.1. The roles of conserved regions of the Tom40 protein in assembly and function of Tom40 in the TOM complex

4.1.1. The *in vitro* assembly phenotype of EKR-AAA and VDH-AAA

Both Tom40 variants EKR-AAA and VDH-AAA showed an *in vitro* assembly defect of being slow to progress past the 100 kDa intermediate stage. The Tom40 precursor is thought be integrated into the membrane at this stage and to be associated with another pre-existing Tom40 subunit (Model *et al.* 2001). Recent data suggest that assembly past the 100 kDa intermediate requires the Mdm10 protein. Mdm10 is required at a late stage in Tom40 assembly and is involved in assembling Tom40 with Tom6, Tom7 and Tom22 but does not affect the formation of the 250 kDa or the 100 kDa intermediates (Meisinger *et al.* 2004). It is possible that the EKR or the VDH residues may be needed for Tom40 to interact properly with Mdm10 or the additional Tom subunits and therefore the Tom40 variant with alanine substitutions at these residues accumulates as the 100 kDa intermediate. The possibility that these Tom40 variants interact poorly with additional Tom subunits is supported by the extreme instability of TOM complexes containing these variants in the presence of DDM. It is also conceivable that the alanine substituted EKR or VDH residues alter Tom40 structure in a way that prevents these interactions. However, large changes in the structure of these variants seem unlikely since they are able to progress along the assembly pathway to the point of being inserted into the MOM.

In contrast to the alanine-substituted form, the ΔEKR Tom40 variant protein was not able to assemble past the 250 kDa intermediate *in vitro* and was unable to rescue the *tom40*^{RIP} nucleus *in vivo*. This suggests that removing these residues may disrupt the structure of Tom40 or its ability to integrate into the membrane. In contrast, the ΔVDH Tom40 protein can still assemble but accumulates at the 250 kDa intermediate stage. *In vivo*, ΔVDH Tom40 provides enough function for a viable strain. This would indicate that the VDH residues could play a role in Tom40 assembly but loss or change of the residues does not cause gross structural changes. The ΔVDH Tom40 and the VDH-AAA protein are delayed at different points in the assembly pathway, with the deletion

accumulating at the earlier 250 kDa stage, while the alanine substituted version progresses to the 100 kDa stage. This may reflect how these two mutations change the ability of the protein to integrate into the membrane, with the deletion slowing integration more than the alanine substitutions do.

4.1.2. Role of the conserved residues which cause lethality when deleted

Mutations in several regions of Tom40 failed to rescue the *tom40*^{RIP} nucleus including ΔSHQ, ΔKK, ΔVTP and ΔEKR. Since all of these variants could assemble at least to the 250 kDa stage *in vitro,* it appears that the deleted residues are not critical for targeting Tom40 to the mitochondria. The deleted residues appear to be important for assembly, especially in ΔKK, ΔVTP and ΔEKR which do not assemble past the 250 kDa intermediate. There are a number of possible roles for residues involved in assembly. For example, they could be important for interacting with other Tom proteins or for interacting with other proteins that assist in Tom40 assembly such as the small Tim proteins in the IMS, or the SAM complex (Wiedemann 2003; Hoppins and Nargang 2004; Wiedemann *et al.* 2004). Inability to progress past the 250 kDa intermediate might point to the importance of these residues in inserting into the membrane, which is proposed to happen after Tom40 interacts with the SAM complex in the 250 kDa intermediate (Wiedemann 2003). The same inability to rescue the *tom40*^{RIP} nucleus was seen for a deletion of the KLG (321-323) residues near the C-terminus of Tom40. It was proposed that these residues lie in a membrane spanning β-strand, where the deletion renders the strand too short to span the membrane resulting in no Tom40 integration into the membrane and preventing rescue of the *tom40*^{RIP} nucleus. On the other hand, the Ala-substituted KLG-AAA variant is able to assemble and rescue the RIP nucleus (Taylor *et al.* 2003). Of the four non-rescuing variants described here, only the SHQ residues are predicted by computer to be part of a β-stand while the others are predicted to be in cytosolic or IMS loops (T. Schirmer, via D. Rapaport, personal communication) (Fig3). However, it should be noted that there is virtually no experimental data to support the computer model, so that errors in the predicted topology of Tom40 are not unlikely.

4.1.3. Strains exhibiting slower growth

Only a small number of strains containing Tom40 mutations exhibited slower growth than the control strain. Initially, growth was measured in liquid cultures and on solid media in race tubes. These methods did not detect any strong growth defects except in the ΔQFEHE Tom40 strain, which was found to be slower by both methods. This could be due to its general defect in importing all three classes of mitochondrial preproteins tested (section 4.1.5), which may slow the rate of mitochondrial biogenesis and ultimately the growth of the strain. The ΔGLRAD strain, which showed slower growth in liquid media, also has import defects. The confusing results seen between the race tubes and liquid media could be due to the fact that the race tubes only measure the mycelial extension but not the density of growth. Thus, liquid growth curves are generally a more sensitive measure of growth. The SHQ-AAA strain had a minor defect in growth as measured in race tubes but was not deficient in import for the precursors tested. The last strain to exhibit a growth phenotype contains the ΔVDH Tom40 variant. This mutant form of the Tom40 protein exhibited the *in vitro* assembly phenotype of accumulating at 250 kDa intermediate. The slower growth could be due to this assembly defect.

Later in these studies, another technique for measuring growth was developed, involving spotting a series of different concentrations of conidia on sorbose-containing plates for the comparison of colony size (Fig. 11). All strains showing an import defect were examined in this fashion. The results confirmed the slower growth of the ΔQFEHE and ΔGLRAD strains. An explanation for the ΔGLRAD and ΔQFEHE mutations resulting in slower growth than the other strains with import defects may relate to additional roles of these residues in the assembly or structure of Tom40. All other strains with import defects showed a slight affect on growth at the lowest concentration of conidia.

4.1.4. TOM complex stability

Three classes of TOM complex stability were found: destabilized in DDM, very destabilized in DDM and severely destabilized in both DIG and DDM. Since the majority of changes to Tom40 caused the TOM complex to be very destabilized in DDM,

these regions may play a part in Tom40 interacting with the other components of the TOM complex, including other Tom40 molecules. All but one of the changes also causes an *in vitro* assembly phenotype of some sort, indicating a role for these regions in the overall assembly of the TOM complex. It does not seem surprising that there is a general relationship between stability and assembly since it is expected that interactions promoting assembly of subunits are also involved in maintaining their stability.

Only two strains have severely destabilized TOM complexes in DIG and DDM: SHQ-AAA and ΔGLRAD. SHQ are the only residues in this study that were previously predicted to be in a membrane spanning β-strand (Fig. 3) (T. Schirmer, via D. Rappaport, personal communication). Since the SHQ deletion is lethal while the alanine replacement version is able to rescue but still results in a severely destabilized complex, it seems likely these mutations occur within a membrane spanning β-strand. These properties are identical to those described previously for the ΔKLG and KLG-AAA variants of Tom40, mentioned previously in section 4.1.3, which are also proposed to be in a membrane spanning β-strand (Taylor *et al.* 2003).

The reason for the severe instability of the TOM complex containing the ΔGLRAD variant is not readily apparent. The region is predicted to be in the IMS and not part of the β-barrel structure in the membrane. Conceivably these residues could interact with Tom22, which is thought to be an organizer of the TOM complex (van Wilpe *et al.* 1999). It is also conceivable that the import defects of ΔGLRAD might be in part due to the disrupted stability of the complex.

4.1.5. Roles of conserved regions in import of preproteins

Of the 17 strains containing Tom40 variants that were tested, six showed import defects: ΔTK, ΔQFEHE, ΔGLRAD, GLRAD-AAAAA, ΔNP and NP-AA. Since all the changes made to Tom40 were in conserved regions of the protein and most did not cause import defects, the conservation of these regions probably reflects their involvement in the assembly of Tom40 into the TOM complex or interactions of Tom40 with the other Tom proteins. All but one of these mutants (ΔGNLD) showed an assembly defect and even ΔGNLD showed a stability problem.

Both ΔTK and ΔQFEHE mitochondria have general import defects for preproteins destined to all the subcompartments tested. This general defect suggests a role for these residues in the actual translocation process. The ΔTK strain has a fairly stable TOM complex and shows no major growth defect. It is difficult to reconcile the significant growth difference in the ΔTK and ΔQFEHE strains when the import defects measured are very similar. The decreased stability of the TOM complex in the ΔQFEHE strain suggests that this defect may play a role in the growth rate. Since the alanine substituted versions of these deletions do not show import defects, it is likely that the effects on import in the deletions are due to structural changes in the protein rather than a specific role for the residues themselves in the import process.

Mitochondria containing Tom40 with a deletion of the NP residues had a major defect in the import of β-barrel proteins while the other classes of preproteins were imported at levels similar to the control strain or only slightly affected. It has been reported that β-barrel proteins use the small Tim proteins in the IMS for their assembly before the SAM complex (Hoppins and Nargang 2004; Wiedemann *et al.* 2004). This could mean that the NP residues are involved in recruiting the small Tim proteins to the TOM complex or directing β-barrel preproteins to the small Tims. In support of this, the NP-AA mutant also appears to have defects in importing inner membrane carrier proteins that also use the small Tim proteins as chaperones to the TIM22 complex (Endres *et al.* 1999; Wiedemann *et al.* 2001). However, this decrease in carrier import was not seen in ΔNP mitochondria. It should also be noted that the NP residues are located in a cytosolic loop in the computer predicted structure, which would contradict a role in recruiting the small Tims in the IMS. However, since the computer prediction has not been experimentally validated, it is conceivable that the NP residues reside in the IMS. This led to another possibility that the NP residues are involved in recognizing the β-barrels before translocation and these residues could be partially responsible for recognition of internal import signals, which are not clearly defined in β-barrel proteins. The internal signals of proteins such as AAC might also be partially recognized in the area of the NP residues. Surprisingly, the deletion does not affect the import of AAC. While this is difficult to explain, it is conceivable that this could be due to structural changes that allow

other residues to compensate for the missing NP residues, which may not occur when the residues are substituted with alanine.

Mitochondria containing the ΔGLRAD Tom40 variant had significant defects importing matrix preproteins and β-barrel proteins, while the GLRAD-AAAAA version only had defects importing the β-barrel proteins. Since only the deletion had a defect in importing matrix proteins, it could be that the deletion disrupts a structure important for this function in Tom40 or in the TOM complex as a whole, therefore indirectly affecting the sorting of the matrix proteins to the TIM23 complex. One study in yeast found that a single amino acid change (W243R) affected import of only matrix preproteins showing that Tom40 does have specific roles in sorting precursors (Gabriel *et al.* 2003). It was suggested that this mutation affected a binding site for matrix preproteins in the IMS. It is possible that the deletion of GLRAD could affect a matrix preprotein binding site since Tom40 does form part of the *trans* binding site (Rapaport *et al.* 1997). Consistent with the deletion causing structural changes in the complex, ΔGLRAD has a more destabilized TOM complex than the GLRAD-AAAAA containing TOM complex.

The fact that both GLRAD variants affect the import of β-barrel proteins suggests that these residues have a specific role, possibly involving passing β-barrel proteins to the SAM complex or small Tim proteins. Interestingly, in the ΔGLRAD strain, there is no defect seen in importing Tom40 itself while the other β-barrel proteins are imported poorly. This might indicate that Tom40 has an altered route of import compared to the other β-barrel proteins. In support of this idea is the observation that mitochondria lacking Mdm10 were shown to have a reduced ability to import Tom40 but not other β-barrel proteins (Meisinger *et al.* 2004). However, our data are difficult to interpret since the GLRAD-AAAAA mutant had a general decrease in import of all β-barrel proteins tested, including Tom40. One explanation could be that when the region is converted to AAAAA residues, the newly imported Tom40 is unable to interact with other residues that can compensate for the function of these residues when GLRAD is absent.

4.2. Role of small Tom proteins in the TOM complex

Prior to the present study, most of the work on the small Tom proteins in the TOM complex was done in yeast, especially for Tom5, which was only recently discovered in *N. crassa* (W. Neupert, personal communication). The data on the small Tom proteins is summarized in Table 7 and compares the roles of the proteins in yeast and in *N. crassa*. The most noticeable difference between the role of the yeast and *N. crassa* small Tom proteins was seen with respect to Tom5. Tom5 has only a minor role in *N. crassa* since no growth rate defects, import defects, or TOM complex destabilization was observed in the *tom5*^{RIP} strain. Even when the lack of Tom5 is combined with the absence of Tom6 or Tom7, the import defects are not exacerbated but remain the same as in the Tom6 or Tom7 single mutants. In yeast, Tom5 was found to have a major role in importing preproteins and to have receptor like functions, with receptor independent import being strongly dependent on Tom5 (Dietmeier *et al.* 1997). In addition, the ΔTom5 combined with either ΔTom6 or ΔTom7 is lethal in yeast (Dietmeier *et al.* 1997). Analogous strains in *N. crassa* have no discernible growth defects. It is likely that in *N. crassa* other TOM complex components have taken over the import functions that Tom5 has in yeast. Nonetheless, Tom5 appears to have at least some role in *N. crassa* that may be redundant with Tom6 and Tom7 since all double mutant combinations are viable, but the triple mutant is not.

Certain aspects of Tom6 function appear to be similar in yeast and in *N. crassa*. The absence of Tom6 in both organisms leads to destabilization of the TOM complex. However, in yeast the import of AAC and $F_1\beta$ are affected in strains lacking Tom6 while the absence of the protein results in only a slight affect on matrix destined proteins in *N. crassa*. The role of Tom6 in yeast and *N. crassa* has been suggested to be in modulating the interactions of the Tom proteins during preprotein translocation (Rapaport *et al.* 1998; Dembowski *et al.* 2001). Although their roles seem to be similar, *in vitro* synthesized yeast Tom6 cannot be assembled into *N. crassa* TOM complexes (Dembowski *et al.* 2001).

Tom7 may behave differently in *N. crassa*. Without Tom7, there is no growth defect but in yeast a Tom7 deletion has slower growth on non-fermentable carbon sources at 30°C and is inviable at 37°C. In *N. crassa* the absence of Tom7 seems to

partially destabilize the TOM complex as seen by the slight loss of Tom40 in both 1% DIG and 1% DDM. This is in contrast to yeast Tom7, which is thought to have a destabilizing effect on the Tom complex. Thus, the absence of Tom7 in yeast is thought to increase interactions between the Tom proteins (Honlinger *et al.* 1996). There is also a difference in import defects when Tom7 is missing. In yeast, the import of porin is more strongly affected by the loss of Tom7 than import of other preproteins (Honlinger *et al.* 1996) but in *N. crassa* both porin and the matrix protein $F_1\beta$ are affected.

The small Tom proteins do not seem to play a similar role in *N. crassa* as in yeast in regards to Tom40 assembly into the Tom complex. In yeast, Tom5 is needed for Tom40 to form the 250 kDa intermediate, Tom6 is needed to form the fully assembled complex of 400 kDa and Tom7 is required for the Tom40 precursor to be released from the receptors early in assembly (Model *et al.* 2001). However, in *N. crassa* assembly in mitochondria lacking Tom5 or Tom7 is similar to or even more efficient than in wild type, as seen by the presence of the 400 kDa fully assembled complex even at 0°C. This phenotype seems to be exaggerated in the double mutant, with even faster assembly of Tom40, with no 250 kDa intermediate left at either temperature after 20 min. It is possible that in wild type mitochondria the presence of these two proteins slows down assembly because Tom40 has to interact with them, making the assembly of the complex more complicated. Without the extra proteins to assemble with, Tom40 may be able to assemble more rapidly. The absence of Tom6 does not appear to affect the assembly of Tom40.

Another striking difference between yeast and *N. crassa* is seen in the relative phenotypes when the combinations of the small Toms are deleted (Honlinger *et al.* 1996; Dietmeier *et al.* 1997). In yeast, the importance of Tom5 is seen in the synthetic lethality with ΔTom6 or ΔTom7 while the double deletion of Tom6 and Tom7 is the least severe, since it is viable on fermentable carbon sources at 30°C. The opposite is true in *N. crassa* as the most severe phenotype is seen in the double mutant of Tom6 and Tom7 while no growth defects are seen in the strains containing any double mutation involving Tom5. Furthermore, in *N. crassa* the stability of the TOM complex is severely affected in mitochondria lacking both Tom6 and Tom7 and the basal levels of Tom5, Tom22 and

Tom20 are decreased. No such phenotypes were observed in *tom5*^{RIP}*tom6*^{RIP} or *tom5*^{RIP}Δ*tom7* double mutants.

4.3. Future directions

Analysis of the Tom40 variants studied here could be supplemented by using SCAM (substituted cysteine accessibility method). Others in our laboratory have used this technique to determine if a residue in a membrane spanning β-strand is embedded in the bilayer or accessible to bind with a cysteine-modifying reagent in a hydrophilic environment. Such an analysis may help clarify the topology of residues which fail to rescue the *tom40*^{RIP} nucleus when mutated. Strains for SCAM have been created for the regions shown in Fig. 5, substituting cysteine residues for each of the residues in the mutated regions, along with four or five flanking residues on either side, and are ready to be used for SCAM (Table 2).

The small Tom mutants should be further examined by showing that the severe phenotype of the *tom6*^{RIP}Δ*tom7* double mutant can be rescued by either *tom7* or *tom6*.

Table 7.

Summary of small Tom protein data in yeast and *N. crassa*.

Tom5	Yeast	*N. crassa*[1]
Proposed role	-Receptor: direct or pass preproteins from receptor to GIP[2,4]	
Phenotype when absent	-Growth defect at 30°C, essential at 37°C on both carbon sources -Defect in importing all classes of preproteins[2,4]	-No growth defects -No import defects
Growth phenotype in combined mutants	-Synthetic lethality with all other Tom proteins [2]	-*tom5*^{RIP}*tom6*^{RIP}, *tom5*^{RIP}*Δtom7* double mutants no phenotype -Inviable when all three mutated
Role in Tom40 assembly	-Needed for Tom40 assembly into the 250 kDa intermediate[3]	-Tom40 assembly faster when Tom5 absent

Tom6	Yeast	*N. crassa*
Proposed role	-Stabilize TOM complex, modulate interactions between Tom proteins during preprotein translocation [5,7]	-Modulate interactions between Tom proteins during preprotein translocation [6] -Stabilize TOM complex
Phenotype when absent	-Minor growth defect at 37°C on non-fermentable carbon source -Import of matrix and carrier proteins affected [5]	-No growth defects -Import of matrix preproteins affected
Growth phenotype in combined mutants	-*tom6tom5*, *tom6tom20* double mutants inviable -*tom6tom7*, *tom6tom70* double mutants inviable at 37°C on any carbon source and growth defect at 30°C on non-fermentable carbon [2,5]	-*tom6*^{RIP}*tom5*^{RIP} double mutant no phenotype -*tom6*^{RIP}*Δtom7* double mutant severe growth defect -Inviable when all three mutated
Role in Tom40 assembly	-Needed for Tom40 to form the fully assembled 400 kDa complex [3]	-No affect on Tom40 assembly when absent

[1] The *N. crassa* column summarizes results from this study.
[2](Dietmeier *et al.* 1997)
[3](Model *et al.* 2001)
[4](Kurz *et al.* 1999)
[5](Alconada *et al.* 1995)
[6](Dembowski *et al.* 2001)
[7](Rapaport *et al.* 1998)
[8](Hönlinger *et al.* 1996)
[9](Esaki *et al.* 2004)

Table 7 continued

Tom7	Yeast	*N. crassa*
Proposed role	-Destabilized TOM complex -C-terminus part of *trans* binding site [8,9]	-Stabilizes complex -Sort preproteins during translocation
Phenotype when absent	-Import of all preproteins is affected but porin import is severely affected -Growth defect only at 37°C on non-fermentable carbon [8]	-No growth defects -Import of matrix proteins and porin is affected
Growth phenotype in combined mutants	-*tom7tom5*, *tom7tom20* double mutants inviable -*tom7tom6* inviable at 37°C and slow growth on non-fermentable carbon at 30°C [2,8]	-$tom5^{RIP}\Delta tom7$ double mutants no phenotype -$tom6^{RIP}\Delta tom7$ double mutant severe growth defect -Inviable when all three mutated
Role in Tom Complex assembly	-Required for release of Tom40 precursor from the receptors before the 250 kDa intermediate is formed [3]	-Tom40 assembly faster when Tom7 absent

[1] The *N. crassa* column summarizes results from this study.
[2](Dietmeier *et al.* 1997)
[3](Model *et al.* 2001)
[4](Kurz *et al.* 1999)
[5](Alconada *et al.* 1995)
[6](Dembowski *et al.* 2001)
[7](Rapaport *et al.* 1998)
[8](Hönlinger *et al.* 1996)
[9](Esaki *et al.* 2004)

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Appendix 1. **Tom40 variant protein assembly *in vitro*.** 50μg of mitochondria were used in the import assays. Imports were performed at 0°C and 25°C for 20 min using ^{35}S-Met labeled Tom40 precursor variants. Following import, mitochondria were solubilized in 1% DIG, and electrophoresed on blue native gels. Proteins were transferred to PVDF, and exposed to X-ray film. Strains are also classified according to their assembly phenotype (see also Table 1). **A.** Wild type assembly of Tom40. **B.** Tom40 stops at the 250 kDa intermediate. **C.** Varying amounts of the 400 kDa form but with significant accumulation of the 250 kDa intermediate. **D.** Accumulation of the 100 kDa intermediate. **E.** Assembly resembles wild type.