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Tom40, the import channel of the mitochondrial outer membrane, plays an active role in sorting imported proteins

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The Translocase of the Outer Mitochondrial membrane (TOM complex) is centred on a channel, created by Tom40, serving as the only means of entry for proteins into the mitochondrion. Proteins destined for internal mitochondrial compartments interact subsequently with one of the two distinct protein Translocases of the Inner Mitochondrial membrane (TIM23 and TIM54 complexes) or follow specialized paths into the intermembrane space. To investigate the sorting of precursor proteins to these various sub-mitochondrial compartments, we created a library of *tom40* mutants and screened for alleles selectively corrupt in protein sorting. One of the *tom40* mutants, *tom40–97*, carries a single point mutation ($W^{243}R$) resulting in an ineffective transfer of precursors to the TIM23 complex. There is no defect on transfer of precursors to the TIM54 complex or insertion of proteins into the outer membrane. The Tom40 channel is not a passive pore, but plays an active role in protein sorting for all sub-mitochondrial locations.

Keywords: mitochondrion/protein sorting/TIM complex/TOM complex

Introduction

Mitochondria are essential organelles consisting of two discrete membranes: the mitochondrial outer membrane encloses a soluble intermembrane space and the mitochondrial inner membrane surrounding a protein-dense matrix space. Almost all mitochondrial proteins are encoded on nuclear genes, translated in the cytosol and imported by the mitochondria, to be sorted specifically to one of these sub-mitochondrial compartments (Neupert, 1997; Schatz, 1997; Pfanner and Geissler, 2001).

Proteins destined for all sub-mitochondrial compartments are inserted into or translocated across the outer mitochondrial membrane by the TOM complex (Translocase in the Outer Mitochondrial membrane), and can thereafter cross the inner membrane by various TIM complexes (Translocases in the Inner Mitochondrial membrane). The TOM complex consists of distinct membrane protein subunits: these include various receptor subunits that recognize and bind mitochondrial precursor proteins, and a core translocase composed of the central protein Tom40 together with a set of small, tightly associated accessory subunits (Neupert, 1997; Schatz, 1997; Voos *et al*., 1999; Gabriel *et al*., 2001). Electron microscopy, electrophysiology and functional assays all suggest protein translocation takes place through an ~20 Å channel formed by Tom40 (Vestweber *et al*., 1989; Hill *et al*., 1998; Ahting *et al*., 1999; Kanamori *et al*., 1997; Schwartz and Matouschek, 1999).

The mechanisms for sorting proteins to their intended sub-mitochondrial location remain poorly understood. From the TOM complex, some inner membrane proteins and all proteins destined for the mitochondrial matrix must be translocated to the TIM23 complex (Neupert, 1997; Voos *et al*., 1999). The receptor domain of the Tim23 subunit of this complex can bind precursor proteins (Bauer *et al*., 1996; Komiya *et al*., 1998; Donzeau *et al*., 2000; Truscott *et al*., 2001) and might, in principle, actively remove precursors from a passive TOM complex. A distinct set of precursor proteins, including the abundant carrier family of transporters, are instead recruited from the TOM complex by a small TIM shuttle for delivery to the TIM54 complex (Kübrich *et al*., 1998; Endres *et al*., 1999; Koehler *et al*., 1999). An alternate specialized route, such as that used by apo-cytochrome *c*, can entail transfer from Tom40 to the intermembrane space with assistance from proteins other than the TIM complexes (Diekert *et al*., 2001).

How are these transfer steps from the TOM complex mediated, and to what extent does the TOM complex actively sort precursors for interaction with subsequent complexes of the import machinery? *In vitro* assays show that Tom40 can provide binding 'sites' for a translocating precursor and assist movement of a precursor from the *cis* (or cytosolic) to the *trans* (intermembrane space) face of the TOM complex (Kanamori *et al*., 1997; Rapaport *et al*., 1997, 1998). This vectorial movement is also facilitated by the *cis* and *trans* receptor domains of Tom22 (Bolliger *et al*., 1995; Kanamori *et al*., 1997; Moczko *et al*., 1997). Subsequent release of the precursor from the TOM complex, and interaction with the appropriate TIM complex, is required to complete translocation across the outer membrane and enable sorting to one of the three inner compartments of the mitochondria.

To test whether active sorting of precursors has already occurred at the level of Tom40, we generated and searched a library of conditional *tom40* alleles. The mutant strains have discrete point mutations in Tom40, and *tom40–97* mutants (which carry the mutation $W^{243}\rightarrow R^{243}$) are defective for the transfer of precursors to the TIM23 complex. However, there is no defect in protein transfer to the TIM54 complex and protein insertion into the outer membrane proceeds efficiently into *tom40–97* mitochondria. Thus, a single point mutation in Tom40 selectively affects the ability of the translocase to discriminate precursors destined for one branch of the sorting scheme for import into mitochondria.

Table I. Twenty novel *tom40* mutants showing clear growth defects, with their non-permissive growth condition

Allele	Non-permissive growth condition	Suppressed by expression of		
		TOM6	*TOM7*	*TOM22*
tom40–47	glycerol at 37°C	+	+	+
tom40–92	glycerol	+	–	+
tom40–97	glycerol	–	–	–
tom40–111	glycerol at 14°C	+	+	–
tom40–132	37°C	–	–	–
tom40–139	37°C	+	–	+
tom40–149	glycerol	–	+	+
tom40–170	37°C	–	–	–
tom40–182	37°C	–	+	–
tom40–185	37°C	+	+	+
tom40–270	14°C	+	+	+
tom40–274	glycerol	+	+	+
tom40–288	37°C	–	–	–
tom40–302	37°C	–	–	–
tom40–321	glycerol	+	+	–
tom40–333	glycerol at 37°C	+	–	–
tom40–342	glycerol	+	+	+
tom40–347	glycerol	+	–	+
tom40–350	37°C	–	–	+
tom40–357	37°C	–	+	–
KKY3.3	37°C	+	–	–

Each of the mutants was transformed with a plasmid inducing overexpression of Tom6, Tom7 or Tom22 and plated for growth at the non-permissive condition. Those cases in which growth was restored are indicated '+'. The previously isolated *tom40* mutant KKY3.3 [Kassenbrock *et al*., 1993; also referred to as KKY3.3 (*tom40–3*) by Krimmer *et al*., 2001] was included in these experiments.

Results

A library of conditional tom40 mutants

PCR-mediated mutagenesis was used to generate a library of *tom40* mutants. Twenty of these had clear conditional growth defects and were selected for further study (Table I). Some of the alleles result in strict temperature sensitivity in growth on all media tested at 37°C (*tom40–132*, *tom40–139*, *tom40–170*, *tom40–182*, *tom40–185*, *tom40–270*, *tom40–288*, *tom40–302*, *tom40–350*, *tom40–357*) or at 14°C (*tom40–270*). Other alleles render respiratory defects that result in failure of cells to grow on plates with glycerol as the carbon source, either at all temperatures tested (Table I, glycerol) or at extreme temperatures only.

In a previous study, Kassenbrock *et al*. (1993) isolated the gene encoding Tom6 because overexpression of the *TOM6* gene suppressed a growth defect in the strain KKY3.3, carrying multiple mutations in the *TOM40* gene. The growth defects of many of the alleles we generated could be suppressed by overexpression of genes encoding discrete combinations of the Tom6, Tom7 or Tom22 subunits of the TOM core complex (Table I), suggesting these mutations might effect specific protein–protein interactions with Tom40 (Kassenbrock *et al*., 1993; Krimmer *et al*., 2001).

To screen the library for selective defects in protein import, mitochondria were prepared from each of the strains and immunoblot analysis was used to determine whether some mitochondrial proteins might have decreased steady-state levels in the *tom40* mutants. The mutant *tom40–97* had a clear defect in steady-state levels of several mitochondrial proteins and was further analysed in detail. The *tom40–97* mutant has a reduced growth rate on media with non-fermentable carbon sources, and the phenotype cannot be restored by over-expression of *TOM6*, *TOM7* or *TOM22*.

tom40–97 mutants have reduced steady-state levels of some mitochondrial proteins

Antibodies to a range of mitochondrial proteins were used in immunoblot analysis. Most proteins, including porin components of the TIM54 complex (Tim54 and Tim22) and the matrix-located HSP70 (mHSP70) were present at wild-type levels in *tom40–97* mitochondria (Figure 1A). While components of the TIM23 complex (Tim23 and Tim44) are slightly increased in the mutants, two proteins of the intermembrane space, cytochrome b_2 and the processed form of NADH–cytochrome b_5 reductase ($Mcr1^{IMS}$), were reduced in the *tom40–97* mutant (Figure 1A). The unprocessed form $Mcr1^{OM}$ in the outer membrane is accumulated in the mutants, where the translocated form $Mcr1^{IMS}$ is barely detectable. There is a very small, but consistently observed, decrease in the steady-state level of the matrix-localized protein $F_1\beta$, and a clear defect in import of an artificial matrix-targeted version of dihydrofolate reductase (DHFR, Figure 1A). Importantly, the steady-state level of Tom40 itself is not reduced in the *tom40–97* mutants, suggesting the mutant form of the protein is stable. Limited proteolysis of isolated mitochondria in the presence of detergent revealed the mutant form of Tom40 to be as stable as wild-type Tom40 (data not shown). Alternatively, when the isolated mitochondria were solubilized in digitonin and committed to blue native PAGE analysis, the detergent-solubilized TOM complex from *tom40–97* cells is as soluble in digitonin (data not shown) but observed to be less stable during electrophoresis (Figure 1B).

DNA sequencing revealed a single mutation, converting $W^{243} \rightarrow R^{243}$, in the coding sequence from the *tom40–97* mutants (see Materials and methods). Comparative analysis of Tom40 from the three species in which the protein has been functionally analysed, *Saccharomyces cerevisiae* (Baker *et al*., 1990), *Neurospora crassa* (Kiebler *et al*., 1990) and *Rattus norvegicus* (Suzuki *et al*., 2000), and a number of highly homologous sequences from plants and invertebrate animals reveals 13 blocks of highly conserved primary structure (represented graphically in Figure 1C). In a membrane-embedded protein like Tom40, these discrete blocks of homology might represent repetitive transmembrane strands or inter-strand loops and the mutation in *tom40–97* maps to a tryptophan residue (W^{243} in the *S.cerevisiae* sequence) adjacent to one of the blocks of conserved sequence. Our preliminary sequencing in this region reveals only two of the other 20 alleles have neighbouring mutations: $N^{252}S$ in *tom40–270* and $Q^{250}R$ in *tom40–347*. Western blots of cell extracts and protein import assays into mitochondria isolated from *tom40–270* and *tom40–347* cells revealed only minor defects; however, a severe and select defect was seen in *tom40–97*.

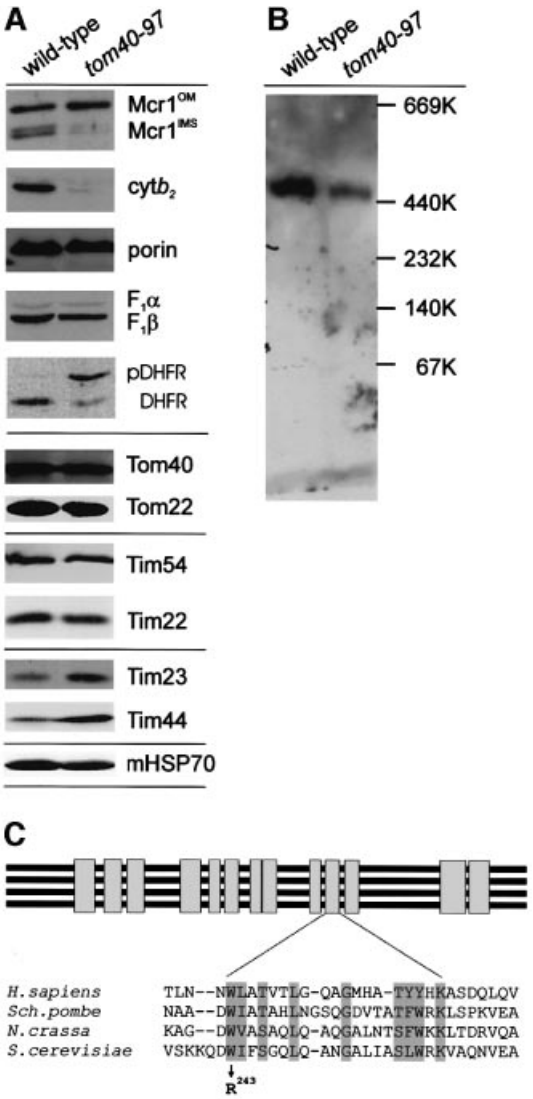

Fig. 1. The $W^{243}R$ mutation in *tom40–97* cells results in changes in the steady-state levels of some mitochondrial proteins. (**A**) Mitochondria (100 μg protein) isolated from wild-type and *tom40–97* mutant cells were analyzed by SDS–PAGE and immunoblotting to determine steady-state levels of the intermembrane space proteins cytochrome b_2 (Cyt b_2) and the processed form of NADH–cytochrome b_5 reductase (Mcr1), the outer membrane protein porin and the matrix-located proteins $F_1\beta$, $F_1\alpha$ and CoxIV–DHFR (George *et al*., 1998), and for various subunits of the TOM complex (Tom40, Tom22 and Tom20), the TIM23 complex (Tim23 and Tim44) and the TIM54 complex (Tim54 and Tim22). (**B**) Mitochondria (200 μg protein) were isolated from wild-type or *tom40–97* mutant cells, solubilized in digitonin and analysed by blue native PAGE. The migration of high molecular weight markers (Amersham) is indicated and the presence of the 450 kDa TOM holocomplex is shown after immunoblotting with antibodies recognizing Tom40. (**C**) ClustalW sequence analysis of Tom40 from various organisms (Macasev *et al*., 2000). Tom40 homologues were identified in plants, fungi, invertebrate and vertebrate animals by iterative BLAST analysis with short segments of *Nc*Tom40 and *Sc*Tom40. The sequence cluster revealed 13 blocks of homology as represented diagrammatically, and expanded in the four species (*Homo sapiens*, *Schizosaccharomyces pombe*, *N.crassa* and *S.cerevisiae*) shown. The arrow denotes W^{243} in the sequence of *Sc*Tom40, converted to R^{243} in the *tom40–97* mutant.

A select protein import defect can be measured in mitochondria isolated from tom40–97 cells

Mitochondria were isolated from wild-type and *tom40–97* mutant cells and incubated with proteins destined for the various mitochondrial sub-compartments. The precursor form of $F_1\beta$ is imported into the mutant mitochondria at <10% the wild-type rate (Figure 2A). Similarly, import of Su9–DHFR, measured from the processing of the precursor to its intermediate and mature forms shows a severe defect in the mutants (Figure 2B). Cytochrome b_2 is translocated across the outer membrane to the TIM23 complex and subsequently processed for release into the intermembrane space (Glick *et al*., 1992), and import of cytochrome b_2 is similarly diminished in mitochondria isolated from *tom40–97* cells (Figure 2C).

To be certain that the TIM23 complex of these *tom40–97* mutants is fully functional for later stages of protein translocation, 'mitoplasts' were prepared where the outer membrane of mitochondria is ruptured by osmotic shock, allowing precursor proteins direct access to the TIM complexes (Ohba and Schatz, 1987). The kinetics of import of Su9–DHFR into mitoplasts was similar in both wild-type or *tom40–97* preparations (Figure 2D). Thus, the transmembrane potential is intact and the TIM23 complex is functional in mitochondria of *tom40–97* cells, and the protein translocation defect observed is at the level of the TOM complex.

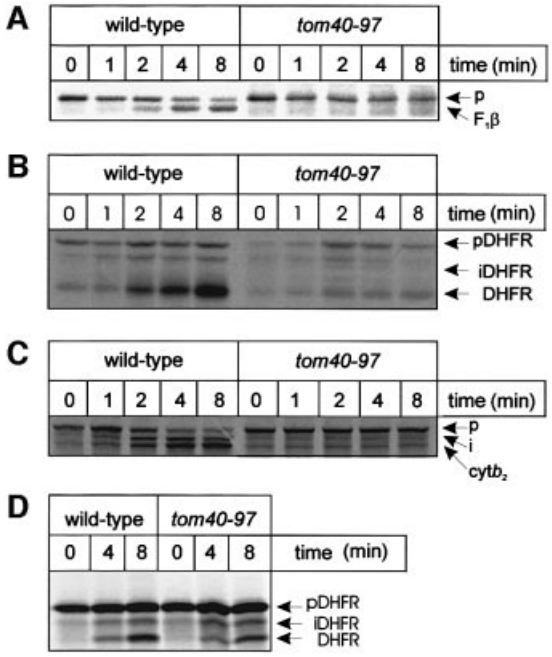

Fig. 2. Precursors using the TIM23 complex import slowly into mitochondria from *tom40–97* cells. (**A**) Mitochondria (25 μg of protein) were incubated with ^{35}S-labeled precursor form of $F_1\beta$ for the time indicated (min) and import stopped with CCCP. Samples were treated with 100 μg/ml trypsin and analyzed by SDS–PAGE and fluorography. $F_1\beta$ precursor (p) is cleaved to the mature form. (**B**) Mitochondria (25 μg of protein) were incubated with ^{35}S-labeled Su9–DHFR for the time indicated (min) and analysed by SDS–PAGE and fluorography. The protein is processed from precursor (p) to and intermediate form (i) before being processed again to reveal a mature protein. (**C**) Mitochondria (25 μg of protein) were incubated with ^{35}S-labeled precursor form of cytochrome b_2 for the time indicated (min) and analyzed by SDS–PAGE and fluorography, to resolve the precursor (p), intermediate form (i) and mature protein. (**D**) Mitochondria were converted to mitoplasts in hypotonic buffer, and the mitoplasts (25 μg protein) incubated with ^{35}S-labeled Su9–DHFR for the time indicated (min).

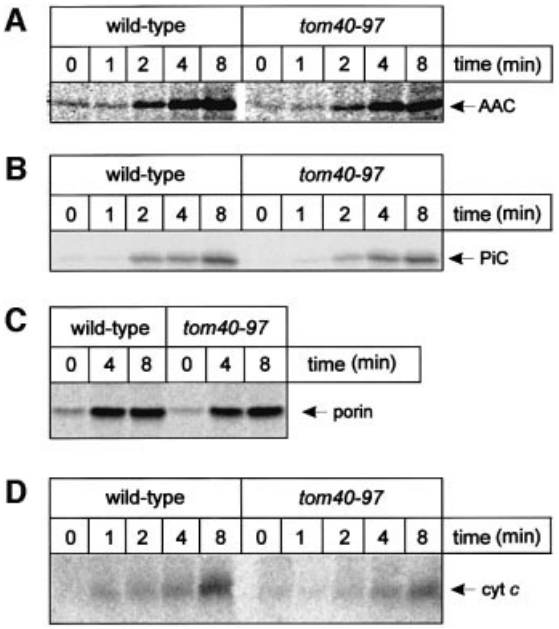

Fig. 3. Import of other precursors into mitochondria from *tom40–97* cells is not affected. (**A**) Mitochondria (25 μg of protein) were incubated with ^{35}S-labeled AAC for the time indicated, then import was stopped with CCCP and samples treated with 100 μg/ml trypsin to remove unimported material. (**B**) *In vitro* import of PiC, and (**D**) import of apo-cytochrome *c* were similarly assayed. (**C**) Mitochondria (25 μg of protein) were incubated with ^{35}S-labeled porin for the time indicated, and import stopped with Na_2CO_3 (pH 11.5). After centrifugation, to collect insoluble material including membrane sheets, and flotation to purify the membranes, porin integrated into the membranes was analysed by SDS–PAGE and fluorography (Krimmer *et al*., 2001).

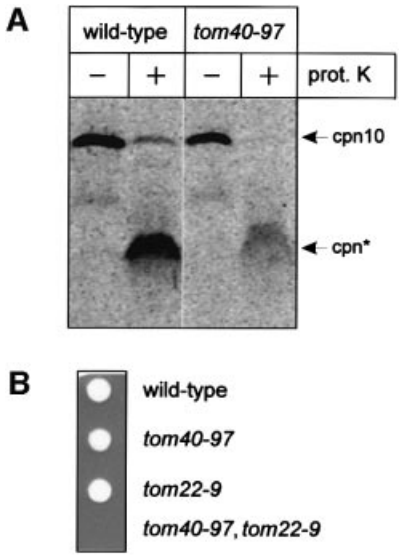

Fig. 4. Mutant *tom40–97* mitochondria cannot trap a precursor and require the function of the Tom22 intermembrane space domain. (**A**) Mitochondria (25 μg protein) isolated from wild-type or *tom40–97* mutant cells were pre-incubated with CCCP and then ^{35}S-labeled Cpn10 was added for 15 min. Where indicated (+) samples were treated with 100 μg/ml proteinase K, and then mitochondria were isolated, trapped and the precursor analyzed by SDS–PAGE and fluorography. The position of intact (Cpn10) and the fragment of Cpn10 protected from protease (cpn*) are indicated. (**B**) A heterozygous diploid strain was induced to sporulated and the meiotic progeny dissected onto plates of rich medium. The identity of the viable colonies was established by marker analysis.

The $W^{243}R$ mutation does not lead to a general block in the translocation pore. Import of the ADP/ATP carrier (AAC) into mitochondria from *tom40–97* cells occurs at a rate similar to wild type (Figure 3A). In addition, *tom40–97* mitochondria import the phosphate carrier (PiC), another protein of the mitochondrial inner membrane, at a rate close to that of wild-type mitochondria (Figure 3B). Porin also requires Tom40 for insertion into the outer membrane (Krimmer *et al*., 2001) and porin insertion is equivalent in wild-type or *tom40–97* mitochondria (Figure 3C). Apo-cytochrome *c* requires Tom40 for translocation across the outer membrane (Diekert *et al*., 2001), and while the $W^{243}R$ mutation does not prevent apo-cytochrome *c* import (Figure 3D), a decrease of ~35% is consistently observed in the import rate.

tom40–97 and the trans site for protein import into mitochondria

The transmembrane potential across the inner membrane can be dissipated chemically by pretreating mitochondria with CCCP or FCCP, and precursors *in transit* across the outer membrane are trapped, transiently, in contact with the TOM complex (Bolliger *et al*., 1995). Figure 4A depicts such a trapping experiment. If the [^{35}S]substrate precursor protein chaperonin 10 (Cpn10) is trapped in this way and the mitochondria then treated with proteinase K, an ~3 kDa fragment (cpn*) representing the N-terminal presequence can be visualized (the only ^{35}S-labelled methionine residues in cpn10 are Met^1 and Met^{15}). The cpn* fragment represents the precursor molecules for which the presequence had entered the TOM complex in wild-type mitochondria to be protected from trypsin. The Cpn10 precursor molecules trapped on mitochondria isolated from *tom40–97* cannot be stably trapped in the complex to be protected from trypsin (Figure 4A).

This phenotype is similar to that seen in mitochondria isolated from *tom22–9* mutants, where the intermembrane space domain of Tom22 has been deleted (Bolliger *et al*., 1995). Accordingly, a cross was set up between haploid cells carrying the *tom40–97* mutation and haploid *tom22–9* mutants. The resulting heterozygous diploid (*TOM22*/*tom22–9*, *TOM40*/*tom40–97*) yeast strain was sporulated and the meiotic haploid progeny dissected onto rich growth media. While the wild-type daughter cells and each of progeny carrying single mutations germinated and grew with the predicted phenotypes, the cell inheriting the double combination *tom40–97*, *tom22–9* is inviable (Figure 4B).

Discussion

Tom40 is a sorting station for mitochondrial protein import

The final events of protein import into mitochondria are mediated by interactions with internal components of the import machinery in processes using ATP hydrolysis and the transmembrane potential across the inner membrane (Pfanner and Geissler, 2001; Neupert and Brunner, 2002), while translocation through the TOM complex does not require ATP hydrolysis or a transmembrane potential (Neupert, 1997; Schatz, 1997; Matouschek *et al*., 2000). A fundamental question has been whether or not the TOM

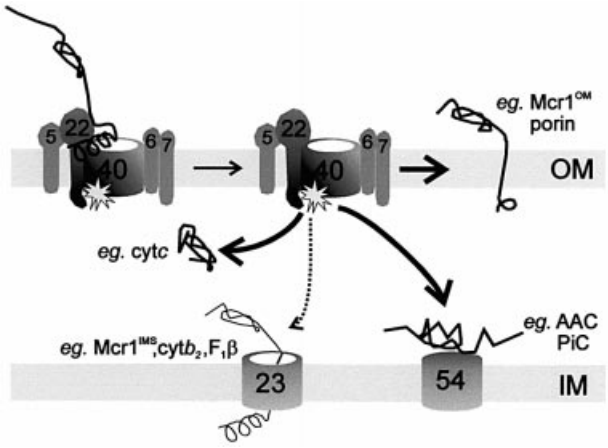

Fig. 5. Tom40 functions as a protein sorting station. Precursor proteins bound to the cytosolic face of the TOM complex can be inserted into the outer membrane or translocated through Tom40 for transfer to the TIM54 complex, or to the TIM23 complex. A deletion of the intermembrane space domain of Tom22 or conversion of $W^{243}R$ in Tom40 effects transfer of precursors to the TIM23 complex. A combination of these two mutations is lethal. The steady-state levels of $Mcr1^{OM}$ accumulate at the expense of $Mcr1^{IMS}$ as a result of the $W^{243}R$ mutation.

complex functions only as a passive pore for protein translocation. Our data suggest that Tom40 functions as a sophisticated sorting station, distinguishing substrates for insertion into the outer membrane, translocation into the intermembrane space, translocation and transfer to the TIM54 complex or translocation and transfer to the TIM23 complex (Figure 5). The point mutation W243R discretely blocks one of these pathways, but not the other three.

In addition to the *in vitro* data, the accumulated steady-state levels of the ~35 kDa $Mcr1^{OM}$ in *tom40–97* cells demonstrate the *in vivo* consequences of failed translocation. In wild-type cells, a proportion of Mcr1 is normally inserted into the outer membrane, while a proportion is translocated through the TOM complex and to the TIM23 complex. After reaching the TIM23 complex, Mcr1 is processed by the Imp protease to release ~32 kDa $Mcr1^{IMS}$ into the intermembrane space (Hahne *et al*., 1994; Haucke *et al*., 1997). Essentially no processed $Mcr1^{IMS}$ is found in *tom40–97* cells, with $Mcr1^{OM}$ accumulating instead.

AAC is an example of a precursor sorted from Tom40 to the TIM54 complex. By manipulating *in vitro* assay conditions, the normal import of AAC can be interrupted, such that AAC is accumulated in the TOM complex almost completely protected from exogenously added protease, yet exposed to the intermembrane space (Ryan *et al*., 1999). To achieve transfer from this position to the TIM54 complex, AAC does not require the presence of the Tom22 *trans* domain (Kübrich *et al*., 1998), nor is this transfer of AAC perturbed by the $W^{243}R$ mutation. Taken together, functional analysis of the *tom40–97* mutant suggests the $W^{243}R$ mutation selectively interferes with the contribution Tom40 normally makes to a discrete precursor-binding site for protein transfer to the TIM23 complex.

Consequences of the $W^{243}R$ mutation

The TOM complex is asymmetrical with respect to precursor protein binding sites. On the cytosolic surface, *cis* receptor domains contributed by Tom20, Tom22 and Tom70 cooperate to bind precursor proteins (Söllner *et al*., 1992; Kiebler *et al*., 1993; Lithgow *et al*., 1994, Bolliger *et al*., 1995; Mayer *et al*., 1995; Kanamori *et al*., 1997; Brix *et al*., 1998; Kurz *et al*., 1999; Wiedemann *et al*., 2001). Once within the channel, targeting sequences gain access to what has been operationally defined as the *trans* site of the TOM complex. There is strong evidence from cross-linking studies and analysis of deletion mutants that both the C-terminal domain of Tom22 oriented to the intermembrane space, and a surface or domain of Tom40 contribute to this *trans* site (Bolliger *et al*., 1995; Kanamori *et al*., 1997; Moczko *et al*., 1997; Rapaport *et al*., 1997, 1998).

As yet, we have no structural details for Tom40 for the final interpretation of how the $W^{243}R$ mutation effects binding and transfer of precursors destined to the TIM23 complex. However, structural and theoretical data from other membrane proteins suggest tryptophan residues are preferentially found as the interfacial residue at the lipid–aqueous interface in α-helix or β-strand transmembrane segments (Reithmeier, 1995; Yuen *et al*., 2000). Mutational scanning analysis has shown tryptophan can determine the position of a transmembrane segment within the phospholipid plane, in effect pushing the segment into the bilayer in order to register the tryptophan at the lipid–aqueous interface (Braun and von Heijne, 1999; de Planque *et al*., 2001). Arginine residues can also be readily accommodated towards the ends of transmembrane segments, where the side-chain can be oriented snorkel-like to expose the terminal amine group to the aqueous environment (Monne *et al*., 1998).

Given that there are no effects on AAC import or porin insertion into *tom40–97* mitochondria, no effect on the stability of Tom40($W^{243}R$) as judged by protease-susceptibility and only a partial decrease in stability of the 450 kDa holocomplex after detergent solubilization of *tom40–97* mitochondria, we suspect that the $W^{243}R$ mutation does not grossly effect the structure of Tom40. Instead, we suggest R^{243} might directly disturb a precursor protein binding surface and we propose the Tom40 subunit of the translocase can discriminate the intended destinations for the wide array of precursors imported into mitochondria. The mutant alleles of *tom40* described here provide a means to map various sites functionally important within the TOM complex, both for precursor passage and the docking of the accessory subunits Tom6, Tom7 and Tom22.

Interestingly, the steady-state levels of subunits of the TIM23 complex, Tim23 and Tim44, are always increased in *tom40–97* mutants and yet not in other *tom40* alleles we have analysed. This regulation of the relative numbers of translocase complexes is presumably an attempt by the mutant cells to compensate for the decrease in protein traffic to the TIM23 complex. The mechanism for such regulation, and the extent to which the TOM and TIM23 complexes physically communicate with each other, remains to be studied.

Materials and methods

PCR mutagenesis on the TOM40 gene

Yeast *tom40* mutants were constructed using low-fidelity PCR to mutate a fragment of DNA corresponding to the *TOM40* gene followed by

recombination of the mutant allele onto a plasmid *in vivo* (Muhlrad *et al*., 1992; Staples and Dieckmann, 1993; Koehler *et al*., 1998). A plasmid encoding the *TOM40* gene (including 5′ and 3′ flanking regions) in pBluescript was a kind gift from Kevin Baker. The fragment was amplified by PCR in independent reactions containing 0.6–1.0 mM $MgCl_2$ and 0.1–0.2 mM $MnCl_2$, using primers complementary to regions ~200 nucleotides away from either side of the multiple cloning region (primer sequences CK3145: 5′-GGCTGCGCAACTGTTG-3′ and CK3143: 5′-CTGAGCGCAACGCAAT-3′). The amplified fragments were combined and co-transformed with linearized centromeric vector pRS315 into the yeast strain YKB14–1a [*tom40*::*HIS4*, *his4-519*, *leu2-3 112*, Δ*ura3, ade2*, YEplac42R (*TOM40*::*URA3*)]. Leu^+ transformants were selected at 25°C and screened for growth at 14, 25 and 37°C on minimal glucose media containing 5-fluoroorotic acid and appropriate growth supplements. A collection of ~100 mutants was screened for growth defects. Complete sequencing of three independent clones of the pRS315-*tom40–97* plasmid determined the mutation coding for a single amino acid substitution ($W^{243}R$).

Protein import

Mitochondria were isolated according to published procedures (Daum *et al*., 1982), but with 18.5% (w/v) Nycondenz used at the bottom of the density gradient. Mitoplasts were prepared as described by Glick *et al*. (1992). Mitochondria (25 μg protein) isolated from either wild-type or mutant strains were resuspended in 100 μl of import buffer (0.6 M sorbitol, 50 mM HEPES, 2 mM potassium phosphate, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT). Precursor proteins were translated in nuclease-treated rabbit reticulocyte lysate (Promega) containing [^{35}S]methionine. ^{35}S-labelled precursor proteins were added to mitochondria in import buffer and incubated at 25°C for the appropriate time (Glick *et al*., 1992). Where indicated, import was terminated by addition of CCCP to a final concentration 100 μM and trypsin or proteinase K added to remove protein that failed to be imported.

Miscellaneous

Strains of *S.cerevisiae* were grown at 30°C on YPAD [2% (w/v) glucose, 1% (w/v) yeast extract, 2% (w/v) peptone supplemented with adenine sulfate] grown until late log phase and harvested by centrifugation. Samples of mitochondrial protein (100 μg) were separated by Tris–glycine SDS–PAGE, Tris–tricine SDS–PAGE or blue native PAGE, and western blots were carried out according to published methods (Beilharz *et al*., 1998; Ryan *et al*., 1999; Sambrook and Russell, 2001).

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