

Biogenesis of Porin of the Outer Mitochondrial Membrane Involves an Import Pathway via Receptors and the General Import Pore of the TOM Complex

Thomas Krimmer,^{**‡} Doron Rapaport,[§] Michael T. Ryan,^{*} Chris Meisinger,^{*} C. Kenneth Kassenbrock,^{||} Elizabeth Blachly-Dyson,[¶] Michael Forte,[¶] Michael G. Douglas,^{||} Walter Neupert,[§] Frank E. Nargang,^{**} and Nikolaus Pfanner^{*}

^{*}Institute for Biochemistry and Molecular Biology, and [‡]Faculty for Biology, University of Freiburg, D-79104 Freiburg, Germany; [§]Institute for Physiological Chemistry, Munich University, D-80336 Munich, Germany; ^{||}Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599; [¶]Oregon Health Sciences University, Portland, Oregon 97201; and ^{**}Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada

Abstract. Porin, also termed the voltage-dependent anion channel, is the most abundant protein of the mitochondrial outer membrane. The process of import and assembly of the protein is known to be dependent on the surface receptor Tom20, but the requirement for other mitochondrial proteins remains controversial. We have used mitochondria from *Neurospora crassa* and *Saccharomyces cerevisiae* to analyze the import pathway of porin. Import of porin into isolated mitochondria in which the outer membrane has been opened is inhibited despite similar levels of Tom20 as in intact mitochondria. A matrix-destined precursor and the porin precursor compete for the same translocation sites in both normal mitochondria and mitochondria whose surface receptors have been removed, suggesting that both precursors utilize the general import pore. Using an assay established to monitor the assembly of in vitro–imported porin into

preexisting porin complexes we have shown that besides Tom20, the biogenesis of porin depends on the central receptor Tom22, as well as Tom5 and Tom7 of the general import pore complex (translocase of the outer mitochondrial membrane [TOM] core complex). The characterization of two new mutant alleles of the essential pore protein Tom40 demonstrates that the import of porin also requires a functional Tom40. Moreover, the porin precursor can be cross-linked to Tom20, Tom22, and Tom40 on its import pathway. We conclude that import of porin does not proceed through the action of Tom20 alone, but requires an intact outer membrane and involves at least four more subunits of the TOM machinery, including the general import pore.

Key words: mitochondria • protein sorting • porin • *Neurospora crassa* • *Saccharomyces cerevisiae*

Introduction

Most mitochondrial precursor proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes and im-

ported into the organelle via the action of complexes comprised of multisubunit translocases in the outer (TOM)¹ and the inner (TIM) mitochondrial membranes (Neupert, 1997; Pfanner et al., 1997; Jensen and Johnson, 1999; Koehler et al., 1999; Bauer et al., 2000). The TOM complex contains surface receptors for the specific recognition of precursor proteins and a general import/insertion pore (GIP) that mediates translocation of precursor proteins into or across the outer membrane. The surface receptors Tom70, Tom22, and Tom20 expose large domains to the cytosol. Precursor proteins are initially recognized by

T. Krimmer and D. Rapaport contributed equally to this work.

Address correspondence to Frank E. Nargang and Nikolaus Pfanner. F.E. Nargang, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2F0 Canada. Tel.: (780) 492-5375. Fax: (780) 492-1903. E-mail: frank.nargang@ualberta.ca

N. Pfanner, Institut für Biochemie und Molekularbiologie, Universität Freiburg, Hermann-Herder-Straße 7, D-79104 Freiburg, Germany. Tel.: 49-761-203-5224. Fax: 49-761-203-5261. E-mail: pfanner@uni-freiburg.de

D. Rapaport's present address is Department of Biochemistry, The Hebrew University, Hadassah Medical School, Jerusalem 91120, Israel.

M.T. Ryan's present address is Department of Biochemistry, La Trobe University 3086, Melbourne, Australia.

C.K. Kassenbrock's present address is Department of Pathology, University of Colorado Health Sciences Center, 4200 East 9th Ave., Denver, CO 80262.

M.G. Douglas's present address is Novacyl, Inc., 1816 Lackland Hill Parkway Suite 220, St. Louis, MO 63146.

¹Abbreviations used in this paper: BN-PAGE, blue native gel electrophoresis; CCHL, cytochrome *c* heme lyase; DHFR, dihydrofolate reductase; DSG, disuccinimidyl glutarate; F₁β, the β subunit of the mitochondrial ATPase; GIP, general import/insertion pore; OMV, outer membrane vesicle; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane.

Tom20 or Tom70 and are transferred to the central receptor Tom22. The pore-forming component Tom40 and the three small Tom proteins Tom7, Tom6, and Tom5 are integrally embedded in the mitochondrial outer membrane. Together with Tom22, the latter four Tom proteins form the stable ~400-kD core of the outer membrane translocase, termed the TOM core complex or GIP complex (Dekker et al., 1998; Hill et al., 1998; Künkele et al., 1998; Ahting et al., 1999; van Wilpe et al., 1999). Matrix-destined precursors are targeted to mitochondria by a cleavable NH₂-terminal presequence, which initially interacts with the receptors of the TOM complex. However, many mitochondrial precursors destined for the inner membrane, the intermembrane space, or the outer membrane are targeted to mitochondria without the aid of a presequence. In several of these proteins such as Tom70 (McBride et al., 1992), Tom22 (Rodriguez-Cousino et al., 1998), BCS1 (Fölsch et al., 1996), and cytochrome *c* heme lyase (CCHL) (Diekert et al., 1999), a mitochondrial targeting/assembly signal has been identified within the sequence of the mature protein.

Porin, also known as voltage-dependent anion-selective channel (VDAC), is the most abundant protein of the mitochondrial outer membrane and is imported without the aid of a presequence. It is predicted to exist as a β barrel structure similar to its bacterial counterparts (Benz, 1989; Mannella et al., 1996) and forms a pore that allows the passage of small molecules across the membrane. Attempts to characterize possible targeting signals in the porin precursor protein have identified certain residues and regions of the protein that are involved in its assembly (Hamajima et al., 1988; Smith et al., 1994; Court et al., 1996). However, very little is known about the mechanism whereby the highly structured porin protein is inserted and properly assembled in the membrane. Although there is no doubt that the import of mitochondrial porin requires the surface receptor Tom20 (Kleene et al., 1987; Pfaller and Neupert, 1987; Söllner et al., 1989; Harkness et al., 1994; Schleiff et al., 1997, 1999), the involvement of other mitochondrial factors, including other Tom proteins and the GIP complex, is a subject of debate. On one hand, the import of precursors destined to various mitochondrial compartments could be inhibited by chemical amounts of water-soluble porin at a stage in the import pathway beyond the protease-accessible binding sites. It has been suggested that porin utilizes components of the GIP complex of the outer membrane (Pfaller et al., 1988; Hönlinger et

al., 1996; Dietmeier et al., 1997; van Wilpe et al., 1999). On the other hand, Schleiff et al. (1999) have recently reported that Tom20 alone is sufficient for the insertion and assembly of porin into mitochondria and lipid vesicles. Blocking of the mitochondrial import pore as well as mutations in Tom40 did not inhibit the import of porin. Schleiff et al. (1999) thus proposed a new mechanism of mitochondrial protein import whereby the receptor Tom20 directly catalyzes the insertion and assembly of porin into the outer membrane. The novel mechanism proposed by Schleiff et al. (1999) raises substantial doubts not only about previous studies on the import of porin (Hönlinger et al., 1996; Dietmeier et al., 1997; van Wilpe et al., 1999) but also on the concept of the GIP in general since water-soluble porin was used as the model precursor protein to define the GIP (Pfaller et al., 1988).

A clarification of the import pathway of porin will thus be of fundamental importance for our understanding of mitochondrial protein import. In the present study, we performed a detailed characterization of porin import in *Neurospora crassa* and the yeast *Saccharomyces cerevisiae* to directly determine the role of GIP and individual Tom proteins in import of porin. We have established an assay specific for the assembly of porin imported into isolated yeast mitochondria. Using a collection of mutant mitochondria, competition of precursor proteins for GIP, and cross-linking of porin in transit, we demonstrate that Tom20 alone is not sufficient for porin import. The physiological biogenesis pathway of porin into mitochondria involves several different Tom proteins including the pore Tom40.

Materials and Methods

Neurospora Strains and Media

Growth and handling of *N. crassa* were as described in Davis and De Serres (1970). Strains used in this study were 74-OR23-1 (*A*), 76-26 (*a*, *his-3*, *mtrR*), and 861 (*a*, *his-3*, *mtrR*, *tom22::hygR*, *tom22⁸⁶¹* [ectopic copy]). The *tom22⁸⁶¹* allele contains mutations resulting in several amino acid substitutions in its cytosolic domain (Nargang et al., 1998). The outer mitochondrial membrane of the 861 strain is easily broken during standard mitochondrial isolation procedures.

Yeast Strains and Media

The *S. cerevisiae* strains used here are listed in Table I. Cells were grown on YPG (1% [wt/vol] yeast extract, 2% [wt/vol] bactopectone, 3% [wt/vol] glycerol) or YPD (1% [wt/vol] yeast extract, 2% [wt/vol] bactopectone, 2% [wt/vol] dextrose) as described (Daum et al., 1982).

Table I. *S. cerevisiae* Strains Used in This Study

| Strain | Genotype | Reference |
|---|--|--|
| YPH499 (WT) | <i>MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801</i> | Sikorski and Hieter, 1989 |
| MM112-C (<i>tom20Δ</i>) | <i>MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom20::URA3 (Yep13-TOM22)</i> | Moczko et al., 1994; Hönlinger et al., 1995; Dekker et al., 1998 |
| AH101 (<i>tom7Δ</i>) | <i>MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom7::TRP1</i> | Hönlinger et al., 1996 |
| OL201 (<i>tom22Δ</i>) | <i>MATa his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3 rho⁰</i> | van Wilpe et al., 1999 |
| KD56 (<i>tom5Δ</i>) | <i>MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom5::HIS3</i> | Dietmeier et al., 1997 |
| MM307 (<i>tom6Δ</i>) | <i>MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom6::URA3</i> | Alconada et al., 1995 |
| M3 (WT) | <i>MATa lys2 his4 trp1 ade2 leu2 ura3</i> | Blachly-Dyson et al., 1990, 1997 |
| M22-2 (<i>por1Δ</i>) | <i>MATa lys2 his4 trp1 ade2 leu2 ura3 por1::LEU2</i> | Blachly-Dyson et al., 1990, 1997 |
| M3-2 (<i>por2Δ</i>) | <i>MATa lys2 his4 trp1 ade2 leu2 ura3 por2::TRP1</i> | Blachly-Dyson et al., 1997 |
| KKY3 | <i>MATa his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52 tom40::HIS3 (pRS316-TOM40)</i> | Kassenbrock et al., 1993 |
| KKY3.2 (TK3) (<i>tom40-2</i>) | <i>MATa his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52 tom40::HIS3 (pRS314-tom40-2)</i> | Kassenbrock et al., 1993 |
| KKY3.3 (TK4) (<i>tom40-3</i>) | <i>MATa his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52 tom40::HIS3 (pRS314-tom40-3)</i> | Kassenbrock et al., 1993 |
| KKY3.4 (TK5) (<i>tom40-4</i>) | <i>MATa his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52 tom40::HIS3 (pRS314-tom40-4)</i> | Kassenbrock et al., 1993 |
| KKY3.7 (TK8) (WT) | <i>MATa his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52 tom40::HIS3 (pRS314-TOM40)</i> | This study |

The strains KKY3.2, KKY3.3, and KKY3.4 carry *tom40* alleles on the plasmid pRS314, whereas the chromosomal copy of *TOM40* has been deleted (Kassenbrock et al., 1993). All mutant alleles were analyzed by DNA sequencing and found to contain several independent mutations. To generate a corresponding wild-type strain (KKY3.7) that similarly expresses *TOM40* from the plasmid, a PCR product of *TOM40* including 334 bp upstream and 345 bp downstream of the *TOM40*-ORF as well as additional restriction sites for XhoI (3'-end) and BamHI (5'-end) was inserted into XhoI/BamHI cut pRS314 and transferred into KKY3 by 5-fluoroorotic acid-based plasmid shuffling.

Protein Import into Isolated Mitochondria

The procedures for standard isolation of *N. crassa* mitochondria (Pfanner and Neupert, 1985) and in vitro import of radiolabeled mitochondrial precursors (Harkness et al., 1994) using trypsin pretreatment in control lanes (Mayer et al., 1993) were as described. In some experiments, chemical amounts of a chimeric precursor were used. The chimeric precursor consists of the NH₂-terminal 69 amino acids of the presequence for *N. crassa* ATPase subunit 9 fused to the coding sequence of mouse dihydrofolate reductase (DHFR) (pSu9-DHFR) (Pfanner et al., 1987). The fusion protein with a hexahistidyl tag at the COOH terminus (pSu9[1-69]-DHFR-his₆) was purified by Ni-NTA affinity chromatography from extracts of the *E. coli* strain BL21 carrying the pQE60-pSu9(1-69)-DHFR-his₆ overexpression vector.

Yeast mitochondria were isolated according to published methods (Daum et al., 1982; Rassow, 1999), resuspended in SEM buffer (250 mM sucrose, 1 mM ethylenediaminetetraacetic acid [EDTA], 10 mM morpholinopropanesulfonic acid [MOPS]-KOH, pH 7.2) to a final concentration of 5 mg protein/ml, and stored at -80°C. Radiolabeled porin was synthesized by in vitro translation in rabbit reticulocyte lysate (Amersham Pharmacia Biotech) in the presence of [³⁵S]methionine/cysteine after in vitro transcription using SP6 polymerase (Promega) (Söllner et al., 1991; Rassow, 1999). The fusion protein b₂-DHFR, consisting of the 167 NH₂-terminal amino acids of the precursor of cytochrome b₂ (with a 19-residue deletion in the sorting sequence) and entire DHFR (b₂[167]_{Δ19}-DHFR), was expressed in *E. coli* and purified as described (Dekker et al., 1997). Import into isolated mitochondria was performed in BSA-containing buffer (3% [wt/vol] fatty-acid free BSA, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS-KOH, pH 7.2, 2 mM NADH). Mitochondria (25 μg protein/100 μl reaction) were added and incubated for 2 min at 25°C before starting the import reaction by adding 5 μl reticulocyte lysate or 1 μg b₂-DHFR and ATP (final concentration 2 mM). After incubation for 5–30 min at 25°C, the import reaction was stopped by incubation for 10 min on ice. Mitochondria were subsequently reisolated and washed once with SEM buffer.

Assessment of Mitochondrial Outer Membrane Integrity

Samples of the *N. crassa* mitochondria isolated for import experiments were used to assess outer membrane integrity. The mitochondria (30 μg) were suspended in 100 μl of SEM buffer and incubated with proteinase K (120 μg/ml) at 0°C for 15 min. The reactions were stopped by direct addition of PMSF to a final concentration of 3 mM. Proteins were immediately precipitated with trichloroacetic acid, pelleted by centrifugation, washed

with 1 ml of acetone, air dried, suspended in cracking buffer (62.5 mM Tris-HCl, pH 6.8; 2.5% [wt/vol] SDS; 5% [vol/vol] β-mercaptoethanol; 5% [wt/vol] sucrose), and boiled for 3 min. Samples were separated by SDS-PAGE, blotted onto nitrocellulose, and proteins from different mitochondrial compartments were detected using specific antisera. The susceptibility of the intermembrane space protein CCHL to proteinase K digestion was used as an indicator of outer membrane integrity.

Blue Native-PAGE

Blue native (BN)-PAGE was performed as described previously (Schägger and von Jagow, 1991; Schägger et al., 1994; Dekker et al., 1997, 1998). In brief, mitochondrial pellets from import reactions (50 μg of protein) were lysed in 45 μl ice cold digitonin buffer (1% [wt/vol] digitonin, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol, 1 mM PMSF) by carefully resuspending 10 times and incubating for 10 min on ice. After separating insoluble material through a clarifying spin (15 min, 16,000 g), 5 μl of sample buffer was added (5% [wt/vol] Coomassie brilliant blue G-250, 100 mM bis-tris, pH 7.0, 500 mM ε-aminocaproic acid), and the samples were electrophoresed through a 6–16% polyacrylamide gradient gel at 4°C. The gel was then either stained with Coomassie brilliant blue G-250 and dried, or proteins were transferred onto PVDF membranes by semidry blotting.

Cross-Linking and Immunoprecipitation

For cross-linking experiments, radiolabeled precursor was incubated with isolated outer membrane vesicles (OMVs) that were prepared according to Mayer et al. (1993) from *N. crassa* strain 74-OR-1A. After an initial incubation for 2 min on ice, the cross-linking reagent disuccinimidyl glutarate (DSG) (Pierce Chemical Co.) was added for 40 min at 0°C. Excess cross-linker was quenched by the addition of 80 mM glycine (pH 8.0) and incubation for 15 min at 0°C. OMVs were reisolated by centrifugation (20 min, 225,000 g), and aliquots, from before and after addition of the cross-linking reagent, were analyzed. For immunoprecipitation, samples that were incubated with DSG were dissolved in lysis buffer (1% [wt/vol] SDS, 0.5% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.2) and incubated for 5 min at 25°C. The lysed material was diluted 40-fold with lysis buffer lacking SDS and subjected to a clarifying spin (15 min, 20,000 g). The supernatant was incubated with antibodies that were coupled to protein A-Sepharose beads.

Miscellaneous Methods

Separation of mitochondrial proteins by SDS-PAGE (Laemmli, 1970) and determination of mitochondrial protein concentration (Bradford, 1976) were performed using previously published procedures. Synthesis and radiolabeling of mitochondrial precursor proteins was performed with rabbit reticulocyte lysate (Amersham Pharmacia Biotech) or the TNT-coupled reticulocyte lysate system (Promega) (Rassow, 1999). Carbonate extraction was performed using a published protocol (Hönlinger et al., 1996). Western blotting was performed as previously described (Harlow and Lane, 1999), and, after immunodecoration, proteins were detected by a chemiluminescent system. Bands from autoradiographs were quantified using a Bio-Rad imaging densitometer (GS-670) and a Molecular Dynam-

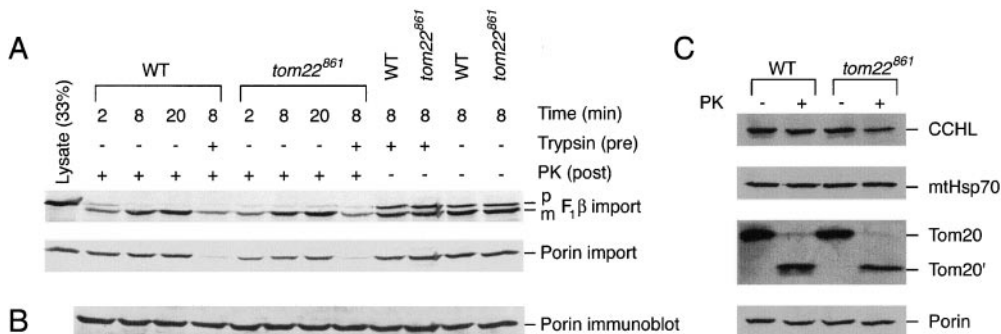


Figure 1. Import of F₁β and porin into *N. crassa* mitochondria with damaged outer membranes. (A) Mitochondria were isolated from mutant strain 861 (*tom22⁸⁶¹*) and the control strain 76-26 (WT) using standard procedures. Import of radiolabeled precursor proteins was conducted at 20°C for the indicated times. After the import reactions, mitochondria were

reisolated and subjected to SDS-PAGE. The gels were blotted to nitrocellulose and subjected to autoradiography. The leftmost lane for each precursor contained 33% of the input lysate used in each import reaction. The precursor (p) and mature (m) forms of F₁β are indicated. (B) After phosphorimaging of the imported proteins, the blot was decorated with antiserum to porin. (C) Effect of proteinase K on proteins from different submitochondrial compartments. Isolated mitochondria were either digested with proteinase K as described in Materials and Methods or were incubated at 0°C for 15 min without proteinase K. After reisolation, electrophoresis, and blotting, the membranes were decorated with antiserum to the indicated proteins. Tom20', proteolytic fragment of Tom20. PK, proteinase K.

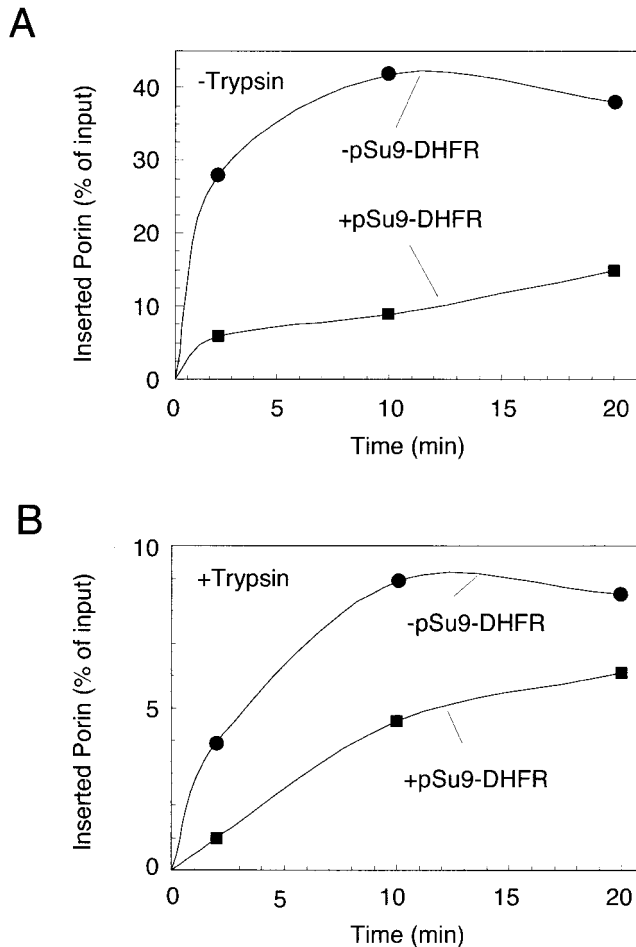


Figure 2. Insertion of porin is inhibited by blocking the translocation pore with import intermediates. *N. crassa* mitochondria (30 µg protein per import reaction) in import buffer containing 2 mM NADH, 2 mM ATP, and 37 µM carbonyl cyanide *m*-chlorophenylhydrazone were incubated for 15 min at 0°C in the absence (A) or presence (B) of 20 µg/ml trypsin. After inactivation of the trypsin by soybean trypsin inhibitor, each sample was split into two, and one half was incubated for 10 min at 0°C with 3.2 µM pSu9(1–69)-DHFR. Radiolabeled porin precursor was then added and further incubated at 15°C (A) or 25°C (B) for the indicated periods. At the end of the import reactions proteinase K (100 µg/ml) was added, proteins were analyzed by SDS-PAGE, and imported porin was quantified.

ics or Fuji PhosphorImager. Sequence alignment was performed with the software MegAlign from DNASTar using the clustal algorithm.

Results

Reduction of Porin Import into Mitochondria with a Fragile Outer Membrane

The mitochondrial outer membrane of certain mutant strains of *N. crassa* is easily broken during standard mitochondrial isolation procedures (Nargang et al., 1998; Grad et al., 1999). In such mitochondria, we found that most precursor proteins were imported with the same efficiency as in mitochondria with intact outer membranes. An exception was the precursor of porin, which was imported at reduced levels. This is shown in Fig. 1 A where mitochondria, isolated by standard procedures from a control strain

(WT) and a *tom22* mutant strain susceptible to opening of the outer membrane during isolation under standard conditions (*tom22*⁸⁶¹) (Nargang et al., 1998), were used to assess the import of the precursors of the β subunit of the mitochondrial ATPase (F₁β) and porin. The import of F₁β was virtually indistinguishable in the two strains, whereas porin import was significantly reduced in the mutant. Quantification of the levels of import in *tom22*⁸⁶¹ relative to the control strain in three separate experiments gave averages of 91% for F₁β and 34% for porin.

To examine mitochondria for outer membrane damage in the preparations used for the import experiments, we determined the levels of proteins from different mitochondrial compartments after the addition of proteinase K. The level of the soluble intermembrane space protein, CCHL, served as a measure for the degree of damage to the outer membrane. Broken outer membranes allow access of externally added proteinase K to the intermembrane space, thereby reducing the amount of CCHL. As shown in the upper panel of Fig. 1 C, the level of CCHL in control mitochondria was virtually unaffected by added proteinase K (13% reduction), whereas in mutant mitochondria the proteinase had a much greater effect (53% reduction). These data demonstrate that little or no damage has occurred to the outer membrane of the control mitochondria, whereas the integrity of the outer membrane in the mutant mitochondria isolated under standard conditions was considerably reduced. The level of a matrix protein, mtHsp70, was unaffected by treatment of mitochondria with proteinase K in both strains, indicating that the inner membrane was intact (Fig. 1 C, second panel). The large hydrophilic domain of the Tom20 receptor protein is exposed to the cytosol and its digestion to characteristic proteolytic fragments (Tom20') serves as a control for the effectiveness of the proteinase digestion in both strains (Fig. 1 C, third panel). Levels of the outer membrane proteins Tom20 and porin (Fig. 1 C, lower panel) were similar in the control mitochondria and the damaged mutant mitochondria, confirming that no significant amount of the outer membrane was lost during isolation of mitochondria from the mutant strain. Therefore, the increased accessibility of the intermembrane space protein CCHL to proteinase K is due only to breaks in the membrane that made the compartment more accessible. Exposure of isolated mitochondria to proteinase K had no effect on endogenous porin levels in either strain, showing that once integrated into the outer membrane the protein was fully protected, even in the mitochondria of *tom22*⁸⁶¹. Direct examination of the import blots for porin levels (Fig. 1 B) verified this observation. Thus, the observed decrease in import of radiolabeled porin cannot be attributed to enhanced degradation of imported porin molecules in mitochondria containing opened outer membranes by the proteinase K treatment that follows the import reaction. We conclude that other mitochondrial factors in addition to Tom20 are required for high efficiency import of porin.

A Matrix-destined Precursor Protein and the Porin Precursor Compete for the Same Translocation Sites

To investigate the role of TOM complex components in porin insertion, we added excess amounts of a matrix-destined precursor protein to saturate import sites and then evaluated the effect on the import of porin into mitochon-

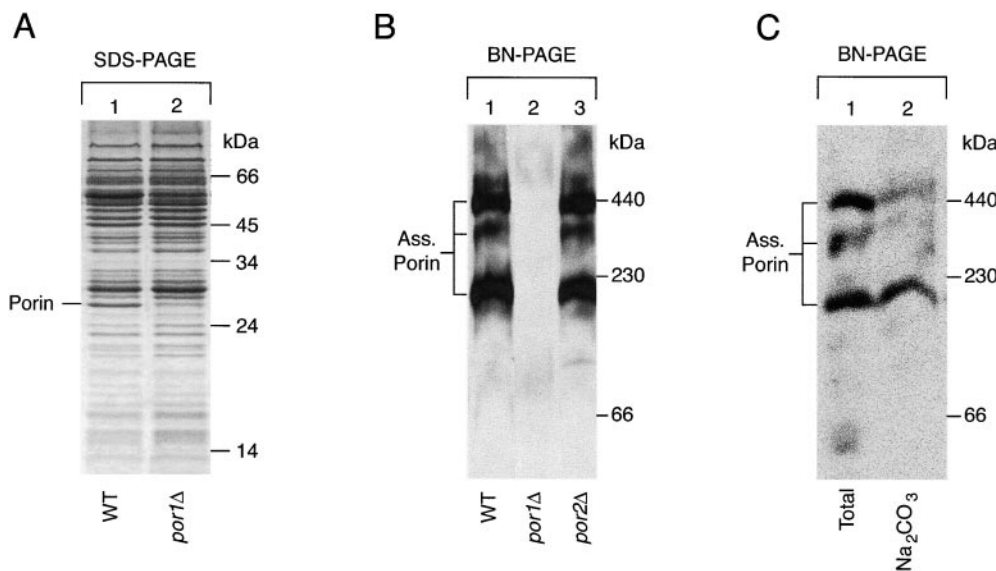


Figure 3. Porin forms high molecular weight complexes in yeast. (A) Purified *S. cerevisiae* mitochondria of a *por1Δ*-strain and the corresponding wild-type (WT) (50 μ g protein per lane) were lysed in SDS sample buffer and subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue G-250. (B) Mitochondria of *por1Δ*, *por2Δ*, and the wild-type (50 μ g protein per lane) were lysed in digitonin buffer and subjected to BN-PAGE. After semidry blotting onto PVDF membranes, immunodecoration with antibodies directed against porin was performed

and the signals were detected by chemiluminescence. (Ass. Porin) Complexes of assembled porin. (C) Wild-type mitochondria (50 μ g protein) were treated with sodium carbonate, and the membrane pellet was subjected to BN-PAGE and immunodecoration with antibodies against porin (lane 2). Nontreated mitochondria are shown as control (lane 1).

dria in vitro. Chemical amounts of a fusion protein consisting of the presequence of F_0 -ATPase subunit 9 and pSu9-DHFR were added to *N. crassa* mitochondria under conditions where completion of import to the matrix was prevented by dissipation of the inner membrane potential with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone. Subsequent import of radiochemical amounts of porin precursor was reduced about fourfold relative to import with no competitor present (Fig. 2 A). This indicates that the pSu9-DHFR and porin compete for similar sites required for insertion. To determine if the inhibitory effect was due solely to competition for binding sites on the receptors of the outer membrane (Tom20/Tom22), or if it also involved the actual import pore, the experiment was repeated using mitochondria pretreated with trypsin to remove the surface receptors. Under these conditions, receptor-dependent precursors enter mitochondria at a lower rate due to “bypass import,” which occurs via direct interaction with the GIP (Pfaller et al., 1989). As expected for trypsinized mitochondria, the import of porin was reduced relative to untreated mitochondria even without the addition of excess amounts of competing pSu9-DHFR precursor (Fig. 2 B). However, the saturation of the pore with excess precursor substantially decreased the level of porin import even via the bypass route. Since the binding of the competing precursor was also reduced in the bypass route, lower levels of competition were expected. As the matrix-destined pSu9-DHFR precursor was found to be in the vicinity of the Tom40 pore component under similar “bypass” conditions (Rapaport et al., 1997), the data suggested that the import of porin is also dependent on components of the TOM complex that make up the translocation pore. Therefore, we decided to perform a detailed analysis for the requirement of individual Tom proteins in import of porin.

Imported Porin Selectively Assembles into Preexisting Porin Complexes

To establish a specific assay for the import of porin, we characterized the properties of yeast mitochondrial porin

(also termed porin1). Mitochondria from yeast wild-type and a porin-deficient strain (*por1Δ*) (Blachly-Dyson et al., 1990) were separated by SDS-PAGE and stained with Coomassie brilliant blue G-250. The mutant mitochondria selectively lacked a protein band at 30 kD, the expected size of porin (Fig. 3 A, lane 2); the identity as porin was confirmed by immunodecoration (not shown). A quantification revealed that porin is 1 of the 10 most abundant proteins of mitochondria.

To assess the native state of porin, isolated yeast mitochondria were lysed with the mild nonionic detergent digitonin and subjected to BN-PAGE. Porin, detected by immunodecoration, migrated mainly in two complexes, an \sim 200-kD complex and an \sim 440-kD complex (Fig. 3 B, lane 1). Additionally, a complex of \sim 400 kD was present with lower abundance (Fig. 3 B, lane 1). In the *por1Δ* mitochondria, all three complexes were missing, indicating that they represent genuine porin-containing complexes (Fig. 3 B, lane 2). Besides porin1, yeast mitochondria contain a homologous protein, termed porin2, that is expressed at low level and does not reveal a detectable pore activity (Lee et al., 1998). The porin complexes of *por2Δ* mitochondria were indistinguishable from those of wild-type mitochondria (Fig. 3 B, lane 3), confirming that they are porin1 specific. The finding of several yeast porin complexes may be related to the observation in mammalian mitochondria that the integral membrane protein porin forms complexes with peripherally associated proteins from the cytosol/intermembrane space (Beutner et al., 1996). To investigate the relative stability of the porin complexes, the mitochondria were extracted with sodium carbonate, at pH 11.5, before BN-PAGE. Under these conditions, only the complex of 200 kD was stable (Fig. 3 C, lane 2 vs. lane 1), indicating that this complex represents the stable membrane-integrated core of the porin complexes.

We used the characteristic behavior of mature endogenous porin on BN-PAGE to establish a specific assay for the correct import and assembly of in vitro-synthesized porin precursors. Porin was synthesized in rabbit reticulocyte lysate in the presence of [35 S]methionine/cysteine and

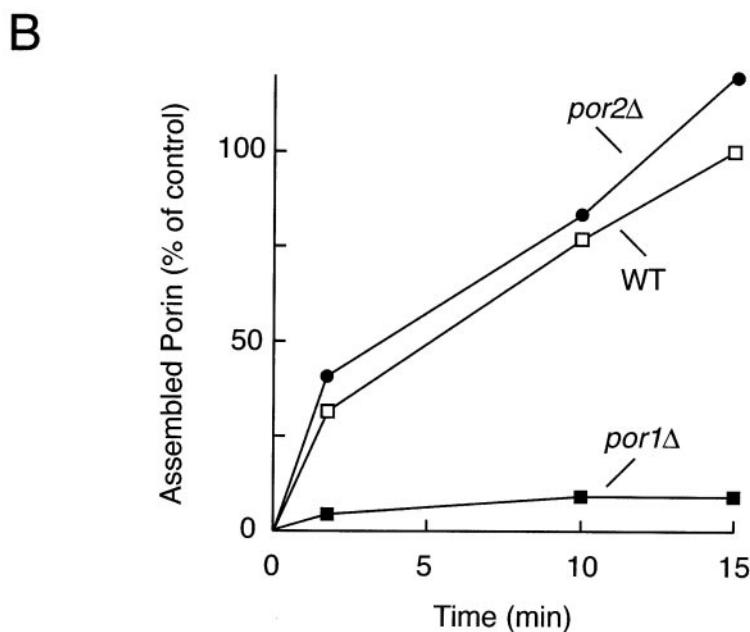
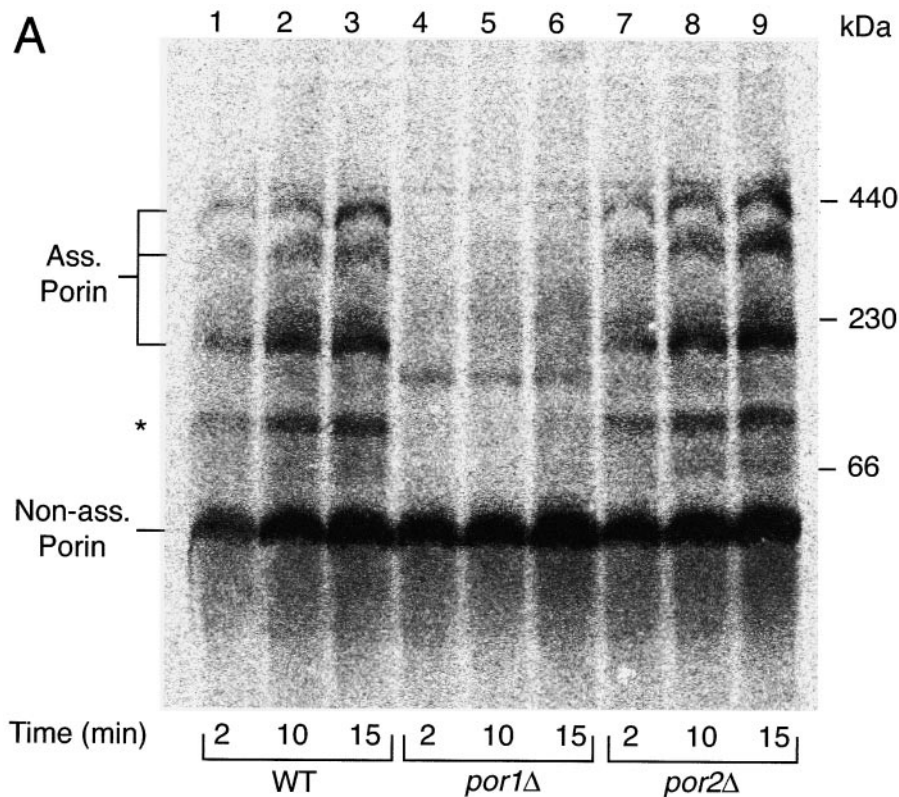


Figure 4. Assembly of in vitro-imported porin into preexisting complexes. (A) Radiolabeled precursor of yeast porin (10 μ l reticulocyte lysate per lane) was incubated with *por1* Δ , *por2* Δ , and wild-type mitochondria (50 μ g protein) at 25°C for the indicated periods. The mitochondria were reisolated, lysed in digitonin-containing buffer, and subjected to BN-PAGE and digital autoradiography. (Ass. Porin) Complexes of assembled porin. Asterisk indicates a putative assembly intermediate of porin. Non-assembled (monomeric) porin is shown in the low molecular weight range. (B) Quantification of the amounts of assembled porin by digital autoradiography. The amount of assembled radiolabeled porin in wild-type mitochondria after 15 min was set to 100% (control).

imported into isolated wild-type mitochondria. The mitochondria were reisolated, lysed in digitonin-containing buffer, and subjected to BN-PAGE and digital autoradiography. The radiolabeled porin was found in three high molecular weight complexes with a mobility indistinguishable from that of endogenous porin (Fig. 4 A, lanes 1–3). When *por1* Δ mitochondria were employed, however, none of the three high molecular weight complexes was observed (Fig. 4 A, lanes 4–6, and B), indicating that preexisting porin was required for the assembly of the radiolabeled porin. *por2* Δ mitochondria behaved like wild-type

mitochondria (Fig. 4 A, lanes 7–9 vs. 1–3, and B). With all three types of mitochondria, a low molecular weight form of porin was found, likely representing the monomeric form of the porin precursor that was associated with mitochondria yet not assembled (Fig. 4 A). Additionally, radiolabeled porin was found at an \sim 100-kD position (Fig. 4 A, asterisk). The formation of this species required the presence of preexisting porin (Fig. 4 A, lanes 4–6), raising the possibility that it represents an assembly intermediate of porin. Since the 100-kD band does not represent a major form of mature porin (Fig. 3 B) and is observed to variable

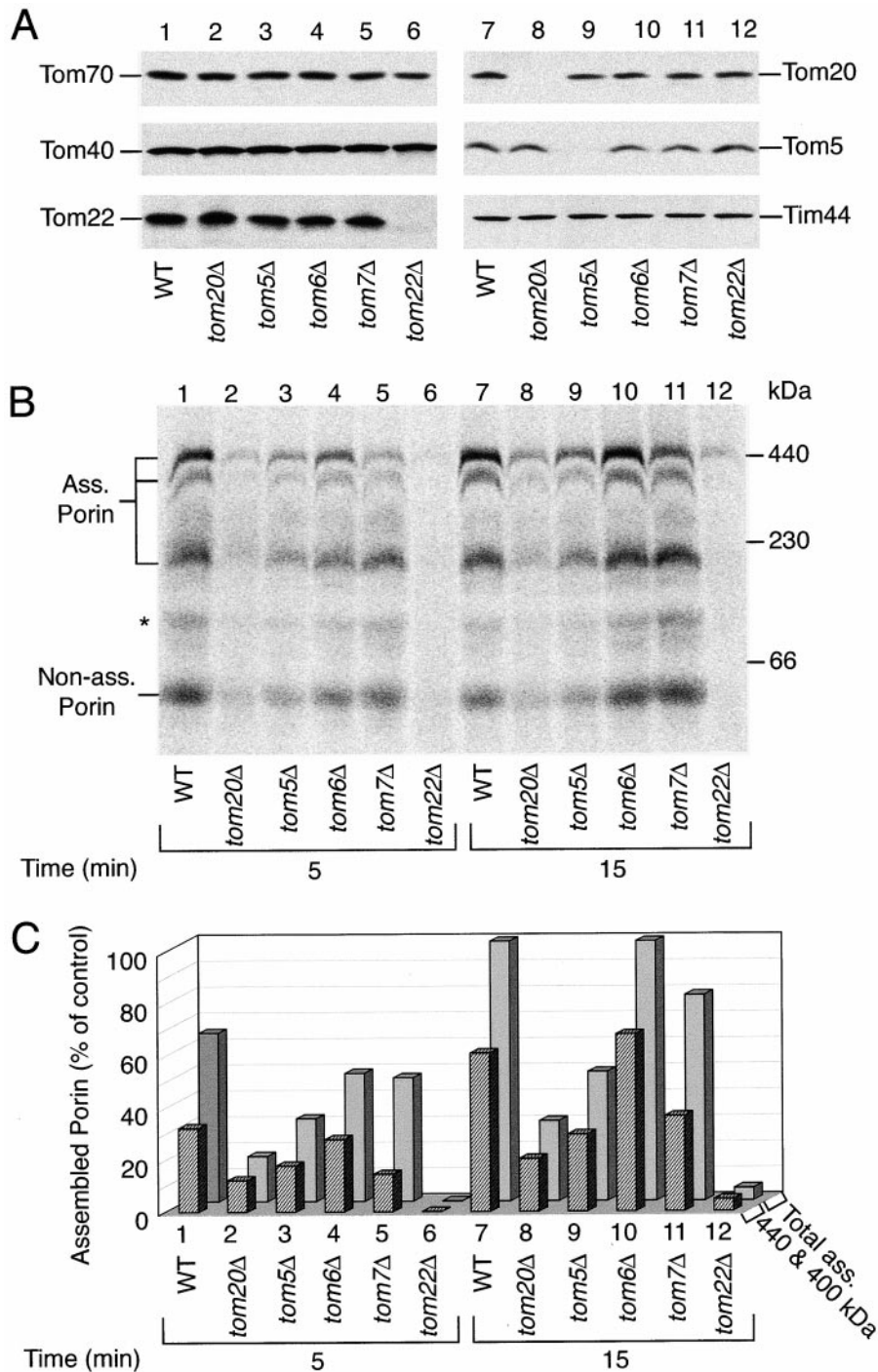


Figure 5. Import of porin requires components of the GIP. (A) Levels of Tom proteins in mitochondria isolated from yeast strains lacking individual *TOM* genes (Table I). Isolated mitochondria (25 μ g protein per lane) were subjected to SDS-PAGE and immunodecoration with antibodies directed against the indicated proteins. Tim44, translocase of inner membrane subunit of 44 kD. (B) Import and assembly of radiolabeled porin. The experiment was performed as described in the legend to Fig. 4 A, except *tom* mutant mitochondria were used, and import was performed for the indicated periods. (Non-ass. Porin) Porin precursor in the low molecular weight range that is associated with mitochondria but not assembled. (C) Quantification of assembled porin. Hatched bars, high molecular weight complexes of assembled porin (440 and 400 kD); white bars, total assembled porin (440, 400, and 200 kD complexes together). The total amount of assembled radiolabeled porin in wild-type mitochondria after 15 min was set to 100% (control).

extent (depending on the yeast strain used), we only used the mature 200–440-kD complexes to determine the efficiency of assembly of radiolabeled porin. We conclude that BN-PAGE of digitonin-lysed mitochondria represents a specific assay to determine correct import and assembly of porin in yeast.

Assembly of Porin Requires *Tom20*, *Tom22*, *Tom5*, and to a Lower Degree *Tom7*

Mitochondria were isolated from yeast strains with single deletions of *TOM22*, *TOM20*, *TOM7*, *TOM6*, or *TOM5*. The steady-state levels of most mitochondrial marker pro-

teins, including the respective other components of the TOM complex and the TIM machinery (shown here with Tim44), were found to be similar to those of wild-type mitochondria (Fig. 5 A) (Alconada et al., 1995; Hönlinger et al., 1996; Dietmeier et al., 1997; Dekker et al., 1998; van Wilpe et al., 1999). This was explained in the following way: mutations impairing the function of the TOM complex result in slower mitochondrial protein import that consequently becomes rate-limiting for mitochondrial and cellular growth. The strain lacking *Tom20*, however, is an exception since *tom20Δ* mitochondria are known to have a strongly reduced level of *Tom22* (Lithgow et al., 1994). Therefore, we used a *tom20Δ* strain that additionally ex-

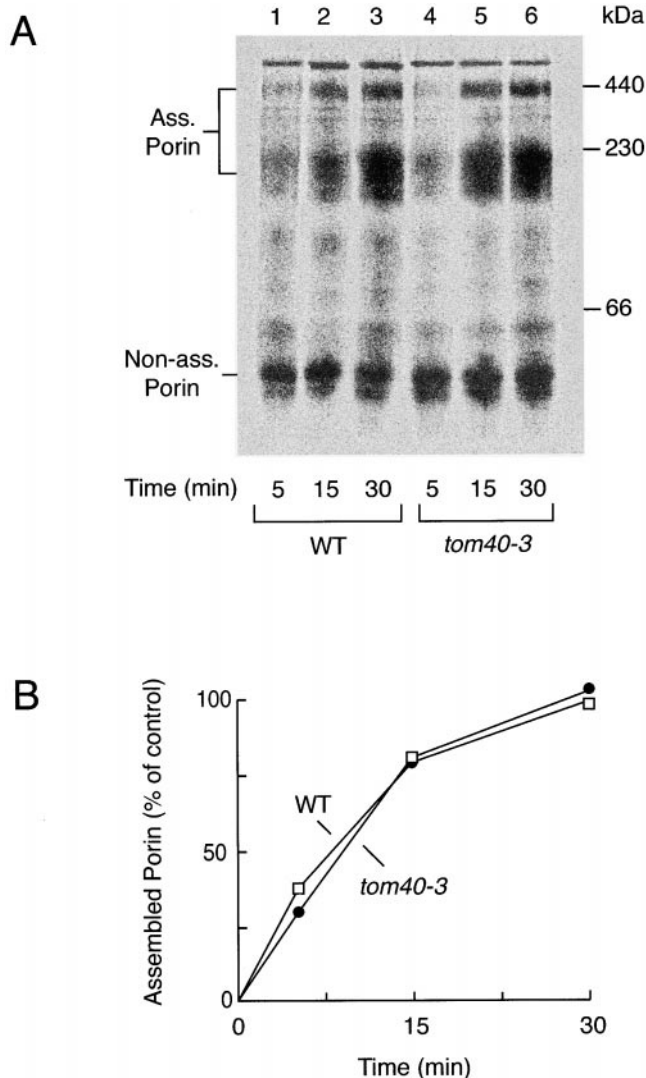


Figure 6. Import of porin is not affected in *tom40-3* mutant mitochondria. (A) The experiment was performed as described in the legend to Fig. 4 A except that mitochondria isolated from the yeast mutant strain *tom40-3* and the corresponding wild-type strain (WT) were used, and import was performed for the indicated periods. (Non-ass. porin) Porin precursor. The assembly of porin in *tom40-3* mitochondria was comparable to that of wild-type mitochondria, independently whether the import was directly performed at 25°C (as in A), or whether the mitochondria were preincubated at 37°C. (B) Quantification of assembled porin. The amount of assembled radiolabeled porin in wild-type mitochondria after 30 min was set to 100% (control).

pressed *TOM22* from a high copy number plasmid (Table I) (Dekker et al., 1998), restoring the mitochondrial content of Tom22 to a level comparable to that of wild-type (Fig. 5 A, lower left panel). Thus, the mutant mitochondria used are selectively defective in only one Tom protein.

Radiolabeled porin precursor was incubated with the isolated mutant mitochondria, followed by BN-PAGE. Assembly of porin was strongly reduced in *tom20Δ* mitochondria (Fig. 5, B and C, lanes 2 and 8) in agreement with the conclusion of Schleiff et al. (1999). However, *tom22Δ* mitochondria were almost completely deficient in the assembly of porin inserted in vitro (Fig. 5, B and C, lanes 6 and 12). Mitochondria lacking one of the small Tom proteins re-

vealed a differential effect. Assembly of porin was clearly reduced in *tom5Δ* mitochondria (Fig. 5, B and C, lanes 3 and 9), although not as strongly as in *tom22Δ* mitochondria. Tom6 and Tom7 promote the dynamics of the TOM machinery; they function at least in part in an antagonistic manner to support assembly (Tom6) and dissociation (Tom7) of the GIP complex (Alconada et al., 1995; Hönlinger et al., 1996; Dekker et al., 1998). Although we did not observe a major porin assembly defect with *tom6Δ* mitochondria (Fig. 5, B and C, lanes 4 and 10), *tom7Δ* mitochondria revealed a moderate reduction of assembly for the two high molecular weight forms (440 and 400 kD) of porin (Fig. 5 B, lanes 5 and 11, and C, first row, lanes 5 and 11). The *tom* mutant mitochondria also displayed a differential effect on the amount of low molecular weight (monomeric) porin precursor that is associated with mitochondria but not assembled. Although the level of monomeric porin was unaltered in *tom7Δ* (and *tom6Δ*) mitochondria, a clear reduction was found with *tom22Δ*, *tom20Δ*, and *tom5Δ* mitochondria (Fig. 5 B). The most severe block was again observed in *tom22Δ* mitochondria (Fig. 5 B, lanes 6 and 12). Therefore, Tom20, Tom22, and Tom5 are involved in the stable association of the porin precursor with mitochondria.

We conclude that the import of porin into mitochondria not only requires Tom20 but also components of the GIP complex, in particular Tom22 and Tom5. Of the two components modulating the dynamics of the TOM machinery, the dissociating component Tom7 stimulates porin assembly. However, an actual requirement for the import pore itself that is formed by Tom40 (Hill et al., 1998; Künkele et al., 1998) cannot be proven with these experiments.

Two New Mutant Alleles of Tom40 Cause a Defect in Porin Import

Tom40 is an essential yeast protein; therefore, deletion mutants are not viable (Baker et al., 1990). A conditional mutant allele of Tom40 has been characterized, termed *tom40-3* (*isp42-3* in the old nomenclature) (Kassenbrock et al., 1993; Pfanner et al., 1996; Schleiff et al., 1999). We used the BN-PAGE assay to analyze assembly of porin in *tom40-3* mitochondria. For comparison, radiolabeled porin was incubated with isolated mitochondria of the corresponding wild-type strain. After lysis in digitonin and BN-PAGE, assembled porin was mainly found in the 200-kD complex and to a smaller degree in the 440-kD complex (Fig. 6 A, lanes 1–3). Immunodecoration of preexisting porin revealed an indistinguishable behavior on BN-PAGE, confirming the specificity of the assay (not shown). (A higher molecular weight species [~500 kD] was a mitochondrial complex not related to porin that became non-specifically radiolabeled.) The two major assembly complexes (200 and 440 kD) of porin are thus observed in different strain backgrounds (with variations in the relative amounts). When radiolabeled porin was incubated with *tom40-3* mitochondria, we did not observe any significant defect in import and assembly of porin (Fig. 6 A, lanes 4–6, and B), demonstrating that *tom40-3* mitochondria indeed are not deficient in porin biogenesis. This is in agreement with results reported by Schleiff et al. (1999).

Two possibilities are conceivable to explain this result. Tom40 is not involved in import of porin at all, or the competence of Tom40 with regard to porin assembly is not compromised in cells harboring the allele *tom40-3*. The

A

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WT  MSAPTPLAEASQIPTIPALSPLTAKQSKGNFFSSNPISSFVVDVTKQLHSHRQSLLELVNPGTVENLNKEVSRDVFLSQYFFFTGLRADLNKAFSMNPAFQTSHTFSGISQALPKYAFSALPANDNLFAQGN 140
tom40-2 MSAPTPAEASQIPTIPALSPLTAKQSKGNFFSSNPISSFVVDTKQLHSHRQSLLELVNPGTVENLNKEVSRDVFLSQYFFFTGLRADLNKAFSMNPAFQTSHTFSGISQALPKYAFSALPANDNLFAQGN 140
tom40-4 MSAPTPLAEASQIPTIPALSPLTAKQSKGNFFSSNPISSFVVGTKQLHSHRQSLLELVNPGTVENLNKEVSRDVFLSQYFFFTGLRADLNSAFSMNPAFQTSHTFSGISQALPKYAFSALPANDNLFYQGN 140

WT  IDNDLSVSGRLNLYGWDKKNISKVNLQISDGGQPTMCLEQDYQASDFSVNVKTLNPSFSEKGEFTGVAVASFLQSVTPQLALGLLETLYSRDGSAPGDAGVSYLTRYVSKKQDWIFSGQLQANGALIASLW 280
tom40-2 IDNDLSVSGRLNLYGWDKKNISKVNLQISDBQPTMCLEQDYQASDFSVNVKTLNPSFSEKGEFTGVAVASFLQSVTPQLALGLLETLYSRDGSAPGDAGVSYLTRYVSKKQDWIFSGQLQANGALIASLW 280
tom40-4 IDNDLSVSGRLNLYGWDKKNISKVNLQISDGGQPTCLEQDYQASDFSVNVKTLNPSFSEKGEFTGVAVASFLQSVTPQLALGLLETLYSRDGSAPGDAGVSYLTRYVSKKQDWIFSGQLQANGALIASLW 280

WT  RKVAQNVEAGIETTLLQAGMVPITDPLMGTPIGIQPTVEGSTTIGAKYBYRQSVYRGTLDNSNGKLVACFLERKVLPTLSVLFCEIDHFKNDTKIGYGLQFETAGNQELMLLQQLDADGNPLQALPQL. 387
tom40-2 RKVAQNVEAGIETTLLQAGMVPITDPLMCPIGIQPTVEGSTIGAKYBYRQSVYRGTLDNSNGKLVACFLERKVLPTLSVLCEIDHFKSDTKIGYGLQFETAGNQELMQQLDADGNPLQALPQL. 387
tom40-4 RKVAQNVEAGIETTLLQAGMVPITDPLMGTPIGIQPTVEGSTTIGAKYBYRQVYRGTLDNSGVACFLERKVLPTLSVLFCEIDHFKNDTKIGYGLQFETAGNQELL. 370
  
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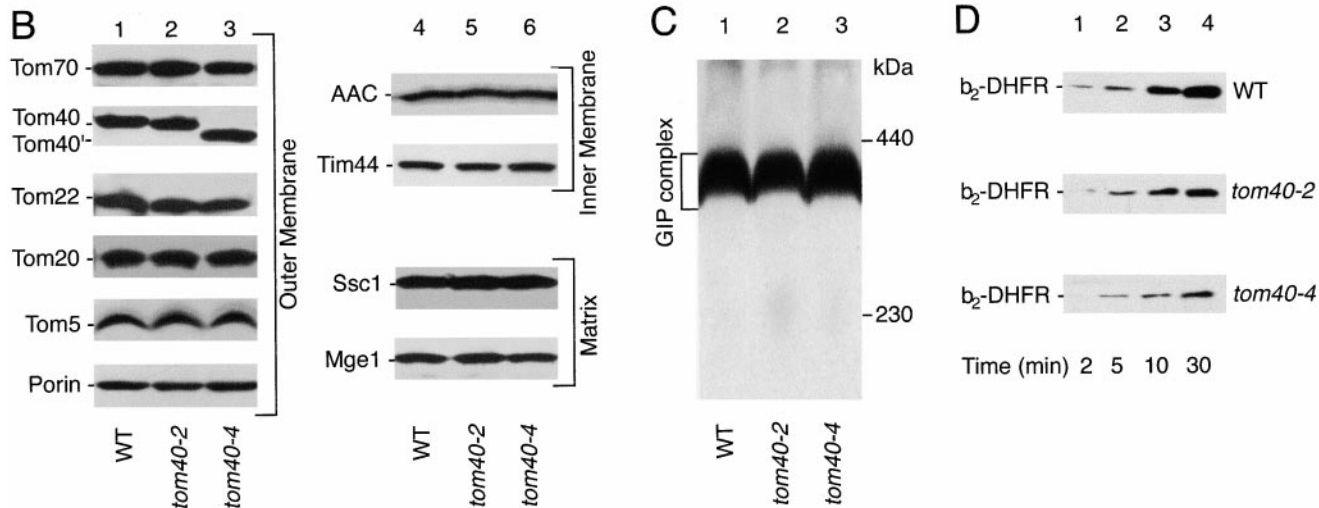


Figure 7. Two new mutant alleles of *TOM40*. (A) Predicted amino acid sequences (single letter code) of wild-type Tom40 and the two mutant alleles *tom40-2* and *tom40-4*. Alterations to the wild-type sequence are indicated in bold. (B) Steady state levels of mitochondrial proteins. Isolated mitochondria (25 μ g protein per lane) from wild-type or *tom40* mutant yeast strains were separated by SDS-PAGE using the Tris-tricine buffer system (Schägger and von Jagow, 1987), followed by immunodecoration with antibodies directed against the indicated yeast proteins. Tom40', truncated form of Tom40. AAC, ADP/ATP carrier; Ssc1, matrix Hsp70; Mge1, matrix co-chaperone of the GrpE-type. (C) Presence of the 400-kD GIP complex in the *tom40* mutant mitochondria. Isolated mitochondria (50 μ g protein per lane) were lysed in digitonin-containing buffer and subjected to BN-PAGE, followed by immunodecoration with antibodies directed against Tom5. (D) Import of a matrix-targeted preprotein is inhibited in the *tom40* mutant mitochondria. The fusion protein b_2 -DHFR (1 μ g protein per lane) was imported into isolated mitochondria (50 μ g protein per lane) for the indicated time points. The processed form of the protein was detected by immunodecoration.

PCR mutagenesis of *TOM40* (Kassenbrock et al., 1993) had yielded several further uncharacterized mutant alleles that confer temperature-sensitive growth. Like *tom40-3* and the corresponding wild-type strain, all these strains harbor a chromosomal deletion of *TOM40* and express Tom40 from a plasmid. We performed an initial screen of the mutant strains in order to select strains where mitochondrial marker proteins, including Tom proteins, are present in normal amounts to minimize indirect effects of the mutations. We then selected two mutant strains, termed *tom40-2* and *tom40-4*. The *TOM40* ORFs of the two new alleles were sequenced, demonstrating their independence (Fig. 7 A). Each allele led to several amino acid alterations; additionally, a premature stop in *tom40-4* led to a Tom40 lacking 17 amino acid residues at the COOH terminus (Fig. 7 A).

Mitochondria were isolated from the *tom40-2* and *tom40-4* strains as well as the corresponding wild-type. We first assessed the steady-state levels of several mitochondrial marker proteins by immunodecoration (Fig. 7 B). All Tom proteins analyzed along with other marker proteins for the outer membrane, inner membrane, and matrix were present in wild-type levels. Notably the levels of Tom40 and preexisting porin were not changed (Fig. 7 B). Moreover, the complexes of preexisting porin of wild-type

and both mutant mitochondria were indistinguishable on BN-PAGE (not shown), excluding that defects in assembly of in vitro-imported porin would be caused by a lack of preexisting porin complexes. As expected, Tom40 from *tom40-4* mitochondria showed a higher electrophoretic mobility consistent with the COOH-terminal truncation. Most importantly for the question addressed in this study, the levels of Tom20 were unchanged by the Tom40 mutations (Fig. 7 B). We further analyzed if the 400-kD GIP complex (TOM core complex) was properly assembled in the mutant mitochondria. The mitochondria were lysed with digitonin, separated by BN-PAGE and decorated with anti-Tom5. No difference in the mobility of the GIP complex was detectable in the mutant mitochondria (Fig. 7 C). We tested if the mutant mitochondria were impaired in the translocation of preproteins through the GIP by using a matrix-targeted fusion protein between an NH₂-terminal portion of the precursor of cytochrome *b*₂ and DHFR (b_2 -DHFR). The import of the preprotein into both mutant mitochondria was significantly inhibited compared with the corresponding wild-type mitochondria (Fig. 7 D).

We then incubated radiolabeled porin with isolated wild-type, *tom40-2*, and *tom40-4* mitochondria and analyzed them by BN-PAGE. The assembly of porin was inhibited in the mutant mitochondria (Fig. 8 A, lanes 4–9). A quantification

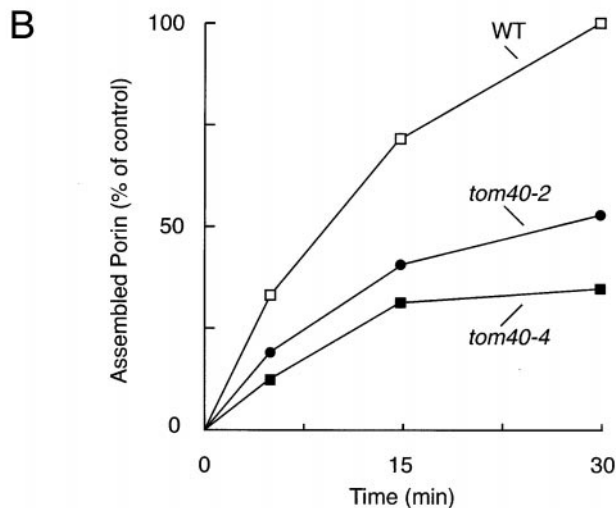
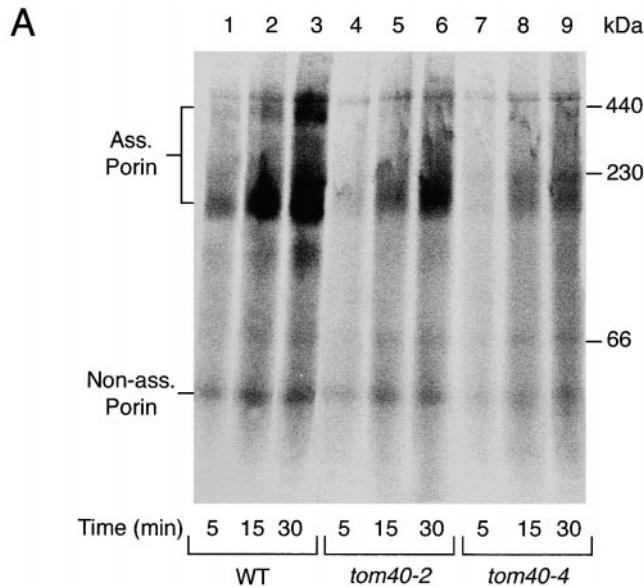


Figure 8. Import of porin is inhibited into *tom40* mutant mitochondria. (A) Radiolabeled porin precursor (10 μ l reticulocyte lysate per lane) was incubated with isolated yeast mitochondria as indicated (50 μ g protein per lane) at 25°C. The mitochondria were re-isolated, lysed in digitonin-containing buffer, and subjected to BN-PAGE and digital autoradiography. (B) Quantification of assembled porin by digital autoradiography. The total amount of assembled radiolabeled porin after 30 min in wild-type mitochondria was set to 100% (control). When the mitochondria were preincubated at 37°C, the assembly of porin in the *tom40* mutant mitochondria was similarly reduced compared with wild-type mitochondria.

showed a 60–70% reduction of porin assembly compared with the wild-type mitochondria (Fig. 8 B). Since the levels of all marker proteins analyzed along with the GIP complex are not disturbed in the mutant mitochondria, we conclude that a functional Tom40 is required for the biogenesis of porin.

Cross-Linking of the Porin Precursor to Tom20, Tom22, and Tom40

To obtain independent evidence for the involvement of components of the GIP complex in porin import, we performed cross-linking of porin precursor molecules during

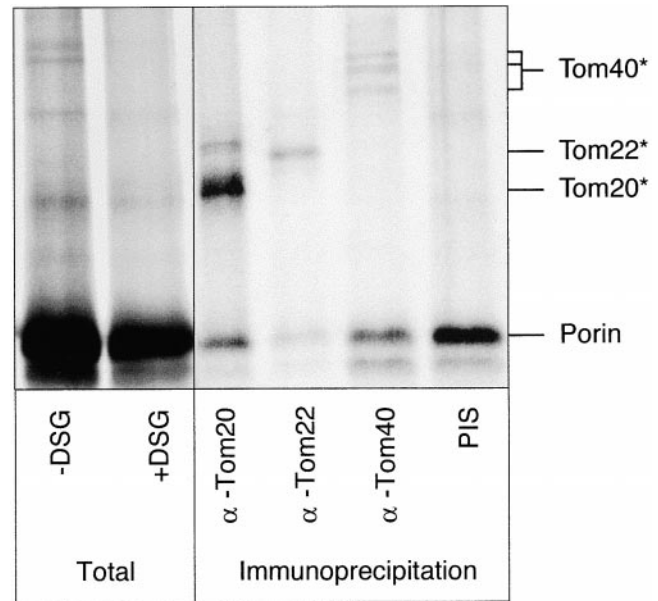


Figure 9. Porin is in the vicinity of Tom20, Tom22, and Tom40 on its insertion pathway. Radiolabeled porin precursor was incubated with isolated OMVs for 2 min at 0°C. One aliquot was left on ice (–DSG) while the chemical cross-linker DSG was added to the others (+DSG) and incubated for 40 min on ice. The cross-linking reagent was quenched with 80 mM glycine (pH 8.0), the OMVs were pelleted, and the first two aliquots (25% of material subjected to immunoprecipitation) were loaded onto SDS-PAGE. The pellets from the other aliquots were first solubilized with SDS- and Triton X-100-containing buffer and then diluted to buffer lacking SDS. Aliquots were subjected to immunoprecipitation with antibodies against either Tom20, Tom22, Tom40, or with preimmune serum (PIS). The immunoprecipitates were solubilized in sample buffer and analyzed by SDS-PAGE and digital autoradiography. *Cross-links of porin to Tom proteins.

the import process. Radiolabeled porin precursor was accumulated as an import intermediate in *Neurospora* OMVs by incubation at 0°C. Addition of the homobifunctional cross-linking reagent, DSG, led to cross-linking of porin to each of Tom20, Tom22, and Tom40 (Fig. 9). Cross-linked Tom40 products tend to appear as multiple bands on SDS-PAGE, probably caused by the existence of different conformations of Tom40 molecules in the membrane (Rapaport et al., 1997, 1998; Kanamori et al., 1999). The abundance of the adduct with Tom20 is at least partly due to the fact that the experiments are performed at 0°C, which insures that cross-linking occurs before the porin precursor becomes fully inserted. These conditions also favor association of porin with the receptor rather than with pore components. Nonetheless, the data clearly show that both Tom22 and Tom40 are in the vicinity of the porin precursor during import. No cross-linking of endogenous porin, the most abundant outer membrane protein, to Tom40 was observed (not shown), demonstrating the specificity of the cross-linking approach and excluding that the observed cross-linking of porin precursor to Tom40 could be explained as random interactions between assembled porin with Tom40. Although the levels of cross-linking of the porin precursor to the GIP complex components are low, they are comparable to those observed previously for the matrix-destined precursor pSu9-DHFR imported under similar conditions (Rapaport et al., 1997) and suggest that the initial stages of import for the two pre-

cursors occur via a similar pathway. As observed with matrix-targeted preproteins (Neupert, 1997; Pfanner et al., 1997), Tom20 may thus interact first with the porin precursor, followed by Tom22 and Tom40.

Discussion

Porin is not only the most abundant protein of the mitochondrial outer membrane, it is 1 of the 10 most abundant proteins found in mitochondria. It has been reported that porin is imported via a pathway that is radically different from that of other mitochondrial proteins since the proposed pathway utilizes only the receptor Tom20 for both recognition and membrane insertion of the precursor protein (Schleiff et al., 1999). It is possible that porin utilizes an unorthodox mechanism because it is a β barrel protein, whereas most mitochondrial proteins, known to be imported via receptors and the GIP, contain α -helical portions. Although another putative β barrel protein, Tom40, was found to be imported via the GIP (Rapaport and Neupert, 1999), its biogenesis pathway may represent a special situation since Tom40 forms the major component of the GIP itself. Therefore, we performed an analysis of the import pathway of porin in *Neurospora* and yeast mitochondria and provide evidence that the physiological import pathway into mitochondria is not mediated by Tom20 alone but requires several components of the GIP complex.

Initially, we observed that *Neurospora* mitochondria with fragile outer membranes were deficient in porin import. Similarly, Smith et al. (1994) reported that the import of porin was reduced in wild-type yeast mitochondria containing ruptured outer membranes. In the previous study with yeast mitochondria, a loss of Tom20 was not excluded. In this report, we analyzed the level of Tom20 in the *Neurospora* mitochondria and found that it was unchanged. Although these experiments cannot discriminate whether factors of the outer membrane or intermembrane space are impaired, the result with *Neurospora* mitochondria indicated that Tom20 alone was not sufficient for porin import and thereby prompted a characterization of the import pathway of porin.

Several observations show that the translocation pore of the TOM complex is involved in the import of the porin precursor. As reported previously, an excess of water-soluble porin competes with the translocation of various precursors at the level of the GIP (Pfaller et al., 1988). We have now demonstrated that the reciprocal is also true. When an excess of a matrix-destined precursor was incubated with mitochondria before addition of porin precursor, the import of porin was decreased. The competing effect is not limited to the receptor binding stage but is also observed in trypsin-treated mitochondria which are devoid of surface receptors. Thus, it appears that trypsin-resistant components of the TOM complex, such as Tom40, are involved in porin import. Furthermore, peptides resembling either matrix targeting signals, or signal-anchor sequences, are also able to inhibit insertion of porin into the outer membrane at a GIP-like step (Millar and Shore, 1996).

We have established a specific assay to directly monitor the assembly of in vitro-imported porin into preexisting porin complexes and thus exclude possible pitfalls that might arise when the import of porin is analyzed only by protease protection or resistance to alkaline pH (Schleiff et al., 1999). Using this assay, we have analyzed two new mutant alleles of yeast

tom40 (*tom40-2* and *tom40-4*) and indeed found that functional Tom40 is required for efficient import of porin. In the *tom40* mutant mitochondria, the other Tom proteins, including Tom20, are present in normal amounts, demonstrating a specific effect of the Tom40 alterations on the import of porin. We also tested the *tom40-3* strain used before by Schleiff et al. (1999) and confirmed that there is no inhibition of porin import in this strain in contrast to mitochondria from strains harboring alleles *tom40-2* and *tom40-4*. Porin precursor accumulated at an intermediate stage of import could be cross-linked to Tom40. Therefore, the precursor must be in the immediate neighborhood of the import channel during its translocation. A similar pattern of cross-linking adducts was seen during low temperature import of pSu9-DHFR (Rapaport et al., 1997); this argues strongly in favor of precursors entering the GIP.

An involvement of the GIP complex in the import of porin is substantiated by analyzing the role of Tom22, the central receptor and organizer of the GIP complex. Mutant mitochondria lacking Tom22 are severely impaired in porin import, and the porin precursor can be cross-linked to Tom22 in wild-type OMVs. Moreover, mitochondria lacking Tom5 are inhibited in porin import, as are mutant mitochondria lacking Tom20. We conclude that the import of porin requires the coordinated action of several Tom proteins: the receptors Tom20 and Tom22, and the small subunit Tom5 that aids in transfer of preproteins to the Tom40 channel. It is not excluded that under certain conditions, e.g., addition of urea or guanidinium chloride (Xu and Colombini, 1996), a fraction of porin may insert into artificial membranes (Schleiff et al., 1999). Our study demonstrates, however, that in a more physiological situation, i.e., with mitochondrial membranes, porin follows a complex import pathway involving two receptors and the GIP complex of the outer membrane.

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