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Tom7 modulates the dynamics of the mitochondrial outer membrane translocase and plays a pathway-related role in protein import

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The preprotein translocase of the outer mitochondrial membrane is a multi-subunit complex with receptors and a general import pore. We report the molecular identification of Tom7, a small subunit of the translocase that behaves as an integral membrane protein. The deletion of *TOM7* inhibited the mitochondrial import of the outer membrane protein porin, whereas the import of preproteins destined for the mitochondrial interior was impaired only slightly. However, protein import into the mitochondrial interior was strongly inhibited when it occurred in two steps: preprotein accumulation at the outer membrane in the absence of a membrane potential and subsequent further import after the re-establishment of a membrane potential. The delay of protein import into *tom7Δ* mitochondria seemed to occur after the binding of preproteins to the outer membrane receptor sites. A lack of Tom7 stabilized the interaction between the receptors Tom20 and Tom22 and the import pore component Tom40. This indicated that Tom7 exerts a destabilizing effect on part of the outer membrane translocase, whereas Tom6 stabilizes the interaction between the receptors and the import pore. Synthetic growth defects of the double mutants *tom7Δ tom20Δ* and *tom7Δ tom6Δ* provided genetic evidence for the functional relationship of Tom7 with Tom20 and Tom6. These results suggest that (i) Tom7 plays a role in sorting and accumulation of the preproteins at the outer membrane, and (ii) Tom7 and Tom6 perform complementary functions in modulating the dynamics of the outer membrane translocase.

Keywords: membrane protein/mitochondrial membrane/protein import/Tom7/translocase

Introduction

The mitochondrial outer membrane recognizes and translocates nuclear-encoded precursor proteins. The preprotein translocase consists of import receptors and a general import pore (GIP), which can assemble to form a high molecular weight complex. The *Saccharomyces cerevisiae* translocase includes four receptor proteins, Tom70, Tom37, Tom22 and Tom20, and at least four additional subunits, Tom40, Tom7, Tom6 and Tom5 (summarized in Kübrich *et al.*, 1995; Lithgow *et al.*, 1995; Ryan and Jensen, 1995). Tom20 and Tom22 cooperate in the recognition of typical hydrophilic preproteins with N-terminal signal sequences (presequences; Haucke *et al.*, 1995; Hönlinger *et al.*, 1995; Mayer *et al.*, 1995). Tom70 together with Tom37 has a preference for preproteins with hydrophobic segments or internal signal sequences (Schlossmann *et al.*, 1994; Gratzer *et al.*, 1995; Hachiya *et al.*, 1995; Lithgow *et al.*, 1995), and transfers the preproteins to Tom22 which seems to function as a central entry gate for the GIP (Kiebler *et al.*, 1993; Lithgow *et al.*, 1994; Hönlinger *et al.*, 1995). Tom40 is the major component of the GIP (Vestweber *et al.*, 1989; Kiebler *et al.*, 1990). However, little is known about the functions of the three small subunits. Only Tom6 has been identified at a molecular level (Kassenbrock *et al.*, 1993; Cao and Douglas, 1995), and it was found to stabilize the interaction between receptors and the general import pore (Alconada *et al.*, 1995b). Tom7 and Tom5 are co-precipitated together with the other subunits of the outer membrane translocase and are components of purified outer membrane vesicles; cross-linking studies suggest that Tom5 may be in close contact with a preprotein in transit (Moczko *et al.*, 1992; Alconada *et al.*, 1995a,b; Hönlinger *et al.*, 1995; A.Hönlinger, M.Kübrich and N.Pfanner, unpublished results). The genes encoding Tom7 and Tom5 have not been identified, and the function of Tom7 is unknown.

Here we report the molecular identification and functional characterization of Tom7, an integral membrane protein. Its function is in part opposite to that of Tom6. While Tom6 stabilizes the interaction between receptors and the GIP, Tom7 supports the dissociation of Tom20, Tom22 and Tom40. A lack of Tom7 preferentially inhibits import of the mitochondrial outer membrane protein porin and the productive accumulation of preproteins at the outer membrane. Our studies suggest a pathway-related role for Tom7 in protein import and a complementary function for Tom7 and Tom6 in promoting the dynamics of the outer membrane translocase.

In the new uniform nomenclature (Pfanner *et al.*, 1996), the subunits of the **t**ranslocase of the **o**uter **m**itochondrial membrane are renamed (new name: former names) as follows: Tom70: Mas70, Mom72; Tom40: Isp42, Mom38; Tom37: Mas37; Tom22: Mas22, Mom22, Mas17; Tom20: Mas20, Mom19; Tom7: Mom7; Tom6: Isp6, Mom8b; and Tom5: Mom8a.

Results

Identification and primary sequence of Tom7

Saccharomyces cerevisiae mitochondrial membranes were lysed with digitonin. The outer membrane translocase

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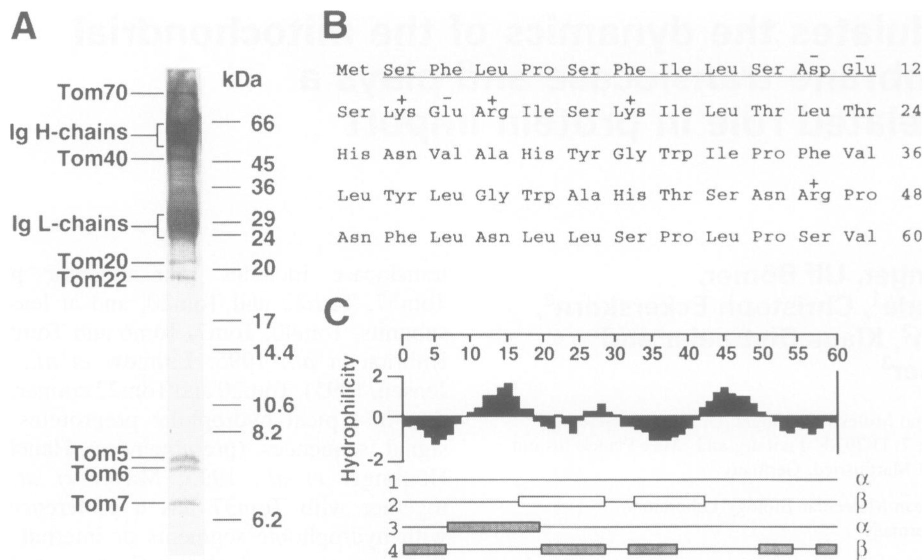

Fig. 1. Identification and primary structure of Tom7. (**A**) The translocase complex of the mitochondrial outer membrane was purified from *S.cerevisiae* wild-type mitochondria by co-immunoprecipitation with antibodies directed against Tom40 that were covalently coupled to protein A–Sepharose. The bound proteins were eluted at pH 2.5, separated by urea–SDS–PAGE and stained with Coomassie Brilliant Blue R250. Some of the Ig heavy (H) and light (L) chains were also released at low pH. The lowest band, marked Tom7, was used for N-terminal amino acid sequencing. (**B**) Predicted primary structure of Tom7. The amino acid sequence of Tom7 is shown in the three-letter code. The underlined part represents the peptide sequence that was identified by N-terminal sequencing. (**C**) Structural predictions. A hydrophilicity plot of Tom7 is shown with structural predictions according to Chou–Fasman (numbers 1 and 2) or Garnier–Robson (numbers 3 and 4). α, α-helix; β, β-regions.

complex was isolated in chemical amounts by co-precipitation with anti-Tom40 antibodies that had been covalently linked to protein A–Sepharose (Moczko *et al.*, 1992; Alconada *et al.*, 1995a,b; Hönlinger *et al.*, 1995). The translocase subunits were eluted from the antibodies at a low pH, separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Figure 1A). The protein band corresponding to Tom7 of the translocase was excised and subjected to N-terminal sequencing. In all, 11 amino acid residues could be determined. A database search revealed that an open reading frame encoding this sequence was determined by two groups in the course of sequencing the dihydrolipoamide acetyltransferase gene (accession number J04096; Niu *et al.*, 1988) and by sequencing cosmid 14–15 of the left arm of chromosome XIV (accession number X86470; R.Poehlmann and P.Philippsen, unpublished results). However, because of its short length, the open reading frame located 394 nucleotides after the stop codon of the dihydrolipoamide acetyltransferase gene was not recognized as a functional gene. The open reading frame contains 60 amino acid codons (Figure 1B). The determined N-terminal peptide sequence of Tom7 is identical to amino acids 2–12 of the predicted sequence (Figure 1B), indicating that the mature protein consists of 59 amino acids (6.7 kDa). After the post-translational removal of the initial methionine, there is no methionine or cysteine in Tom7, in agreement with the previous observation that Tom7 was not labeled in the outer membrane translocase isolated from ^{35}S-labeled yeast mitochondria (Moczko *et al.*, 1992). The primary structure of Tom7 did not show a significant similarity to any known protein.

Secondary structure predictions do not indicate the presence of a hydrophobic α-helix of sufficient length to function as a membrane anchor sequence. While a prediction according to Chou and Fasman (1978) reveals no α-helix (Figure 1C, prediction 1), a prediction according to Garnier *et al.* (1978) suggests the presence of a short highly charged α-helix in the N-terminal region (Figure 1C, prediction 3). However, both prediction methods strongly suggest the presence of β-strands in Tom7, particularly in segments with a more hydrophobic character (Figure 1C). The middle portion of Tom7 contains 27 amino acid residues which are largely uncharged (only three histidines) and are predicted to form two β-strands.

Tom7 is an integral membrane protein of the outer membrane translocase

To confirm that *TOM7* encodes the protein band of the purified outer membrane translocase, designated Tom7, a deletion mutant was constructed by replacing the open reading frame of *TOM7* with the *TRP1* gene. The resulting mutant cells were viable on both fermentable and non-fermentable carbon sources, yet showed a slight reduction of growth at higher temperatures (37°C) on non-fermentable medium (see below). Mitochondria were isolated from the *tom7*Δ cells, and the outer membrane translocase was purified. The protein pattern shown in lane 2 of Figure 2A demonstrates the complete lack of Tom7.

The coding region of *TOM7* was cloned into the vector pGEM4Z, and Tom7 was synthesized *in vitro* by transcription and translation in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. After incubation with isolated *S.cerevisiae* mitochondria, *in vitro*-synthesized Tom7 associated with the mitochondria and assembled with the outer membrane translocase, as assessed by the co-precipitation with antibodies directed against Tom40, Tom20 or Tom22 (Figure 2B, lanes 3–5). No co-precipitation was observed with preimmune antibodies, and only a very weak co-precipitation was observed with antibodies directed against Tom70 (Figure 2B, lanes 1 and 2). To enhance the signal of *in vitro*-synthesized Tom7 (a major

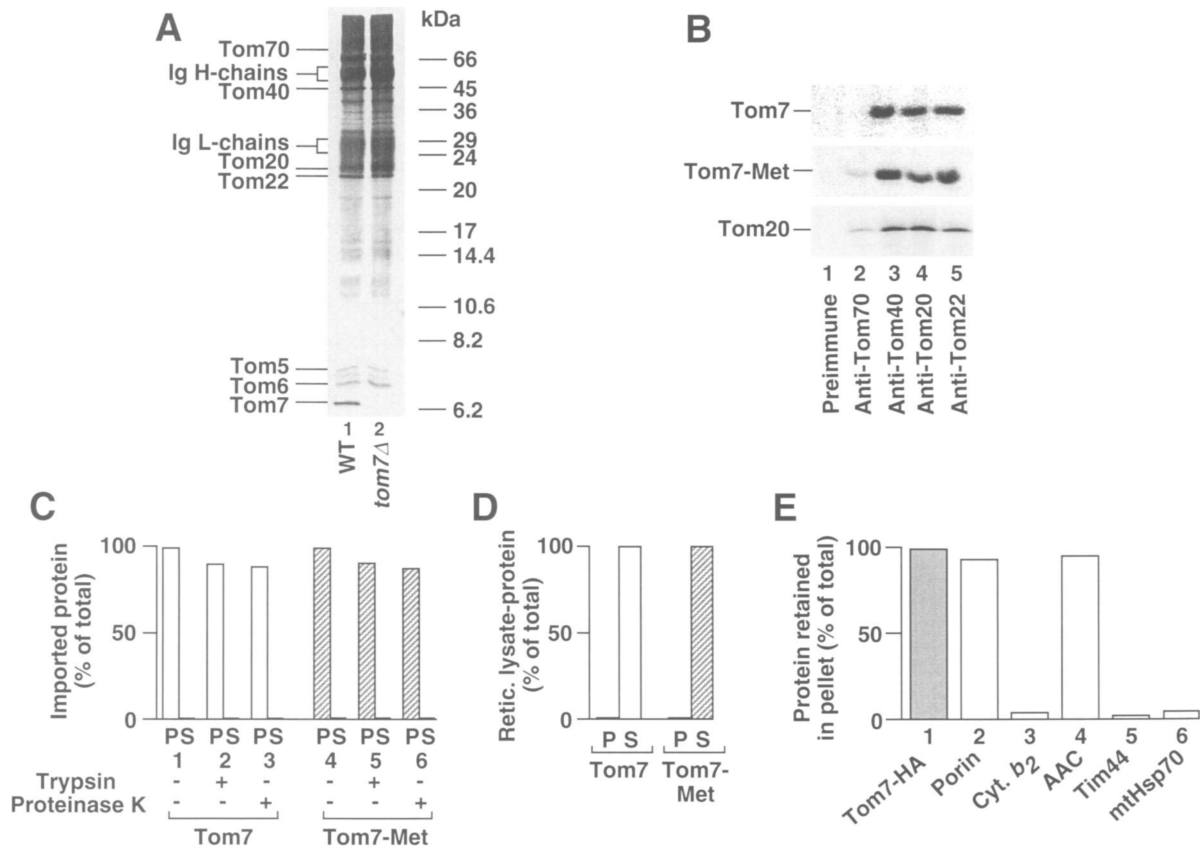

Fig. 2. Tom7 is an integral protein of the outer membrane translocase. (**A**) Outer membrane translocase of *tom7Δ* mitochondria. Mitochondria were isolated from wild-type (WT; sample 1) and the *tom7Δ* deletion strain AH101 (Table I) (sample 2) and solubilized with digitonin. The outer membrane translocase was precipitated with anti-Tom22 antibodies covalently coupled to protein A–Sepharose. The proteins were stained with silver. (**B**) *In vitro*-synthesized Tom7 and Tom7-Met (Tom7 with a methionine at the C-terminus) assemble with the outer membrane translocase. The ^{35}S-labeled precursors of Tom7, Tom7-Met and Tom20 were incubated with wild-type mitochondria for 15 min at 25°C, followed by lysis in digitonin-containing buffer and co-immunoprecipitation with the indicated antibodies. The exposure time for Tom7 was five times longer than for Tom7-Met. (**C**) *In vitro*-imported Tom7 and Tom7-Met are not extracted at alkaline pH. ^{35}S-labeled Tom7 and Tom7-Met were incubated with wild-type mitochondria for 15 min at 25°C. The mitochondria were then treated with 30 μg/ml trypsin or proteinase K for 15 min at 0°C, followed by the addition of 900 μg/ml soybean trypsin inhibitor and, in the case of proteinase K, treatment with 1 mM PMSF (10 min at 0°C). Mitochondria were then treated with 100 mM Na_2CO_3 and separated into supernatant (S) and membrane pellet (P). (**D**) The precursors of Tom7 and Tom7-Met in reticulocyte lysate are extracted at alkaline pH. Reticulocyte lysates containing ^{35}S-labeled Tom7 or Tom7-Met were treated with Na_2CO_3 and separated into supernatant (soluble; S) and pelleted (aggregated; P) protein fractions. (**E**) HA-tagged Tom7 is not extracted at alkaline pH. Mitochondria from strain AH201 were treated with 100 mM Na_2CO_3, separated into pellet and supernatant, and immunodecorated with antibodies directed against the HA tag (12CA5 monoclonal antibody; Boehringer Mannheim). The same result was obtained when mitochondria from the strain AH301 were used. As control, immunodecorations with antibodies directed against porin, cytochrome b_2, ADP/ATP carrier (AAC), Tim44 and mtHsp70 were performed.

fraction of which has probably lost the initial methionine), a methionine codon was placed immediately before the stop codon of *TOM7* in the vector pGEM4Z. This Tom7-Met was imported into isolated mitochondria and assembled with the translocase like the authentic Tom7 precursor (Figure 2B). To control for the specificity of the *in vitro* assembly assay, we synthesized the precursor of Tom20 *in vitro* and incubated it with isolated mitochondria. The specific association of Tom20 with the translocase complex (Schneider *et al.*, 1991; Seki *et al.*, 1995) yielded the same pattern in the co-precipitation as that of Tom7 or Tom7-Met, i.e. efficient co-precipitation with antibodies directed against Tom40 and Tom22 (Figure 2B, lanes 3 and 5). As discussed below, only a minor fraction of Tom70 is associated with the isolated translocase, explaining the very low efficiency of co-precipitation with antibodies directed against Tom70. We conclude that *in vitro*-synthesized Tom7 assembles with the mitochondrial outer membrane translocase.

To assess the membrane association of Tom7, mitochondria with *in vitro*-imported Tom7 or Tom7-Met were subjected to a treatment with sodium carbonate at pH 11.5 (Fujiki *et al.*, 1982a,b; Blom *et al.*, 1993). This treatment extracts soluble protein (e.g. cytochrome b_2 of the intermembrane space and matrix Hsp70; Figure 2E, columns 3 and 6) and peripheral membrane proteins [e.g. the inner membrane protein Tim44 (previously termed Isp45 or Mim44); Figure 2E, column 5], but leaves integral membrane proteins in the membrane sheets (e.g. porin of the outer membrane and the ADP/ATP carrier of the inner membrane; Figure 2E, columns 2 and 4). *In vitro*-imported Tom7 and Tom7-Met were fully resistant to extraction by sodium carbonate (Figure 2C). In contrast, the precursors of Tom7 and Tom7-Met in reticulocyte lysate were fully

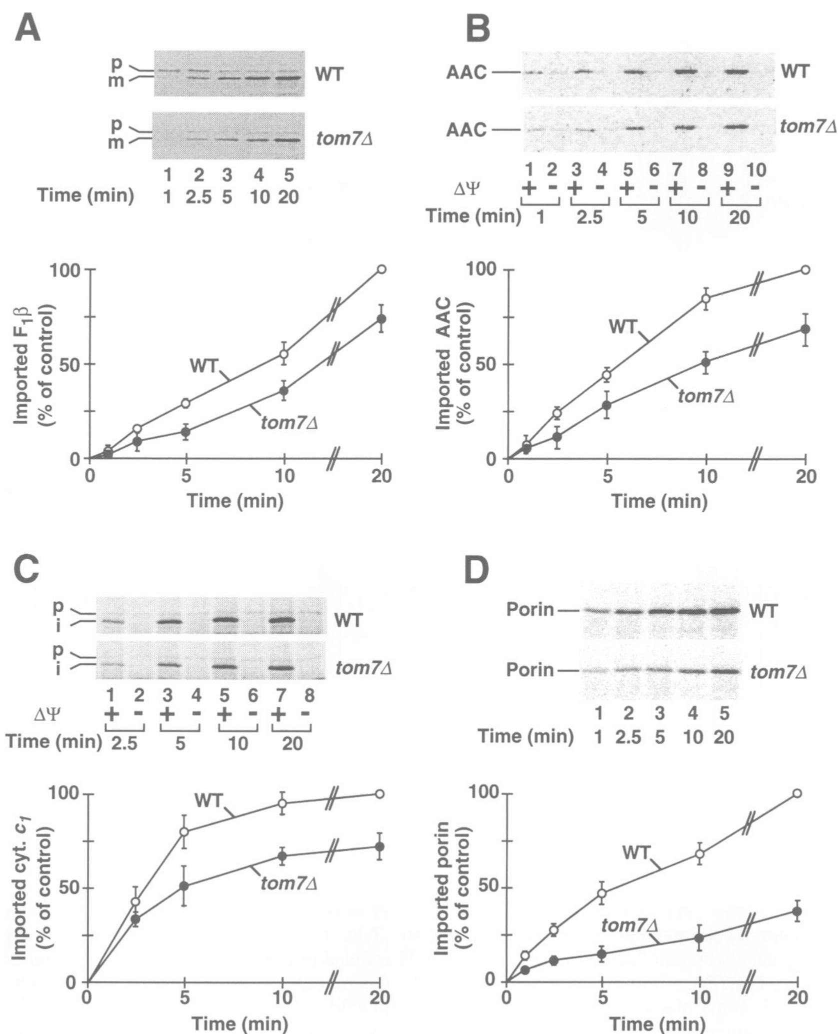

Fig. 3. *tom7Δ* mitochondria are significantly impaired in the import of porin but are only moderately impaired in the import of proteins transported to the inner membrane or matrix. Rabbit reticulocyte lysate with radiolabeled precursors was incubated with wild-type mitochondria (WT) and *tom7Δ* mitochondria (50 μg protein/ml) for the indicated times. Mitochondria were treated with proteinase K (100 μg/ml), reisolated and analyzed by SDS–PAGE and storage phosphor imaging technology. (**A**) Import of F_1-ATPase subunit β ($F_1β$). (**B**) Import of ADP/ATP carrier (AAC). Imported assembled (dimeric) AAC was assessed by hydroxylapatite chromatography (see Materials and methods). (**C**) Import of cytochrome c_1 (cyt. c_1); under the conditions used, imported cytochrome c_1 was processed to the intermediate-sized form. (**D**) Import of porin. p, i and m, precursor, intermediate and mature forms of a protein. The amount of protein imported into wild-type mitochondria after 20 min was set to 100% (control). Bars indicate the standard errors of the means (from three to four independent experiments).

soluble at pH 11.5 (Figure 2D). This shows that during import into the mitochondrial outer membrane, Tom7 undergoes a change from a carbonate-soluble form to a carbonate-resistant form, indicating an insertion into the lipid phase of the outer membrane.

Moreover, we expressed Tom7 with an epitope tag at the C-terminus [the nine-residue hemagglutinin A tag (HA)] in yeast cells with a deletion of the *TOM7* gene. The Tom7-HA was able to complement the growth defect caused by deletion of the authentic *TOM7* gene. Because a deletion of *TOM7* only leads to a partial growth defect, the substitution of Tom7 function by Tom7-HA was analyzed in the double mutant *tom6Δ tom7Δ*, which shows a strong synthetic growth defect (see below). The expression of Tom7-HA fully complemented the lack of *TOM7* (leading to a *tom6Δ* growth phenotype), indicating that Tom7-HA was correctly assembled into the outer membrane translocase and thus functional. Tom7-HA was fully resistant to extraction at pH 11.5 (Figure 2E, column 1), confirming the tight membrane association of Tom7.

Mitochondria lacking Tom7 are only slightly impaired in the import of preproteins destined for the mitochondrial interior but are more strongly impaired in the import of the outer membrane protein porin

To characterize functions of Tom7, mitochondria were isolated from *tom7Δ* cells and analyzed for their import of preproteins. To exclude an indirect effect of the deletion of *TOM7* on the mitochondrial membrane potential $\Delta\psi$, we assessed $\Delta\psi$ by using a fluorescent dye. We found that *tom7Δ* mitochondria were fully competent in the generation of a $\Delta\psi$ which was indistinguishable from that of wild-type mitochondria (data not shown).

Preproteins destined for different mitochondrial subcompartments were synthesized in rabbit reticulocyte

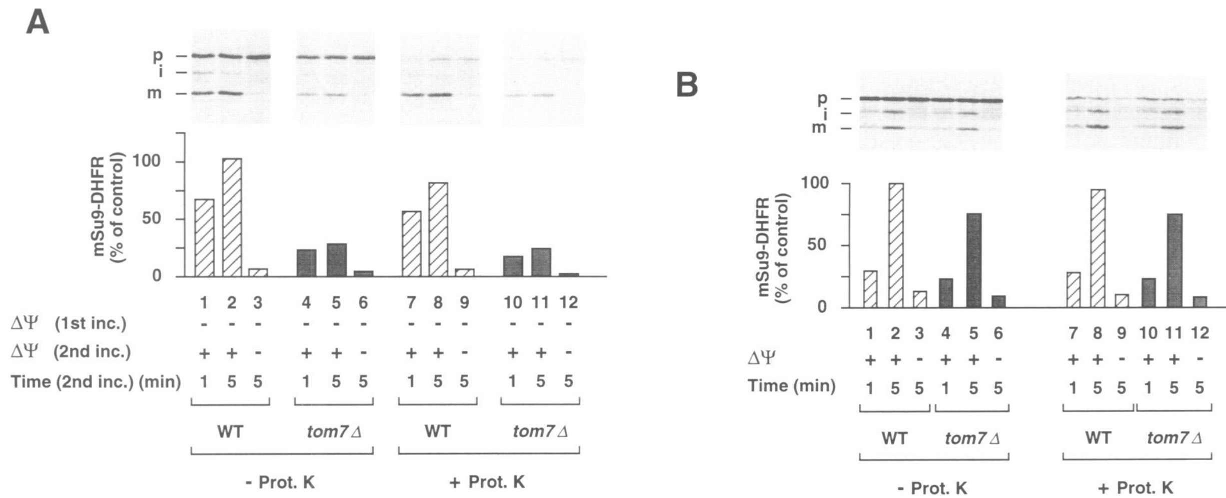

Fig. 4. Impairment of the two-step import of Su9–DHFR into *tom7*Δ mitochondria. (**A**) Two-step import. The fusion protein Su9–DHFR (urea-denatured) was prebound to isolated wild-type (WT) and *tom7*Δ mitochondria in the absence of a membrane potential $\Delta\psi$ for 5 min at 0°C (1st inc.), as described in Materials and methods (valinomycin; ~40 mM potassium ions in the buffer). The samples were split into three parts and the mitochondria were reisolated. One aliquot was resuspended in the same buffer [$-\Delta\psi$ (2nd inc.); samples 3, 6, 9 and 12]; the other two aliquots were resuspended in chase buffer (valinomycin; no potassium) to generate a membrane potential driven by potassium diffusion [$+\Delta\psi$ (2nd inc.)]. The reactions were incubated at 25°C for the times indicated (2nd inc.) and stopped by adding KCl to dissipate the membrane potential. All samples were split into halves. One of each was treated with proteinase K (+ Prot. K, samples 7–12). Mitochondria were reisolated and analyzed by SDS–PAGE and digital autoradiography. The amount of mature-sized Su9–DHFR (mSu9–DHFR) generated by direct (one-step) import into wild-type mitochondria for 5 min in the presence of a potassium diffusion potential (without proteinase K treatment) was set to 100% (for this control sample, an aliquot was treated as described above except that the radiolabeled precursor was added to the second incubation of 5 min instead of to the first incubation). Standard errors of the means (typically from five independent experiments) were $\leq$5%. The comparable result was obtained when Su9–DHFR was bound to mitochondria out of reticulocyte lysate (without urea denaturation). (**B**) Direct import of Su9–DHFR driven by a potassium diffusion potential is only slightly impaired with *tom7*Δ mitochondria. In a first incubation on ice, wild-type and *tom7*Δ mitochondria were preloaded with potassium ions (as in A) and reisolated. Mitochondria were incubated with Su9–DHFR in reticulocyte lysate in the absence of potassium ions to establish a potassium diffusion potential ($+\Delta\psi$); after the indicated times, the reactions were stopped by the addition of KCl. Where indicated, mitochondria and Su9–DHFR were incubated in potassium-containing buffer ($-\Delta\psi$). One half of each sample was treated with proteinase K (+ Prot. K). The mitochondria were reisolated and analyzed as described in (A). The amount of Su9–DHFR processed to the mature form in wild-type mitochondria after 5 min was set to 100% (control). p, i and m, precursor, intermediate and mature forms of Su9–DHFR. A similar result (only a small difference in the one-step import efficiency between wild-type and *tom7*Δ mitochondria) was observed when Su9–DHFR was denatured in 8 M urea prior to import.

lysate in the presence of [^{35}S]methionine and incubated with isolated energized *tom7*Δ mitochondria and wild-type mitochondria (Söllner *et al.*, 1991; Alconada *et al.*, 1995a). The preproteins were F_1-ATPase subunit β (destined for the matrix side of the inner membrane; Figure 3A), the ADP/ATP carrier (destined for the inner membrane; Figure 3B), cytochrome c_1 (destined for the inner membrane/intermembrane space side; Figure 3C) and porin (destined for the outer membrane; Figure 3D). F_1-ATPase subunit β and cytochrome c_1 were synthesized with cleavable N-terminal presequences, whereas the other two preproteins were not cleavable and were targeted by internal targeting sequences. Import of the three preproteins destined for the inner membrane or matrix was reduced only slightly in the mutant compared with wild-type mitochondria (reduction of 20–30%; Figure 3A–C). However, the import of porin was more strongly affected. It was reduced by ~70% when assayed in the kinetically linear import range (Figure 3D).

Mitochondria lacking Tom7 are impaired in the two-step import of a matrix protein

Schatz and colleagues (Hines and Schatz, 1993; Haucke *et al.*, 1995) reported that a two-step import of preproteins via accumulation at the outer membrane revealed a dependence on receptor proteins of the mitochondrial outer membrane that was not apparent in direct import experiments. Preproteins are accumulated at the outer membrane after dissipation of the membrane potential $\Delta\psi$ across the inner membrane by the addition of the protonophore CCCP. They are then chased into or across the inner membrane after re-establishing a membrane potential by the inactivation of CCCP and the addition of an energy source, allowing the determination of productive (i.e. import-competent) binding. In the analysis of receptor function, the assay for productive binding has a significant advantage over the determination of the total amount of preprotein bound to mitochondria; it permits the exclusion of the unspecific binding which is typically high and variable for many preproteins (Pfanner *et al.*, 1987a; Hines and Schatz, 1993).

We questioned if an assay for two-step import (productive binding) revealed new information about a possible role for Tom7 in the import of a preprotein transported into the mitochondrial interior. We used the matrix-targeted preprotein Su9–dihydrofolate reductase (DHFR), a fusion protein between the presequence of F_0-ATPase subunit 9 and the passenger protein DHFR (Pfanner *et al.*, 1987b), and established conditions for a two-step import. Su9–DHFR was first incubated with isolated mitochondria in the presence of the potassium ionophore valinomycin and an external potassium concentration of 40 mM. Thus the membrane potential $\Delta\psi$ of the mitochondria was completely dissipated (Nicholls, 1982; Pfanner and

Neupert, 1985), and Su9–DHFR was accumulated at the outer membrane (Figure 4A, lanes 3 and 6) and remained accessible to added protease (Figure 4A, lanes 9 and 12). The mitochondria were re-isolated and incubated in a buffer without potassium in the presence of valinomycin, leading to the generation of a membrane potential across the inner membrane of the same orientation as the physiological mitochondrial membrane potential (because of export of the potassium ions across the inner membrane; Nicholls, 1982; Pfanner and Neupert, 1985; Martin *et al.*, 1991). Thereby the bound Su9–DHFR was imported, as assessed by proteolytic processing (Figure 4A, lanes 1, 2, 4 and 5) and protection of the processed form against added protease (Figure 4A, lanes 7, 8, 10 and 11). Under these conditions, the amount of Su9–DHFR imported into *tom7*Δ mitochondria (Figure 4A, lanes 4, 5, 10 and 11) was reduced significantly compared with wild-type mitochondria (Figure 4A, lanes 1, 2, 7 and 8).

For comparison, we analyzed the one-step (direct) import of Su9–DHFR into mitochondria driven by a potassium diffusion potential (Figure 4B). As observed for the other preproteins transported into the mitochondrial interior (F_1-ATPase subunit β, ADP/ATP carrier and cytochrome c_1; Figure 3A–C), the lack of Tom7 only moderately impaired the direct import of Su9–DHFR (Figure 4B, lanes 4, 5, 10 and 11, compare to lanes 1, 2, 7 and 8). We conclude that the direct import of Su9–DHFR is affected only slightly by a lack of Tom7, and that the two-step import via an outer membrane intermediate is sensitive to the lack of Tom7.

It should be noted that the two-step import assay with valinomycin may be of use in future studies on mitochondrial protein import because the valinomycin-induced potassium diffusion potential for the efficient two-step import of a cleavable preprotein provides a simple method for the precise modulation of the magnitude of a regenerated membrane potential (Nicholls, 1982; Pfanner and Neupert, 1985).

The lack of Tom7 impairs an import step beyond the initial recognition of preproteins by surface receptors

At which stage of outer membrane protein transport is Tom7 involved? Previous work (Pfanner and Neupert, 1987; Pfaller *et al.*, 1988, 1989; Söllner *et al.*, 1992) has allowed the experimental distinction of two successive stages at the outer membrane: (i) the interaction of preproteins with surface receptors and (ii) transport through the GIP. After removal of the cytosolic domains of the surface receptors by a pretreatment with protease, preproteins are still imported into mitochondria, although with a clearly reduced efficiency (~20–30% of that of intact mitochondria *in vitro*; Figure 5, lanes 4 and 12; Pfaller *et al.*, 1989). This so-called bypass import is thought to reflect the properties of the GIP, i.e. the outer membrane translocase without the cytosolic domains of surface receptors. When the direct (one-step) import of Su9–DHFR into energized mitochondria was studied, the bypass import into *tom7*Δ mitochondria was reduced only moderately compared with the bypass import into wild-type mitochondria (Figure 5, compare lanes 8 and 16 with lanes 4 and 12).

We analyzed whether the two-step import of Su9–DHFR

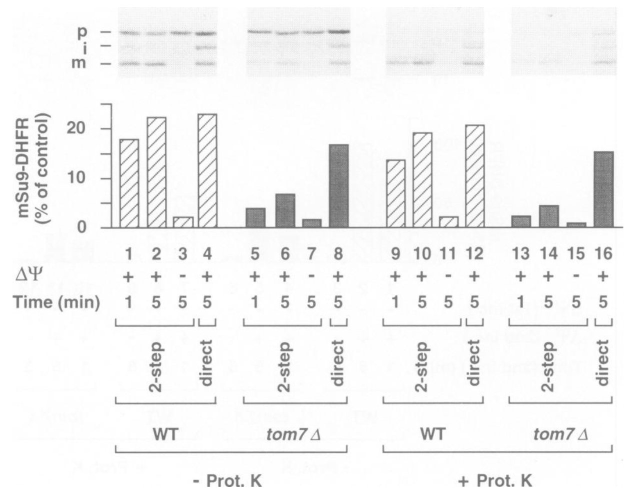

Fig. 5. Two-step import of Su9–DHFR into trypsin-pretreated mitochondria. Wild-type (WT) or *tom7*Δ mitochondria were pretreated with trypsin to remove the cytosolic domains of the import receptors on the mitochondrial outer membrane. After reisolation, the two-step import of urea-denatured Su9–DHFR was performed and analyzed as described in Figure 4A. Mitochondria were resuspended in incubation buffer (valinomycin; ~40 mM potassium ions) to dissipate the membrane potential. Precursor was bound to the mitochondria in a first incubation of 5 min. After division into three parts, mitochondria were reisolated. Productively bound Su9–DHFR was chased by resuspension in chase buffer [+Δψ (2nd inc.)] and incubation at 25°C for the times indicated (2nd inc.). One aliquot was resuspended in potassium-containing incubation buffer [–Δψ (2nd inc.), lanes 3, 7, 11 and 15] and incubated at 25°C without establishing a potassium diffusion potential. A further sample, which did not receive radiolabeled precursor in the first incubation (direct import, lanes 4, 8, 12 and 16), was resuspended in chase buffer and incubated with the radiolabeled precursor in the second incubation. Reactions were stopped and samples divided into halves. One of each was treated with proteinase K (+ Prot. K, lanes 9–16). After reisolation of the mitochondria, proteins were separated by SDS–PAGE and transferred to nitrocellulose. Imported proteins were analyzed by digital autoradiography. Quantitative removal of the surface domains of the import receptors was controlled by immunodecoration with antibodies directed against Tom20, Tom22 and Tom70 (results not shown). In parallel, the same experiment was performed with mock-treated mitochondria instead of trypsin-treated mitochondria. The amount of mature-sized Su9–DHFR in mock-treated wild-type mitochondria after 5 min of two-step import (without proteinase K treatment) was set to 100% (control).

was still possible after a pretreatment of mitochondria with trypsin, i.e. whether it could occur via accumulation at a post-receptor stage. Lanes 1, 2, 9 and 10 in Figure 5 demonstrate that this was indeed the case. The efficiency of a two-step bypass import into wild-type mitochondria was ~20–30% that of a two-step import into non-trypsinized mitochondria, indicating that the pretreatment with trypsin reduced the direct import and the two-step import into wild-type mitochondria by about the same factor (the trypsin treatment quantitatively removed the cytosolic domains of import receptors; Moczko *et al.*, 1993; legend to Figure 5). A two-step bypass import into *tom7*Δ mitochondria was inefficient (Figure 5, lanes 5, 6, 13 and 14), demonstrating a significant effect of the lack of Tom7 on protein import in the absence of the cytosolic domains of import receptors.

Is the bypass import of porin similarly sensitive to a lack of Tom7? Wild-type and *tom7*Δ mitochondria were pretreated with trypsin, and the precursor of porin was

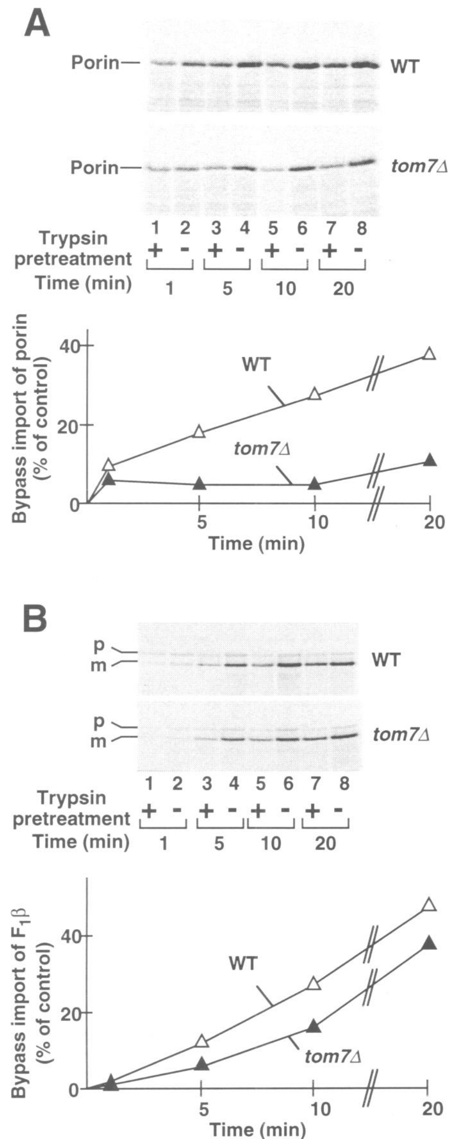

Fig. 6. The bypass import of porin is strongly impaired by a lack of Tom7. Outer membrane surface receptors were removed prior to the import reaction by treatment with 20 μg/ml trypsin for 20 min at 0°C, followed by inactivation of the trypsin with a 30-fold weight excess of soybean trypsin inhibitor (10 min at 0°C), where indicated. (**A**) The bypass import of radiolabeled porin into wild-type and *tom7Δ* mitochondria was compared at the indicated times (the mitochondria were treated with proteinase K after the import reaction). The amount of protein imported into non-trypsinized wild-type mitochondria after 20 min was set to 100% (control). The standard errors of the means (from four independent experiments) were ≤4%. (**B**) The bypass import of F_1-ATPase subunit β was performed and analyzed as described in (A). p and m, precursor and mature forms of F_1β.

imported. The deletion of Tom7 strongly reduced the rate of bypass import (Figure 6A). In comparison, the bypass import of F_1-ATPase subunit β was affected only slightly by the lack of Tom7 (Figure 6B), as was observed for the import of F_1-ATPase subunit β into non-trypsinized mitochondria (Figure 3A).

We conclude that Tom7 is involved in the import of porin and the two-step import of Su9–DHFR at a step after the function of the cytosolic domains of import receptors.

The lack of Tom7 stabilizes the interaction between Tom20, Tom22 and Tom40

We asked if the lack of Tom7 affected the association of Tom proteins in the outer membrane translocase. Isolated mitochondria from wild-type and *tom7Δ* cells were lysed in digitonin. Co-immunoprecipitations with antibodies directed against distinct Tom proteins, Tom40, Tom22 or Tom20, were performed. The amount of Tom20 co-precipitated with anti-Tom40 or anti-Tom22 antibodies from *tom7Δ* mitochondria was increased compared with the co-precipitation from wild-type mitochondria (Figure 7A, lanes 2 and 6; Figure 7C, columns 1 and 2). Similarly, the amount of Tom22 co-precipitated with anti-Tom40 or anti-Tom20 antibodies (Figure 7A, lanes 2 and 4; Figure 7E, columns 1 and 2), and the amount of Tom40 co-precipitated with anti-Tom20 or anti-Tom22 antibodies, were increased when comparing *tom7Δ* with wild-type mitochondria (Figure 7A, lanes 4 and 6). Because the total mitochondrial amounts of Tom40, Tom22 and Tom20 are unchanged between wild-type and *tom7Δ* mitochondria (Figure 7B), these results indicate that the stability of association between these Tom proteins is increased with *tom7Δ* mitochondria.

Alconada *et al.* (1995b) reported that mitochondria lacking Tom6 showed a destabilization of the outer membrane translocase (weakened association of Tom20, Tom40 and Tom70). Therefore we directly compared co-immunoprecipitations from *tom6Δ* mitochondria with those from *tom7Δ* mitochondria. The association of Tom20 with Tom40 and of Tom22 with Tom40 was decreased with *tom6Δ* mitochondria (Figure 7C, column 3; Figure 7D, column 4; Figure 7E, column 3), in contrast to the increased association observed with *tom7Δ* mitochondria (Figure 7A, C and E). In addition, we assessed the association of Tom20 with Tom22 by co-immunoprecipitations from *tom6Δ* and wild-type mitochondria, and did not observe a decrease with *tom6Δ* mitochondria (Figure 7C, column 4; Figure 7E, column 4).

The total mitochondrial amount of Tom70 is comparable with that of Tom20, yet Tom70 is associated only very loosely with most of the subunits of the translocase when assayed in a co-precipitation from digitonin-lysed mitochondria (Kiebler *et al.*, 1990; Alconada *et al.*, 1995b; Hönlinger *et al.*, 1995). In co-precipitations with anti-Tom20 or anti-Tom22 antibodies we observed only background levels of Tom70 (data not shown). With anti-Tom40 antibodies the co-precipitated amounts of Tom70 represented 10–15% that of Tom20 (wild-type mitochondria). As reported by Alconada *et al.* (1995b), we found that a lack of Tom6 further decreased the association between Tom70 and Tom40 (Figure 7D, column 3). However, with *tom7Δ* mitochondria the association of Tom70 with Tom40 was indistinguishable from wild type under both assay conditions, the co-precipitation of Tom70 with anti-Tom40 antibodies (Figure 7D, column 1) and the co-precipitation of Tom40 with anti-Tom70 antibodies (Figure 7D, column 2).

In summary, co-immunoprecipitations reveal a stabilization of the interactions between Tom20, Tom22 and Tom40 with *tom7Δ* mitochondria in comparison with wild-type mitochondria, whereas the interaction of Tom70 with Tom40 is unchanged. With *tom6Δ* mitochondria, the interactions between Tom20/Tom22, Tom40 and Tom70

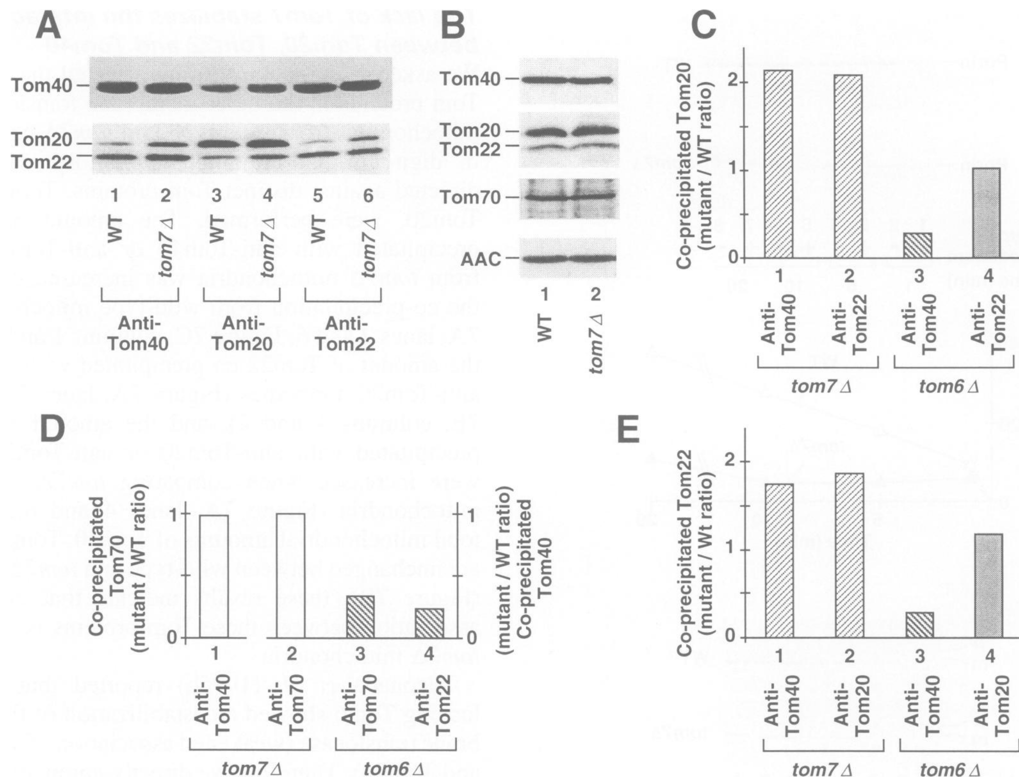

Fig. 7. Deletion of Tom7 stabilizes the interactions between Tom20, Tom22 and Tom40. (**A**) Co-immunoprecipitations of Tom40, Tom22 and Tom20. Mitochondria (250 μg/ml) were isolated from strains YPH499 and AH101 grown on YPGal medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) galactose], lysed in 0.5% digitonin and subjected to co-immunoprecipitation with anti-Tom40 antibodies (lanes 1 and 2), anti-Tom20 antibodies (lanes 3 and 4) and anti-Tom22 antibodies (lanes 5 and 6) covalently coupled to protein A–Sepharose. The co-precipitated proteins were separated by SDS–PAGE, transferred onto nitrocellulose and immunodecorated with antisera directed against Tom40, Tom22 and Tom20. (**B**) Total amounts of Tom70, Tom40, Tom22, Tom20 and ADP/ATP carrier (AAC) are not changed in mitochondria lacking Tom7. Wild-type and *tom7*Δ mitochondria (50 μg of protein) were separated by SDS–PAGE, transferred to nitrocellulose and immunodecorated with antisera directed against Tom70, Tom40, Tom22, Tom20 and ADP/ATP carrier. (**C**) Co-precipitation of Tom20 with anti-Tom40 and anti-Tom22 antibodies, respectively. Wild-type, *tom7*Δ and *tom6*Δ mitochondria were lysed with digitonin, subjected to co-immunoprecipitation with anti-Tom40 and anti-Tom22 antibodies, and immunodecorated with anti-Tom20 antibodies. The Tom20 band was quantified by laser densitometry, and the ratio of Tom20 co-precipitation from mutant versus wild-type mitochondria is shown. (**D**) Co-precipitation of Tom70 with anti-Tom40 antibodies, and of Tom40 with antibodies directed against Tom70 and Tom22, respectively, was performed as described in (C). (**E**) Co-precipitation of Tom22 with anti-Tom40 and anti-Tom20 antibodies was performed as described in (C). The standard errors of the means for (C)–(E) (typically from three to four independent experiments) were ≤0.1.

are destabilized (but not the interaction between Tom20 and Tom22).

Synthetic phenotypes of double deletions tom7Δ tom20Δ and tom6Δ tom7Δ

Genetic evidence for the functional cooperation of components can be obtained by a search for synthetic phenotypes, i.e. mutations in two genes (or deletions) lead to a phenotype not observed after the mutation or deletion of one gene (Huffaker *et al.*, 1987; Kaiser and Schekman, 1990; Rassow *et al.*, 1994). For example, synthetic growth defects were observed with *tom6*Δ *tom20*Δ and *tom6*Δ *tom70*Δ (Alconada *et al.*, 1995b).

We asked for the growth phenotype of a double deletion *tom7*Δ *tom20*Δ. The *tom7*Δ strain was crossed with a *tom20*Δ strain (which is viable on a fermentable carbon source; Ramage *et al.*, 1993; Moczko *et al.*, 1994). The heterozygous diploid was sporulated, and viable and inviable spores were obtained on fermentable medium at 30°C (Figure 8A). An analysis of the genotypes of the viable spores demonstrated that the inviability was caused by the loss of both genes. The double-deficient spores stopped growth after ~10 divisions, even when streaked out on new plates. The same inviability was observed at 23°C. These results indicate the synthetic lethality of a double deletion *tom7*Δ *tom20*Δ.

Similarly, we crossed a *tom7*Δ strain with a *tom70*Δ strain (like *tom7*Δ cells, *tom70*Δ cells are viable on both fermentable and non-fermentable carbon sources and are only moderately reduced in growth at higher temperatures on non-fermentable medium; Hines *et al.*, 1990; Steger *et al.*, 1990). We obtained a double mutant *tom7*Δ *tom70*Δ that was viable on both fermentable and non-fermentable carbon sources. In contrast with *tom6*Δ *tom70*Δ cells that stopped growth on non-fermentable medium at 37°C (Alconada *et al.*, 1995b), *tom7*Δ *tom70*Δ cells grew at 37°C on non-fermentable medium (Figure 8B); the ~2-fold reduction in the growth rate in comparison with wild-type cells can be explained by the sum of the growth reductions of the single-deletion mutants. Thus we did not observe evidence for a synthetic growth defect of a double deletion *tom7*Δ *tom70*Δ.

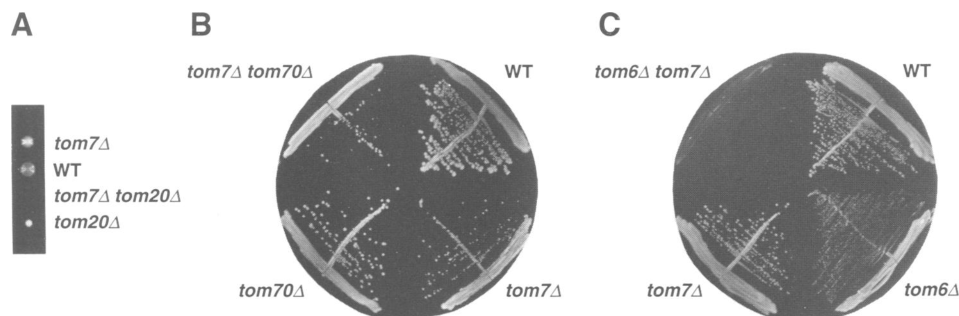

Fig. 8. Synthetic growth defects of *S.cerevisiae* cells with deletions of two *TOM* genes. (**A**) Synthetic lethality of *tom7Δ tom20Δ*. A heterozygous diploid strain containing one disrupted allele of *TOM7* and one disrupted allele of *TOM20* was sporulated, and the asci were dissected. The viable spores were analyzed for their phenotype to determine the genotypes. A total of 52 tetrads were analyzed. A typical tetratype tetrad is shown. (**B**) No synthetic growth defect of *tom7Δ tom70Δ*. Strains YPH499, AH101, MM208 and AH410 were grown on a YPG (3% glycerol) plate at 37°C. (**C**) Synthetic growth defect of *tom6Δ tom7Δ*. Strains YPH499, MM307, AH101 and AH610 were grown on a YPG plate at 37°C.

Next we generated a double mutant *tom6Δ tom7Δ*. The mutant cells grew very slowly at 30°C on non-fermentable medium and stopped growing at 37°C on both fermentable and non-fermentable media, in contrast to the single mutants (Figure 8C). As a control, *tom6Δ tom7Δ* cells were transformed with a plasmid expressing Tom7, leading to a growth phenotype like the *tom6Δ* single-mutant cells. We conclude that a double deletion *tom6Δ tom7Δ* leads to a strong synthetic growth defect.

Discussion

At a molecular level we have identified Tom7, a small subunit of the preprotein translocase of the outer mitochondrial membrane. The primary sequence of Tom7 does not reveal a significant similarity to any known protein. Tom7 contains a hydrophobic core with predicted β-strands and behaves as an integral membrane protein. Like other outer membrane proteins, Tom7 is not proteolytically processed during import. Amino acid sequencing demonstrates that the initial methionine is post-translationally removed, yielding a protein of 59 amino acid residues.

Saccharomyces cerevisiae cells lacking the *TOM7* gene were constructed. The resulting *tom7Δ* cells were viable. Mitochondria lacking Tom7 revealed an import phenotype that was not observed with any of the numerous mutants in other components of the mitochondrial protein import machinery studied so far (summarized in Kübrich *et al.*, 1995). The import of preproteins destined for the mitochondrial interior, such as the inner membrane or matrix, was affected only slightly with *tom7Δ* mitochondria, regardless of whether or not the preproteins were synthesized with a cleavable presequence. However, import of the outer membrane protein porin was reduced significantly with *tom7Δ* mitochondria. The import of porin was previously found to involve the import receptor Tom20. Anti-Tom20 antibodies or a deletion of Tom20 inhibited the import of porin, as well as the import of typical presequence-carrying preproteins, to the same degree (Söllner *et al.*, 1989; Mayer *et al.*, 1993; Ramage *et al.*, 1993; Moczko *et al.*, 1994). The preferential inhibition of porin import into *tom7Δ* mitochondria suggests that the import impairment may occur beyond the initial recognition by Tom20. This was demonstrated experimentally by analyzing the so-called bypass import, i.e. import into mitochondria devoid of the cytosolic domains of import receptors because of pretreatment with trypsin (Pfaller *et al.*, 1989). Bypass import occurs with an efficiency of ~20–30% compared with import into intact mitochondria, and is thought to reflect the import properties of the general import pore of the mitochondrial outer membrane. The bypass import into *tom7Δ* mitochondria revealed a similar preference for the inhibition of porin import as non-trypsinized mitochondria, indicating that an import defect caused by a lack of Tom7 was effective beyond the action of surface domains of import receptors.

To investigate whether *tom7Δ* mitochondria may also significantly affect the import steps of preproteins destined for the mitochondrial interior, we studied a two-step import reaction (productive binding; Hines and Schatz, 1993). A matrix-targeted preprotein was accumulated first at the outer membrane in the absence of a membrane potential, followed by chase into mitochondria by the establishment of a membrane potential. In contrast to the direct one-step import into energized mitochondria, the two-step import was reduced strongly with *tom7Δ* mitochondria. We conclude that Tom7 is involved in the productive accumulation of preproteins at the outer membrane.

How can the unique import phenotype of *tom7Δ* mitochondria be explained? Three stages of porin transfer through the outer membrane translocase can be envisaged: (i) recognition by the cytosolic domains of surface receptors (Söllner *et al.*, 1989; Mayer *et al.*, 1993; Moczko *et al.*, 1994), (ii) insertion via the GIP (Pfaller *et al.*, 1988), and (iii) lateral movement out of the GIP for sorting into the outer membrane. Because an impairment of stages (i) or (ii) would reduce the import of porin and the one-step import of preproteins destined for the mitochondrial interior to a similar degree, it is likely that Tom7 influences stage (iii). Moreover, we found that the inhibitory effect of a lack of Tom7 on the two-step import of a cleavable preprotein still took place after removal of the surface receptor domains, supporting the hypothesis that Tom7 also influences a later stage beyond recognition by surface receptor domains. We speculate that a 'sorting site' of the outer membrane translocase is of critical importance for the lateral movement of porin into the outer membrane. Such a site can be used for the productive accumulation of transport intermediates. This 'sorting site' could either be different from the GIP, i.e. a site of the

translocase which is located as a sideshoot from the main import route into the mitochondrial interior, or represent a distinct functional state of the GIP. Tom7 would influence the function of this 'sorting site'.

Then we questioned how Tom7 exerts its effect on protein import. Accumulated preproteins (including Su9–DHFR) could be cross-linked to Tom5 and several of the large Tom proteins, but not to Tom7 or Tom6 (Hönlinger *et al.*, 1995; A.Hönlinger, M.Kübrich and N.Pfanner, unpublished results). Thus there is no experimental evidence for a direct interaction of Tom7 with preproteins. However, we found that Tom7 modulates the association of Tom proteins in the outer membrane translocase. A lack of Tom7 stabilized the interactions between the receptors Tom20 and Tom22 and the import pore component Tom40. The interaction of the receptor Tom70 with Tom40 was not affected by a lack of Tom7. These observations are in contrast to the influence of Tom6 on the translocase. A lack of Tom6 destabilizes the interactions between Tom20/Tom22, Tom70 and Tom40; the interaction between Tom20 and Tom22 is not destabilized in *tom6*Δ mitochondria (a lack of Tom6 or Tom7 does not affect the total mitochondrial amounts of the other Tom proteins).

These observations suggest that Tom7 favors a dissociation of subunits of the outer membrane translocase, whereas Tom6 favors an association of subunits. Tom6 promotes the association of both receptor subcomplexes of Tom20/Tom22 (Lithgow *et al.*, 1994; Hönlinger *et al.*, 1995; Mayer *et al.*, 1995) and Tom70 (probably together with Tom37; Gratzer *et al.*, 1995) with Tom40, which is assumed to represent the central component of the outer membrane translocase (Vestweber *et al.*, 1989; Kiebler *et al.*, 1990; Baker and Schatz, 1991; Pfanner *et al.*, 1991; Alconada *et al.*, 1995b). Tom6 does not influence the interaction between Tom20 and Tom22, indicating that Tom6 is important for the interactions occurring with Tom40. Tom7 promotes a dissociation of Tom20 and Tom22 from Tom40, as well as a dissociation of the subcomplex between Tom20 and Tom22. Tom7 does not seem to influence the interaction of Tom70 (probably together with Tom37) with Tom40. The dissociating effect of Tom7 thus focuses on the interactions of and within the Tom20/Tom22 subcomplex.

The biochemical evidence for a functional relationship of Tom6 and Tom7 with subsets of larger Tom proteins is supported by genetic evidence, i.e. synthetic growth defects of double mutants. The genetic data indicate a functional relationship of Tom6 with both Tom20 and Tom70 (Alconada *et al.*, 1995b), and a functional relationship of Tom7 with Tom20; a synthetic growth defect was not observed for double mutant *tom7*Δ *tom70*Δ, in agreement with the biochemical data.

We conclude that Tom6 and Tom7 modulate the dynamics of the outer membrane translocase. They have opposite effects on the interactions between Tom20/Tom22 and Tom40. We propose that cycles of assembly and disassembly of subunits of the translocase are important for the efficient action of the outer membrane import machinery. Furthermore, we suggest that the complementary functions of Tom6 and Tom7 promote such cycles. Indeed, a double deletion *tom6*Δ *tom7*Δ showed a strong synthetic growth defect. It may be speculated that a dissociation of several subunits, as favored by Tom7, is important for the function of the 'sorting site' which is involved in the import of porin and the accumulation of transport intermediates (whereas the direct transport through the GIP is influenced only slightly). The dissociation of domains of receptors that are not exposed on the cytosolic side (membrane anchors, intermembrane space domain of Tom22; Kiebler *et al.*, 1993; Lithgow *et al.*, 1994; Hönlinger *et al.*, 1995) may be critical for the function of the 'sorting site', because the pathway-related import inhibition, caused by a lack of Tom7, was still effective after removal of the cytosolic domains of the import receptors.

Recent studies with various membrane systems suggest that dynamic interactions of subunits of preprotein translocases are important for the ordered action of protein transport machineries (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schnell *et al.*, 1994; Wickner, 1994; Berthold *et al.*, 1995; Brodsky *et al.*, 1995; Horst *et al.*, 1995; Panzner *et al.*, 1995; Soll, 1995). Our studies with the translocase of the outer mitochondrial membrane indicate that very small subunits can function as modulators of the dynamics of the protein transport machinery, and suggest a network of interactions between individual subunits of a translocase.

Materials and methods

Construction of plasmids and S.cerevisiae mutants

Construction of pGEM4Z–TOM7 and pGEM4Z–TOM7-Met. For the *in vitro* synthesis of Tom7, the coding region of the yeast *TOM7* was amplified by PCR using 1 μg yeast genomic DNA. Amplification was performed with the sense primer 5′-GAATTCAGATCTATGAGTTT-CCTACCATCTTTTATC-3′ with a *Bgl*II site and the antisense primer 5′-CTGGACGTCGACTTAAACACTTGGTAACGGAGACAGTAA-3′ with a *Sal*I site. To construct *TOM7-Met*, the oligonucleotide 5′-CTGG-ACGTCGACTTACATAACACTTGGTAACGGAGACAGTAA-3′ containing a *Sal*I site was used as the second antisense primer to introduce a methionine codon before the stop codon of *TOM7*. Both amplified DNA fragments were cleaved with *Bgl*II and *Sal*I and ligated into the *BamH*I–*Sal*I linearized vector pGEM4Z (Promega), resulting in pGEM4Z–*TOM7* and pGEM4Z–*TOM7-Met*. The accuracy of the amplified sequences was confirmed by double-strand sequencing.

Construction of pRS415–TOM7-HA. A DNA sequence encoding the HA epitope YPYDVPDYA (Kolodziej and Young, 1991) was added to the C-terminus of the *TOM7* cDNA by manipulation with PCR. To enable the synthesis of Tom7-HA under the control of its own promoter, the amplification was performed using the sense primer 5′-AAGCTTG-AGCTCGGTTAGAATGAAAATAAAATTCGCCAG-3′ starting immediately after the stop codon of the dihydrolipoamide acetyltransferase gene with a *Sac*I site and the antisense primer 5′-TTTGTCGACTTAA**GCGTAGTCTGGGACGTCGTATGGGTA**AACACTTGGTAAGG-3′ with a *Sal*I site; the HA motif (12CA5)-encoding part is in bold. The amplified DNA fragment was cleaved with *Sac*I and *Sal*I, and ligated into the *Sac*I–*Sal*I linearized vector pRS415 (Sikorski and Hieter, 1989). The resulting plasmid pRS415–*TOM7-HA* was transformed into the haploid strains AH101 (*tom7*Δ) and AH610 (*tom6*Δ *tom7*Δ). For detection, the 12CA5 antibody (Boehringer Mannheim) was used according to the user's manual.

Disruption of TOM7. To disrupt *TOM7*, a 270 bp fragment of the 5′ non-coding region was amplified using the sense primer 5′-GGATTCG-AATTCTACTTCGCTTGTGTTCGAATC-3′ with an *Eco*RI site and the antisense primer 5′-AGCTGTCGACCCCGGGGGTTTATTTGTTAAA-AGCTAATTT-3′ with a *Sal*I and *Sma*I site. The amplified fragment was cleaved with *Eco*RI and *Sal*I and ligated into an *Eco*RI–*Sal*I linearized vector pGEM4Z (Promega), resulting in pGEM4Z-5-7. A 160 bp fragment of the 3′ non-coding region of *TOM7* was amplified using the sense primer 5′-GAATTCCCCGGGAAACATCAGTTCGATACAA-CTT-3′ containing a *Sma*I site and the antisense primer 5′-GGATTCGT-

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference
PK82	*MATα his4-173 lys2 ura3-52 Δtrp1 leu2-3,112*	Gambill *et al.* (1993)
YPH501	*MATa/α ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 lys2-801/lys2-801*	Sikorski and Hieter (1989)
YPH499	*MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801*	Sikorski and Hieter (1989)
MM112	*MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom20::URA3*	Moczko *et al.* (1994)
MM208	*MATa ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom70::HIS3*	Moczko *et al.* (1994)
MM307	*MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom6::URA3*	Alconada *et al.* (1995b)
AH101	*MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom7::TRP1*	this study
AH201	*MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom7::TRP1, pRS415(LEU2)-TOM7-HA*	this study
AH301	*MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom6::URA3 tom7::TRP1, pRS415(LEU2)-TOM7-HA*	this study
AH410	*MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom7::TRP1 tom70::HIS3*	this study
AH520	*MATa/α ade2-101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 lys2-801/lys2-801 TOM7/tom7::TRP1 TOM20/tom20::URA3*	this study
AH610	*MATα ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom6::URA3 tom7::TRP1*	this study

CGACCAATATGCAAAATGAATTTTATATTTT-3′ with a *Sal*I site. The amplified fragment was cleaved with *Sma*I and *Sal*I and subcloned into the *Sma*I–*Sal*I linearized plasmid pGEM4Z-5-7, resulting in pGEM4Z-53-7. A *Sma*I fragment encoding the *TRP1* gene was ligated into the *Sma*I site of pGEM4Z-53-7. The disrupted *TOM7* gene was excised with *Eco*RI and *Sal*I and transformed into the diploid strain YPH501. Tryptophan-prototrophic cells were subjected to a spore analysis. The *S.cerevisiae* strains used are listed in Table I.

For the construction of double-deficient yeast cells, heterozygous diploids were constructed with the strains MM112 (*tom20Δ*), MM208 (*tom70Δ*) and MM307 (*tom6Δ*) (Moczko *et al.*, 1994; Alconada *et al.*, 1995b). The diploid cells were sporulated and the genotypes of the spores were analyzed by growth on selective media. Standard procedures were used for manipulation of the DNA and yeast strains (Ausubel *et al.*, 1989; Guthrie and Fink, 1991).

In vitro import of preproteins into isolated mitochondria

After *in vitro* transcription, preproteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine, as described previously (Söllner *et al.*, 1991). Mitochondria were isolated according to Daum *et al.* (1982) and Hartl *et al.* (1987). Import reactions were performed in bovine serum albumin (BSA)-containing buffer [250 mM sucrose, 3% (w/v) fatty acid-free BSA, 80 mM KCl, 5 mM $MgCl_2$ and 10 mM MOPS/KOH, pH 7.2], including 2 mM ATP, 2 mM NADH and 50 μg mitochondrial protein per ml (Söllner *et al.*, 1991; Gärtner *et al.*, 1995a). Import reactions were performed at 25°C for the times indicated. Mitochondria were reisolated by centrifugation at 16 000 *g* for 10 min at 2°C. Samples were treated with proteinase K (100 μg/ml) for 15 min at 0°C where indicated. Proteinase K was inactivated by the addition of 1 mM phenylmethylsulfonyl fluoride and incubation for 10 min at 0°C. After a washing step with SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2), pelleted mitochondria were either analyzed by SDS–PAGE or lysed in digitonin buffer (Alconada *et al.*, 1995a,b) and subjected to co-immunoprecipitation with antibodies covalently coupled to protein A–Sepharose, as described previously (Rassow *et al.*, 1994; Voos *et al.*, 1994). Surface domains of receptors were removed by the treatment of mitochondria prior to the import reaction with 20 μg/ml trypsin for 20 min at 0°C. The protease was inactivated by the addition of a 30-fold weight excess of soybean trypsin inhibitor and a 10 min incubation at 0°C. Dissipation of the membrane potential was accomplished by the addition of a mixture of valinomycin, oligomycin and antimycin A (final concentrations 0.5, 20 and 8 μM, respectively).

For the assessment of the import of the ADP/ATP carrier, radiolabeled protein was imported into mitochondria. Mitochondria were reisolated, washed in SEM and resuspended in 100 μl lysis buffer [2.5% (w/v) Triton X-100-Puffer, 110 mM NaCl, 20 mM MOPS/KOH, pH 7.2] containing 10 μM atractyloside (Sigma). Samples were incubated for 10 min at 4°C. Insoluble material was separated by centrifugation at 16 000 *g* for 5 min at 2°C. The supernatant was applied onto columns containing 40 μg hydroxylapatite pre-equilibrated with 0.5% (w/v) Triton X-100, 50 mM NaCl, 10 mM MOPS/KOH, pH 7.2 (Schleyer and Neupert, 1984). Assembled, dimeric ADP/ATP carrier was found in the flowthrough fraction and was precipitated with 7.5% trichloroacetic acid in the presence of 0.0125% sodium deoxycholate.

Standard procedures were used for Western blotting onto nitrocellulose. Bands were detected by the Enhanced Chemiluminescence System (Amersham). Membrane potential $\Delta\psi$ was assessed using the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$; Molecular Probes Inc.; Sims *et al.*, 1974; Eilers *et al.*, 1987; Gärtner *et al.*, 1995b].

Two-step import of Su9–DHFR

Mitochondria (100 μg protein) were incubated with valinomycin in 300 μl incubation buffer [3% (w/v) BSA, 250 mM sucrose, 5 mM $MgCl_2$, 5 mM sodium malate, 20 mM KP_i, 1 μM valinomycin, 10 mM MOPS/KOH, pH 7.2] for 3 min at 0°C. 1/20 volume of reticulocyte lysate or urea-denatured precursor (Ostermann *et al.*, 1989; Kang *et al.*, 1990) was added, and incubation was continued for 5 min. After dividing the samples into three aliquots, mitochondria were reisolated and resuspended in a corresponding volume of incubation buffer ('$-\Delta\psi$') or chase buffer ['$+\Delta\psi$'; 3% (w/v) BSA, 250 mM sucrose, 5 mM $MgCl_2$, 5 mM sodium malate, 20 mM NaP_i, 1 μM valinomycin, 10 mM MOPS/NaOH, pH 7.2]. After incubation at 25°C, import was stopped by the addition of KCl (20 mM final concentration) and rapid cooling to 0°C. Samples were divided in two: one half was treated with proteinase K (50 μg/ml) for 15 min at 0°C. As a control, one aliquot was treated as described above but received the precursor protein in the second incubation step instead of the first.

Alkaline extraction of mitochondria

Mitochondria (100–250 μg protein) were incubated in 100 mM Na_2CO_3 for 60 min at 0°C. Separation of the pellets and supernatants was achieved by centrifugation at 266 000 *g* for 90 min at 2°C. Supernatants and pellets were either precipitated with 7.5% trichloroacetic acid in the presence of 0.0125% sodium deoxycholate, or treated according to the method described by Wessel and Flügge (1984).

Amino acid sequence analysis

For N-terminal sequencing, outer membrane translocase was isolated from *S.cerevisiae* strain PK82 in chemical amounts by lysis of the mitochondria with digitonin and affinity purification with protein A–Sepharose containing covalently coupled antibodies against Tom40. After several washing steps, the proteins were eluted at pH 2.5 (Moczko *et al.*, 1992; Hönlinger *et al.*, 1995), separated by urea–SDS–PAGE (Alconada *et al.*, 1995a,b) and transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore). The band corresponding to Tom7 was sequenced using a pulsed liquid-phase 477A sequencer equipped with an on-line model 120A PTH amino acid analyzer (Applied Biosystems; Eckerskorn *et al.*, 1988).

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