The EMBO Journal vol.12 no.8 pp.3035–3041, 1993

Protein import into yeast mitochondria: the inner membrane import site protein ISP45 is the *MPI1* gene product

Martin Horst, Paul Jenö, Nafsika G.Kronidou, Luca Bolliger, Wolfgang Oppliger, Philipp Scherer[1], Ute Manning-Krieg[2], Thomas Jascur[3] and Gottfried Schatz[4]

Biocenter, University of Basel, CH-4056 Basel, Switzerland

[1]Present address: The Whitehead Institute, Nine Cambridge Center, Cambridge, MA 02142, USA
[2]Present address: SANDOZ AG, Pharma Division, Preclinical Research, PO Box, CH-4002 Basel, Switzerland
[3]Present address: Hoffman-La Roche Ltd, Signal Transduction Group, PO Box, CH-4002 Basel, Switzerland
[4]Corresponding author

Communicated by G.Schatz

Protein import across both mitochondrial membranes is mediated by the cooperation of two distinct protein transport systems, one in the outer and the other in the inner membrane. Previously we described a 45 kDa yeast mitochondrial inner membrane protein (ISP45) that can be cross-linked to a partially translocated precursor protein (Scherer *et al.*, 1992). We have now purified ISP45 to homogeneity and identified it as the product of the nuclear *MPI1* gene. Identity of ISP45 with the *MPI1* gene product was shown by microsequencing of three tryptic ISP45 peptides and by demonstrating that an antibody against an Mpi1p–β-galactosidase fusion protein specifically recognizes ISP45. Antibodies monospecific for ISP45 inhibited protein import into right-side-out mitochondrial inner membrane vesicles, but not into intact mitochondria. On solubilizing mitochondria, ISP45 was rapidly converted to a 40 kDa proteolytic fragment unless mitochondria were first denatured with trichloroacetic acid. The combined genetic and biochemical evidence identifies ISP45/Mpi1p as a component of the protein import system of the yeast mitochondrial inner membrane.

Key words: mitochondrial inner membrane/protein translocation/submitochondrial vesicles/yeast

Introduction

There is now strong evidence that the mitochondrial inner membrane has a protein import system distinct from that in the outer membrane (Glick *et al.*, 1991; Pfanner *et al.*, 1992; Horst *et al.*, 1993; Schatz, 1993). After protein import into intact yeast mitochondria has been blocked by inactivating the outer membrane system either with proteases, with antibodies against outer membrane proteins or with partly translocated precursors, import can be restored by selectively rupturing the outer membrane (Ohba and Schatz, 1987; Hwang *et al.*, 1989). Also, right-side-out inner membrane vesicles import precursor proteins nearly as efficiently as do intact mitochondria (Hwang *et al.*, 1989). The separate operation of the two import systems can also be demonstrated with intact mitochondria. For example, when mitochondria are depleted of matrix ATP, precursors are transported across the outer membrane and get stuck across the inner membrane (Hwang *et al.*, 1991; Jascur *et al.*, 1992). Furthermore, uncoupled mitochondria have been shown to transport an artificial precursor across only the outer membrane; if the electrochemical potential across the inner membrane is then restored, the precursor is transported from the intermembrane space into the matrix (Segui-Real *et al.*, 1993). Thus, the two translocation systems are not permanently linked, but can interact reversibly.

While several proteins of the outer membrane system have been identified (Baker and Schatz, 1991; Pfanner *et al.*, 1992), little is known about the inner membrane system. Biochemical as well as genetic approaches have been used in attempts to find components of this system in *Saccharomyces cerevisiae*. The biochemical approach was based on two separate methods (Scherer *et al.*, 1992). In the first method, antisera raised against glutaraldehyde-fixed mitochondrial inner membrane vesicles were screened for their ability to inhibit protein import into these vesicles. The target antigens responsible for the inhibition were then identified by antibody quenching tests. In the second method, antisera directed against different subsets of mitochondrial proteins were tested for their ability to immunoprecipitate a protein that was cross-linked to a radioactive precursor protein stuck across the inner membrane of ATP-depleted mitochondria (Scherer *et al.*, 1992). As before, the target antigen was subsequently tracked down by antibody quenching assays. These methods identified two inner membrane proteins: a 45 kDa protein termed ISP45 (import site protein 45; Scherer *et al.*, 1992) and a 40 kDa protein (M.Horst, unpublished). However, as neither of these proteins was purified to homogeneity, their properties and detailed function could not be defined.

The genetic approach was based on a screen designed to select yeast mutants defective in transporting an artificial mitochondrial precursor protein across the inner membrane (Maarse *et al.*, 1992). This screen netted a gene (termed *MPI1*) that potentially encoded a 48.8 kDa protein. The gene was essential for viability and a mutation in it partly inhibited proteolytic cleavage of some mitochondrial precursor proteins *in vivo*. However, this approach did not show directly that the *MPI1* gene encoded a component of the inner membrane protein import system.

In this study, we purified ISP45 and the 40 kDa protein to homogeneity and obtained partial amino acid sequences of both proteins. We found that ISP45 is encoded by the *MPI1* gene and that the 40 kDa protein is a proteolytic fragment of ISP45. In addition, we observed that antibodies monospecific for ISP45 immunoprecipitate the cross-linked inner membrane translocation intermediate and that they

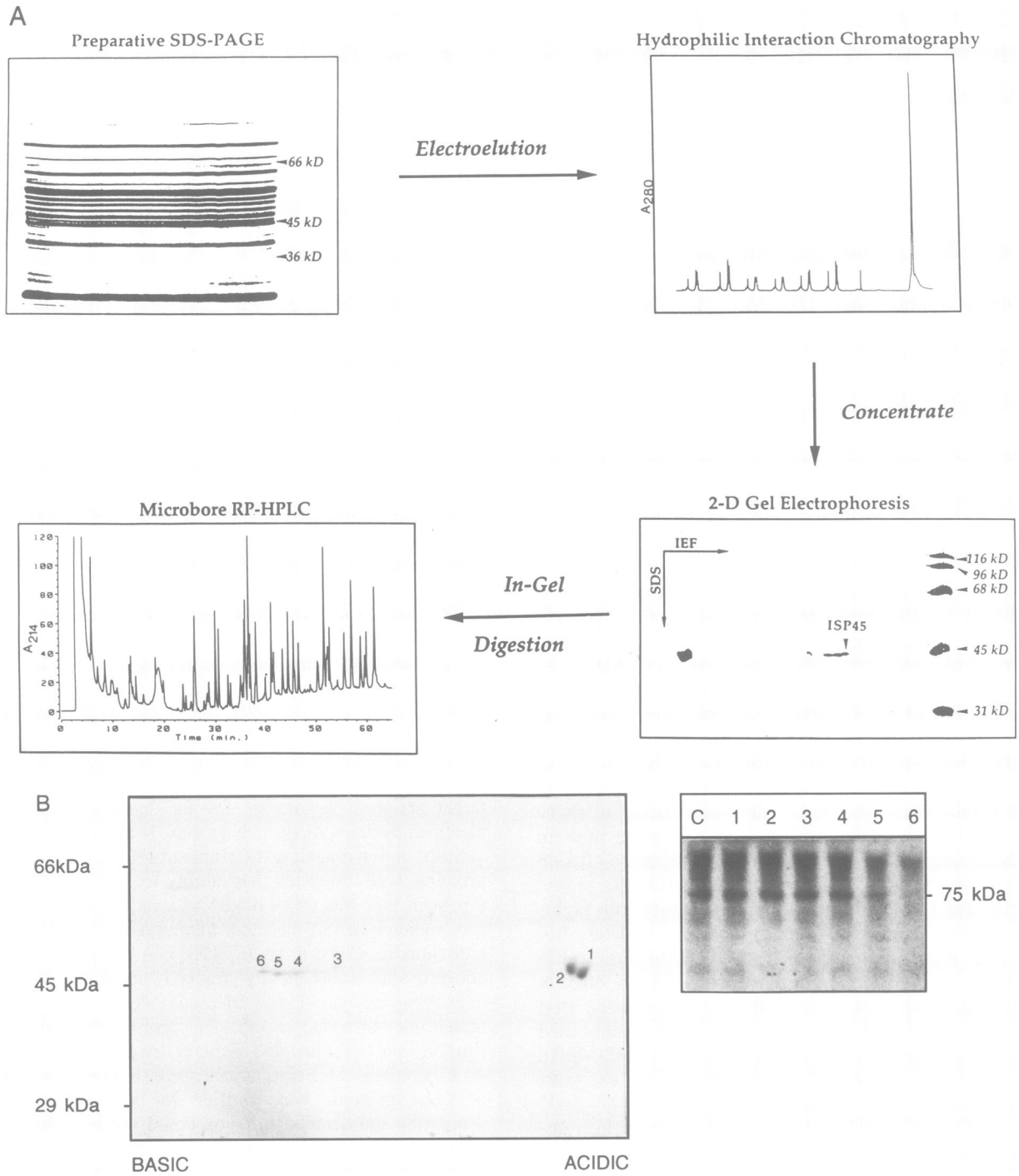

Fig. 1. Purification of ISP45. (**A**) Yeast mitochondria were purified on a Nycodenz gradient, precipitated with 10% trichloroacetic acid and subjected to preparative SDS−10% PAGE. The gels were lightly stained with Coomassie brilliant blue and the 45 kDa region was excised. Proteins were electroeluted from the gel slices, precipitated with 80% ethanol, purified by hydrophilic interaction chromatography, concentrated in a Speed-Vac desiccator and resolved by two-dimensional gel electrophoresis. The spot containing ISP45 was excised, digested within the gel piece with trypsin, and the resulting tryptic ISP45 fragments were separated by reversed-phase microbore HPLC. (**B**) Purified ISP45 quenches immunoprecipitation of the 75 kDa cross-linking product. Top: 2D-gel electrophoresis (isoelectric focusing from pH 7−10, followed by SDS−10% PAGE) of the 45 kDa proteins eluted from the hydrophilic interaction column (see above), stained briefly with Coomassie blue. The six major spots are numbered 1−6. Bottom: each of the stained spots was excised and tested for quenching of the immunoprecipitation of the 75 kDa crosslink by antiserum A. (**C**) Immunoprecipitation by untreated antiserum A. The position of the crosslinked product on the SDS gel is marked on the right.

inhibit protein import into inner membrane vesicles, but not into intact mitochondria. Therefore, the combined biochemical and genetic data now provide strong evidence that ISP45/Mpi1p is a component of the protein import system of the inner membrane.

Results

Purification of ISP45

We previously reported that a rabbit antiserum raised against yeast mitochondrial inner membrane vesicles inhibited import

Table I. Amino acid sequences of tryptic peptides derived from ISP45 and its 40 kDa fragment

Sequence	Correspondence to *MPI1* open reading frame
Peptides derived from ISP45	
NH_2 - E - Y - I - V - P - E - I - L - E - A - Y - V -	295–306
NH_2 - W - F - S - E - A - P - F - N - V - Y - A - A -	316–327
NH_2 - L - L - A - P - Q - D - I - P - V - L - V - V - G - C - R - COOH	356–370
Peptides derived from the 40 kDa protein	
NH_2 - L - G - E - S - E - A - Y - K - COOH	92–99
NH_2 - T - G - E - T - M - E - H - I - A - T - K - COOH	112–122
NH_2 - K - V - E - D - F - K - E - K - COOH	221–228

Peptides whose sequences are terminated by four dots were only partially sequenced

of the artificial precursor protein pCOXIV–DHFR into these vesicles (Scherer *et al.*, 1992). In that study, we attempted to identify the target antigen(s) as follows. We had solubilized mitochondria with the nonionic detergent octyl-polyoxyethylene (OPOE), fractionated the proteins by anion exchange chromatography and coupled the proteins from each column fraction to cyanogen bromide-activated Sepharose. The immobilized proteins were then used to affinity purify the cognate antibodies from the crude serum and each antibody fraction was tested for inhibition of protein import into inner membrane vesicles. This approach identified four column fractions that contained putative target antigens, but were still contaminated by other proteins. Two of the fractions contained many different proteins and were not studied further. The other two fractions were less heterogeneous. One of them contained a 40 kDa target antigen. The target antigen in the remaining fraction was ISP45 (Scherer *et al.*, 1992).

Although the ability of ISP45 to quench immunoprecipitation of the cross-linked immobilized precursor was a very sensitive assay, ISP45 was difficult to purify as it was extremely protease-sensitive and present in low amounts. Once mitochondria had been solubilized with OPOE, ISP45 was rapidly degraded even in the presence of protease inhibitors. To minimize proteolysis, we purified yeast mitochondria on a Nycodenz density gradient (to remove contaminating vacuolar proteases; B. Glick, unpublished observation), denatured them in 10% trichloroacetic acid and solubilized them in hot SDS. The mitochondrial proteins were then separated on preparative SDS–polyacrylamide gels and proteins migrating in the 45 kDa region were excised and electroeluted from the gel. They were further purified by hydrophilic interaction chromatography and two-dimensional gel electrophoresis in the presence of urea. ISP45 migrated as a single spot at around pI 9 (Figure 1A and Materials and methods). The protein constituting this spot was ISP45 because it quenched the ability of antiserum A to immunoprecipitate the crosslinked, stuck precursor (Figure 1B).

ISP45 is encoded by the MPI1 gene

To check whether ISP45 was an already known protein, purified ISP45 was digested with trypsin, the resulting peptides were separated by reversed phase HPLC (Figure 1A, lower left) and three of them were sequenced by automated Edman degradation. Each of the three sequences corresponded exactly to parts of the *MPI1* open reading frame (Table I, upper part). As mentioned above, this essential yeast gene encodes a putative component of the mitochondrial protein import system (Maarse *et al.*, 1992).

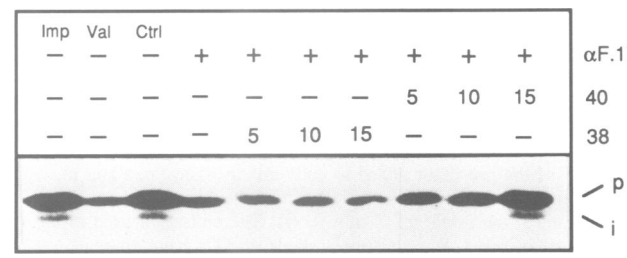

Fig. 2. A 40 kDa mitochondrial protein is a target antigen for import-inhibiting antibodies. The 40 kDa and the 38 kDa proteins were purified from anion exchange chromatography fraction 17 (Scherer *et al.*, 1992) by SDS–PAGE and electroelution, and tested for their ability to quench the import-inhibiting activity of antibodies that had been affinity purified from antiserum MH596 against this column fraction. Import of pCOXIV–DHFR into mitochondrial inner membrane vesicles was performed as described in Materials and methods. Import was assayed as potential-dependent processing of radiolabelled pCOXIV–DHFR to the intermediate sized form. Imp, no additions; Val, in presence of 1 mM valinomycin; Ctrl, after preincubation with 25 μg IgG against yeast cytochrome c_1; + αF.17, after preincubation with 25 μg IgGs from antiserum MH596 that had been affinity-purified against fraction 17; where indicated, this affinity-purified IgG fraction has been preincubated with the indicated amounts (in μg) of purified 40 kDa (40) or 38 kDa (38) protein. p and i, precursor form and intermediate (imported) form of pCOXIV–DHFR.

A 40 kDa inner membrane protein is a target antigen for antibodies inhibiting protein import into mitochondrial membrane vesicles

As mentioned above, chromatography of mitochondrial proteins had yielded another column fraction which contained a putative target antigen for the import-inhibiting antiserum. Analysis by SDS–PAGE showed that this fraction, like all the others, contained several proteins. However, by comparing this fraction with the adjacent ones, only a 38 kDa and a 40 kDa protein band peaked together with antigen(s) recognized by the import-inhibiting antibodies. Further purification of the two proteins by SDS–PAGE followed by electroelution showed that only the 40 kDa protein band quenched the inhibitory activity of the antibodies which had been affinity-purified against this column fraction (Figure 2).

Antisera raised against the electroeluted 40 kDa protein reacted with a single 40 kDa band when tested by immunoblotting against total mitochondrial proteins (Figure 3B) and IgGs prepared from this antiserum strongly inhibited protein import into inner membrane vesicles, but not into intact

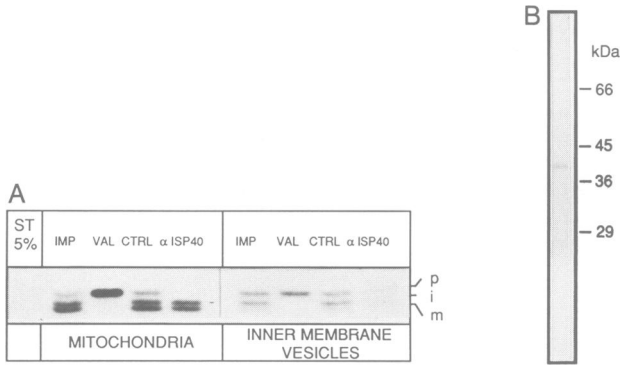

Fig. 3. IgGs against the purified 40 kDa protein block protein import into mitochondrial inner membrane vesicles, but not into intact mitochondria. (**A**) Mitochondria or mitrochondrial inner membrane vesicles (10 μg each) were tested for their ability to import the radiolabelled pCOXIV−DHFR fusion protein under the following conditions: IMP, no additions; VAL, in presence of 1 mM valinomycin; CTRL, after preincubation for 1 h at 0°C with 25 μg IgGs against yeast cytochrome c_1; αISP40, after preincubation for 1 h at 0°C with 25 μg of IgGs against the purified 40 kDa protein. STD 5%, 5% of the precursor added to each import assay; p, i and m, precursor, intermediate and mature form of the fusion protein. A fluorogram of the dried gel is shown. (**B**) The antiserum used in the experiment of panel A recognizes a single 40 kDa band among mitochondrial proteins separated by SDS−10% PAGE. Immunoblotting was performed with 1000-fold diluted antiserum raised against purified 40 kDa protein as described by Haid and Suissa (1983) except that the immune complex was identified by staining for alkaline phosphatase. The corresponding lane of the SDS−10% polyacrylamide gel had been loaded with 50 μg of total mitochondrial protein. Molecular size markers are indicated on the right. In this experiment, the mitochondria had not been denatured with 10% trichloroacetic acid before being solubilized in SDS; most of the ISP45 was therefore converted to the 40 kDa proteolytic fragment (see Figure 5 and 6).

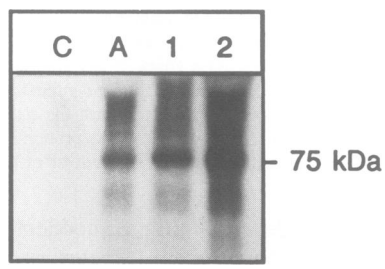

Fig. 4. Antibodies monospecific for the 40 kDa protein immunoprecipitate ISP45 crosslinked to a stuck import intermediate. Different antisera were tested for their ability to immunoprecipitate the 75 kDa crosslinked product. C, control immunoprecipitation for 3 h at 4°C with 15 μl of preimmune serum. A, immunoprecipitation with 15 μl of antiserum A (Materials and methods; Scherer *et al.*, 1992). This antiserum was originally used for monitoring the purification of ISP45 by the quenching assay. 1 and 2, immunoprecipitation with 10 and 50 μg IgGs of an antiserum that had been raised against purified 40 kDa protein and then affinity purified against the Mpilp−β-galactosidase fusion protein. The marker identifies the position of the 75 kDa crosslinked product in the SDS−polyacrylamide gel.

mitochondria (Figure 3A). IgGs against cytochrome c_1, an abundant inner membrane protein, did not inhibit import (Figure 3A, lane CTRL). These results suggested that the 40 kDa protein, or a related antigen, was present at the protein import site of the inner membrane.

The 40 kDa protein is a proteolytic fragment of ISP45

The 40 kDa protein was purified by anion exchange chromatography and SDS−PAGE, digested with trypsin and

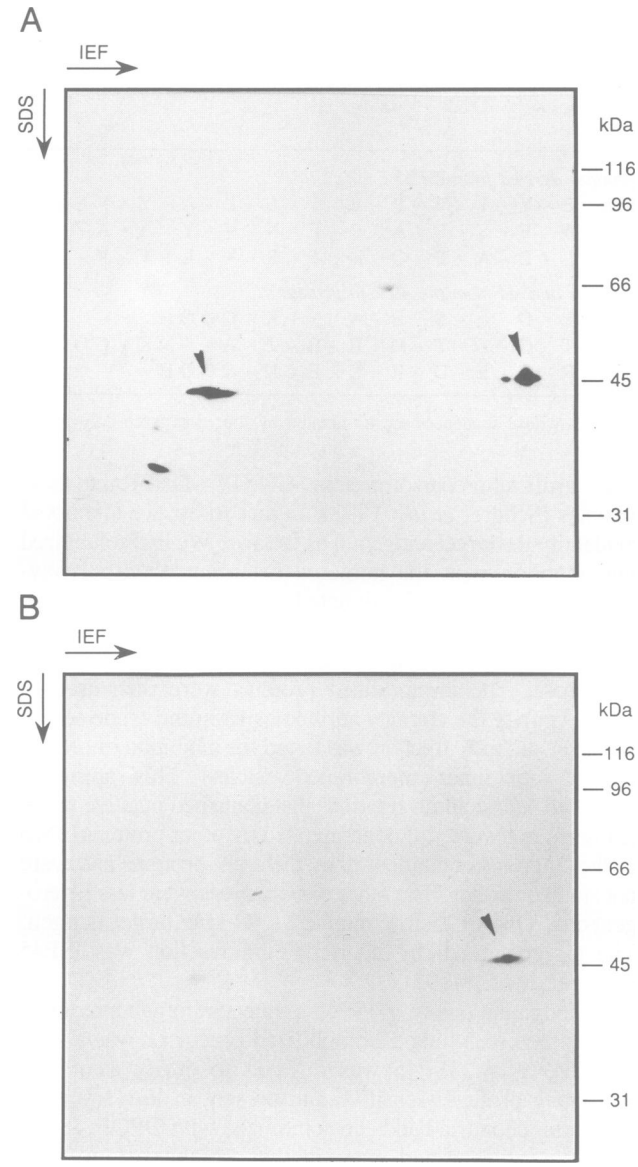

Fig. 5. If proteolysis is minimized, antibodies directed against the *MPI1*-encoded protein detect only a 45 kDa mitochondrial protein. (**A**) Mitochondria (100 μg protein) were solubilized in SDS at 95°C, subjected to two-dimensional gel electrophoresis (isoelectric focusing from pH 7−10, followed by SDS−10% PAGE) and analysed by immunoblotting with antiserum against the Mpi1−β-galactosidase fusion protein. Immune complexes were visualized with the ECL-system according to the manufacturer's instructions; molecular size markers are given on the right. The right arrow points to ISP45, the left arrow points to the 40 kDa fragment of ISP45. (**B**) Same as in panel A, except that the mitochondria were precipitated with 10% trichloroacetic acid before being dissociated in hot SDS. The arrow points to ISP45.

the amino acid sequence of three tryptic peptides was determined. To our surprise, these sequences also corresponded exactly to parts of the *MPI1* open reading frame (Table I, lower part).

The similarity between ISP45 and the 40 kDa protein was confirmed by the observation that antibodies raised against the 40 kDa protein and then affinity-purified against an Mpi1p−β-galactosidase fusion protein immunoprecipitated the cross-linked complex containing ISP45 and the stuck import intermediate (Figure 4). ISP45 and the 40 kDa protein are thus different forms of the same protein.

In order to decide whether the 40 kDa protein represented

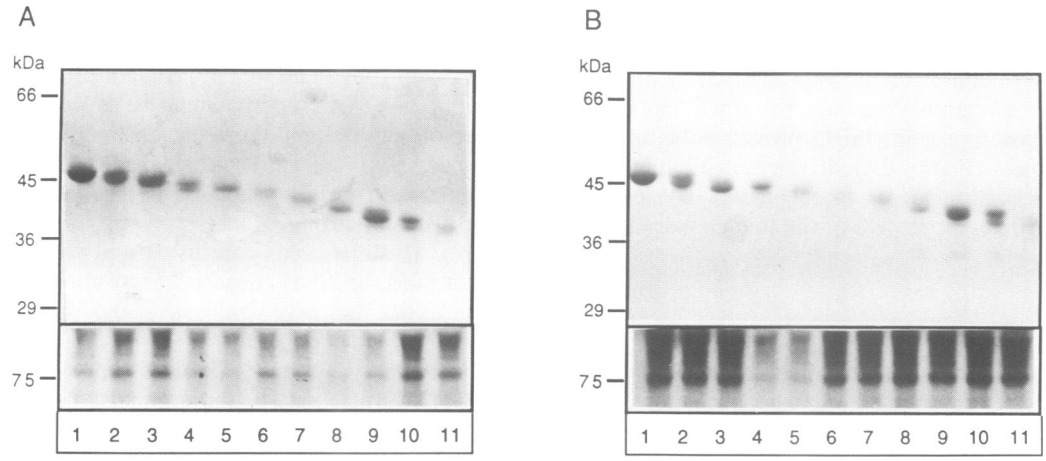

Fig. 6. If proteolysis is minimized, only a 45 kDa mitochondrial protein quenches immunoprecipitation of the crosslinked stuck precursor. (**A**) Mitochondria (7.5 mg protein) were subjected to SDS−10% PAGE on five preparative gels. The gels were briefly stained with Coomassie brilliant blue and each visible band in the 35−50 kDa region was excised (see Figure 1A, upper left panel). Proteins were electroeluted from each slice; 10% of each eluate was analysed by SDS−10% PAGE and stained for protein with Coomassie brilliant blue (upper part of each panel); another 10% was used for quenching the immunoprecipitation of the 75 kDa cross-linked product consisting of ISP45 cross-linked to an immobilized radioactive precursor protein (lower part of each panel). Molecular size markers are given on the right. (**B**) Same as in panel A, except that mitochondria were precipitated with 10% trichloroacetic acid before SDS−PAGE.

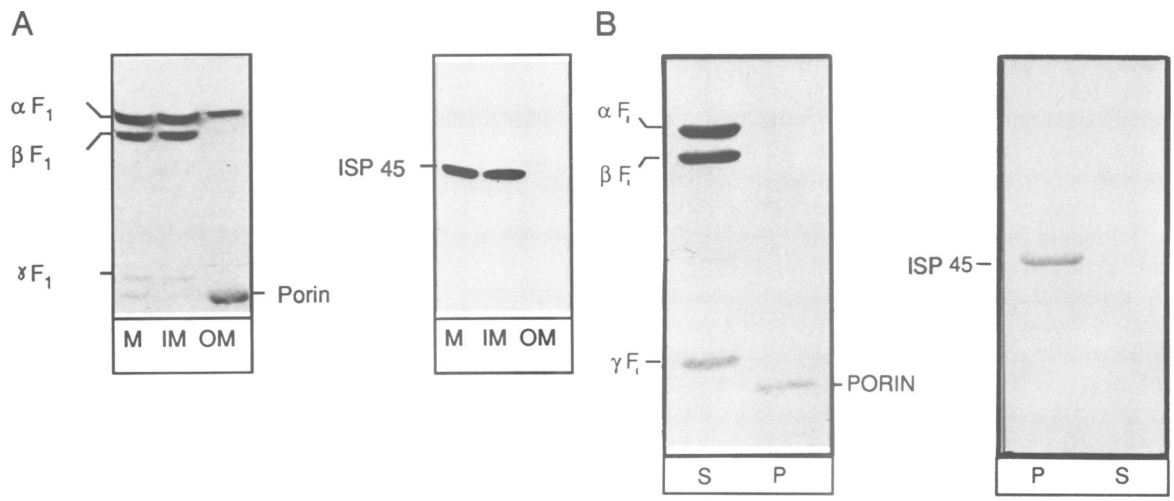

Fig. 7. ISP45 is an integral protein of the mitochondrial inner membrane. (**A**) ISP45 is associated with the inner membrane. Total mitochondria (M) and isolated inner membranes (IM) and outer membranes (OM) (100 μg protein each) were precipitated with 10% trichloroacetic acid and analysed by immunoblotting with antibodies against porin (outer membrane marker), the α, β and γ-subunits of the F_1-ATPase (inner membrane marker) and the Mpilp−β-galactosidase fusion protein (ISP45). Immune complexes were visualized with the ECL System (Amersham, UK). In this experiment, ~25% of the F_1-ATPase α-subunits were found associated with the outer membrane, probably because they had been released from the inner membrane and then bound nonspecifically to the outer membrane. (**B**) ISP45 is an integral membrane protein. Mitochondria (100 μg protein) were extracted with 0.1 M Na_2CO_3 pH 11.5. Soluble (S) and insoluble proteins (P) were separated by centrifugation for 30 min at 100 000 *g* in a Beckman Airfuge, precipitated with 10% trichloroacetic acid and analyzed by SDS−10% PAGE and immunoblotting with the antisera described in panel A.

the physiological mature form of ISP45, or whether it was only a proteolytic fragment generated upon solubilization of mitochondria, we monitored the two protein species during different purification protocols. When mitochondria were first denatured with trichloroacetic acid and then subjected to two-dimensional gel electrophoresis and immunoblotting, antiserum against the Mpi1p−β-galactosidase fusion protein detected essentially a single, basic 45 kDa spot (Figure 5B). In contrast, when denaturation with trichloroacetic acid was omitted, the antiserum detected two main spots on the two-dimensional gel: the basic 45 kDa protein and a more acidic 40 kDa protein (Figure 5A).

Similarly, when mitochondria were first denatured by trichloroacetic acid and then subjected to SDS−PAGE, immunoprecipitation of the cross-linked immobilized precursor was quenched only by proteins eluted from the 45 kDa region of the gel (Figure 6B). Without prior denaturation by trichloroacetic acid, quenching activity was found both in the 45 kDa as well as in the 40 kDa region (Figure 6A).

Both tests clearly showed that the 40 kDa protein cannot be detected under conditions that minimize proteolytic degradation. We conclude that ISP45 is the mature form of the protein encoded by the *MPI1* gene and that the 40 kDa protein is a proteolytic fragment generated from ISP45 upon solubilization of the mitochondria.

ISP45 is an integral protein of the mitochondrial inner membrane

When submitochondrial fractions were analysed by immunoblotting with an antiserum against the Mpi1p–β-galactosidase fusion protein, ISP45 proved to be an inner membrane protein (Figure 7A). It was not solubilized by exposing mitochondria to pH 11.5 (Figure 7B) and thus seems to be an integral protein of the mitochondrial inner membrane.

Discussion

We previously identified two putative components of the protein import system associated with the mitochondrial inner membrane. The apparent molecular weights of these proteins were 40 and 45 kDa. Both were the target antigens of complex antisera which inhibited protein import into mitochondrial inner membrane vesicles. In addition, antibodies against the 45 kDa protein (termed ISP45) immunoprecipitated a precursor that had become stuck across the inner membrane and then cross-linked to adjacent mitochondrial proteins (Scherer *et al.*, 1992). Based on these findings we proposed that ISP45 was a component of the protein import system in the mitochondrial inner membrane. A role for the 40 kDa protein in this system remained somewhat speculative, however, as it was suggested only by antibody inhibition experiments.

Our initial attempts to purify ISP45 were unsuccessful because the protein was unusually sensitive to proteolysis and present in very low amounts. Even when we started from highly purified yeast mitochondria and used a variety of protease inhibitors, solubilization of the mitochondria by detergent rapidly triggered proteolysis of ISP45. These problems explain the unusual purification protocol adopted in the present work: the mitochondria were first denatured with trichloroacetic acid to inactivate proteases and then were subjected to preparative SDS–PAGE on as many as 160 separate gels. This 'brute force' approach was based on our earlier work (Scherer *et al.*, 1992); it was tedious, but successful. Further purification involved hydrophilic interaction chromatography and two-dimensional gel electrophoresis. ISP45 recovered from two-dimensional gels quenched immunoprecipitation of ISP45 cross-linked to a stuck precursor and yielded amounts of tryptic peptides sufficient for microsequencing. These sequences yielded two surprises. First, they exactly matched parts of the recently discovered *MPI1* gene (Maarse *et al.*, 1992). Second, they were identical to the sequences of tryptic fragments derived from the 40 kDa protein.

Initially we suspected that ISP45 might be a physiological precursor form of the 40 kDa protein. However, the evidence reported here leaves little doubt that the 40 kDa protein is a proteolytic fragment of ISP45 which is formed only upon solubilization of the mitochondria. This conclusion is also supported by an inspection of the *MPI1* coding sequence. As the predicted size of the *MPI1*-encoded protein precursor is 48.8 kDa, a mature size of 40 kDa would imply an unusually long transient presequence for an inner membrane protein. In contrast, a mature size of 45 kDa is compatible with a typical mitochondrial presequence of 3–4 kDa.

By tagging Mpi1p with a C-terminal c-*myc*-epitope and localizing the resulting fusion protein with a c-*myc*-specific antibody, Maarse *et al.* (1992) had concluded that Mpi1p was associated with mitochondrial membranes. We now confirm and extend this conclusion by showing that ISP45/Mpi1p is an integral protein of the mitochondrial inner membrane. This property would be expected for a component of the protein transport channel across the inner membrane. In our earlier experiments, ISP45 appeared to be released from mitochondria at alkaline pH, suggesting that it was a peripheral membrane protein (Scherer *et al.*, 1992). This erroneous conclusion was probably caused by the fact that the ISP45 quenching assay also responds to small peptides that may have been generated during alkaline extraction of the mitochondria.

In summary, three independent approaches suggest that ISP45/Mpi1p is part of the protein import system in the mitochondrial inner membrane. First, ISP45 can be cross-linked to a precursor protein whose translocation across the inner membrane has been arrested by depletion of matrix ATP. Second, antibodies against ISP45 inhibit protein import into mitochondrial inner membrane vesicles. Third, mutation or genetic depletion of the protein impairs import of precursors from the cytoplasm into the mitochondrial matrix. While each of these approaches has its individual weaknesses, the combined evidence strongly suggest that ISP45/Mpi1p is indeed the first identified component of the protein import system in the inner membrane.

Materials and methods

Preparation of antisera

Yeast mitochondria were purified on a Nycodenz gradient (Lewin *et al.*, 1990). Right-side-out inner membrane vesicles were prepared (Hwang *et al.*, 1989), fixed for 30 min with 2% glutaraldehyde in PBS (0.15 M NaCl, 10 mM sodium phosphate pH 7.4), washed twice with PBS by centrifugation and injected into rabbits. The resulting antiserum (MH 596) was chromatographed on protein A–Sepharose in order to purify the IgGs for the import inhibition studies.

The antiserum used for immunoprecipitation of the 75 kDa cross-linked product has been raised against SDS-denatured proteins of mitochondrial inner membrane vesicles that equilibrated at an intermediate density in a sucrose gradient (Pon *et al.*, 1989). This serum is referred to as antiserum A (Scherer *et al.*, 1992).

An antiserum was also raised against the 40 kDa protein that had been purified by anion exchange chromatography and SDS–PAGE. Where indicated, the antiserum was affinity-purified against Mpi1p–β-galactosidase fusion protein. The Mpi1p–β-galactosidase fusion protein was also directly injected into rabbits, generating another antiserum specific for ISP45 and the 40 kDa protein. IgGs were purified from the sera by chromatography on protein A–Sepharose.

Purification of ISP45 and generation of tryptic fragments

Nycodenz-purified yeast mitochondria (640 mg) were suspended in 0.6 M sorbitol –20 mM HEPES–KOH, pH 7.4 to 4 mg/ml and precipitated with 10% trichloroacetic acid. The proteins were sedimented at 13 000 *g*, resuspended at 95°C in 3-fold concentrated sample buffer containing 100 mM dithiothreitol and heated for a further 5 min at 95°C. Aliquots of the solubilized proteins were separated on 160 preparative SDS–10% polyacrylamide gels (1.5 × 130 × 120 mm). The gels were stained for 10 min with 0.1% Coomassie brilliant blue in 40% methanol–1% acetic acid, destained for 10 min with 40% methanol–1% acetic acid and washed five times for 5 min with water. The 45 kDa region was excised. The proteins were electroeluted in standard electrode buffer for 3 h at 70 V at room temperature, precipitated for 12–16 h at –20°C with 80% ethanol, washed twice with 70% ethanol, dried in a Speed-Vac desiccator, resuspended in 300 μl of 4-fold concentrated stacking gel buffer and chromatographed at room temperature on a poly-hydroxyethylaspartamide column (Alpert, 1990) that had been equilibrated in buffer A [Poly LC column, 150 × 4.6 mm, pore size 20 nm (Poly LC Corp., Elliott, MD), connected to a Merck–Hitachi HPLC system consisting of a L-4200 UV-VIS Detector, a L-6200 pump and an D-2000 integrator]. Bound proteins were eluted at a flow rate of 500 μl/min with the following gradient program: 5 min with 100% buffer A, 10 min linear gradient with 100% buffer A to 100% buffer

B. Buffer A was 80% *n*-propanol containing 50 mM formic acid; buffer B was 50 mM formic acid. The column effluent was monitored at 280 nm. Peaks were collected manually. All proteins eluted as a single peak at ~14 min. The organic solvents were evaporated in a Speed-Vac desiccator. The proteins were solubilized in 2D-sample buffer containing 6 M urea and 2% of the non-ionic detergent NP40, and electrofocused in tube gels for 12–14 h (5 min at 100 V, 10 min at 200 V, 30 min at 300 V, 10–12 h at 400 V and 1 h at 800 V). The tube gels were then applied on top of preparative SDS–10% polyacrylamide gel slabs and the assembly was subjected to SDS–PAGE. The gel slabs were washed with water six times for 10 min, stained for 10 min with Comassie brilliant blue, destained for 10 min and washed with water five times for 5 min as described above. The gel pieces containing ISP45 were excised, extracted three times with 150 μl 40% *n*-propanol for 5 min each, washed twice with 150 μl 0.2 M NH_4HCO_3–50% acetonitrile for 10 min each and semi-dried at room temperature for 30 min. They were then incubated with 50 μl 0.1 M NH_4CO_3 containing 0.5 μg trypsin for 10 min at room temperature. An additional 50 μl aliquot of 0.1 M NH_4CO_3 was then added and digestion was continued for 16 h at 37°C. The supernatant was removed and the gel pieces were extracted twice with 100 μl 0.1 M NH_4CO_3 for 30 min each, twice with 100 μl 50% acetonitrile, 0.1% trifluoroacetic acid (TFA) for 30 min at room temperature. The supernatant of the digestion and all extracts were pooled, concentrated in a Speed-Vac desiccator to a volume of 20 μl and acidified with 5 μl of 5% TFA. The tryptic peptides were chromatographed on a C18 Vydac 218-TP51 reversed-phase HPLC column (250 × 1 mm; VYDAC Corp., Hesperia CA) at a flow rate of 50 μl/min. Buffer A was 0.05% TFA: buffer B was 0.05% TFA in 80% acetonitrile. Peptides were eluted with the following gradient program: 5 min 2% buffer B; 90 min gradient 2% buffer B to 75% buffer B. The column effluent was monitored at 214 nm. Selected peaks were sequenced on an Applied Biosystems 473A or 477A protein sequencer.

Preparation of an Mpi1p–β-galactosidase fusion protein

The *MPI1* gene was cloned by polymerase chain reaction (PCR) using genomic yeast DNA according to standard procedures (94°C for 3 min; 40 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1 min) with the following oligonucleotides: sense 5′-GAATTCATGCACAGATCCAC-TTTTATCAGG-3′ (corresponding to nucleotides 825–848 in the *MPI1* gene) and antisense 5′-GGTGAATTGTCTAGAACCCCC-3′ (corresponding to nucleotides 2097–2117 in the *MPI1* gene). The sense oligonucleotide contained an *EcoRI* restriction site for in-frame cloning into a pUEX2 vector (Bressan and Stanley, 1987). The PCR product was purified by electrophoresis in an agarose gel and cloned into a pCR vector (Invitrogen, San Diego, CA) according to the manufacturers's instructions. The 1.4 kb insert containing the *MPI1* open reading frame was then subcloned into vector pUEX2 using *Eco*RI. Positive clones were checked by immunoblotting *Escherichia coli* extracts with antibodies against either the 40 kDa fragment or against β-galactosidase.

To prepare the Mpi1p–β-galactosidase fusion protein, bacteria transformed with the fusion gene were grown at 30°C to OD_{600} = 0.7 and the fusion protein was induced by shifting the culture to 42°C for 3 h. The inclusion bodies were isolated using standard techniques, dissolved in threefold concentrated SDS–PAGE sample buffer, heated for 5 min to 95°C and subjected to SDS–8% PAGE. The fusion protein was localized by brief staining with Coomassie brilliant blue, excised, electroeluted, precipitated with 80% ethanol, washed twice with 70% ethanol and dried in a Speed-Vac desiccator. It was then dissolved in PBS, mixed with an equal volume of 1:1 Freund's complete adjuvants and injected into rabbits. The fusion protein was also coupled to CNBr-activated Sepharose beads (Pharmacia) according to the manufacturer's instructions and used for affinity purifying antibodies against ISP45 and its 40 kDa proteolytic fragment.

Submitochondrial localization of ISP45

Mitochondrial inner and outer membranes and right-side-out mitochondrial inner membrane vesicles were isolated as described (Hwang *et al.*, 1989; Pon *et al.*, 1989). For extraction of proteins at alkaline pH (Fujiki *et al.*, 1982), mitochondria were incubated at 1 mg/ml in 100 mM Na_2CO_3 pH 11.5, 1 mM phenylmethyl sulfonyl fluoride (PMSF) for 20 min on ice; mitochondrial membranes were then isolated by centrifugation for 30 min at 4°C at 100 000 *g* and resuspended in an equal volume of 100 mM Na_2CO_3 pH 11.5. Both the 100 000 *g* supernatant and the pellet were precipitated with 10% trichloroacetic acid and analysed by SDS–12% PAGE followed by immunoblotting.

Miscellaneous

Published methods were used for the isolation of mitochondria (Daum *et al.*, 1982), purification of ^{35}S-labeled DV12 (a variant of the COXIV–DHFR fusion protein; Vestweber and Schatz, 1988), import into mitochondria (Manning-Krieg *et al.*, 1991); import into mitochondrial inner membrane vesicles (Hwang *et al.*, 1989), SDS–PAGE and fluorography (Hurt *et al.*, 1984), immunoblotting (Haid and Suissa, 1983), two-dimensional gel electrophoresis (O'Farrell, 1975; Cabral and Schatz, 1978), generation and use of affinity resins (Ey *et al.*, 1978) microsequencing (Hewick *et al.*, 1981) and production of antisera in rabbits (Daum *et al.*, 1982). Generation of cross-links, immunodepletion of antisera and immunoprecipitation and affinity purification of anti-ISP45 antibodies were performed as described (Scherer *et al.*, 1992). Detection of immune complexes in immunoblotting experiments by alkaline phosphatase or light emission was performed as described in the company brochure distributed by Bio-Rad or by Amersham Corp. Protein was measured by the BCA assay (Pierce Chemical Co.). Efrapeptin was a gift from Eli Lilly Co.

Acknowledgements

We thank Hildegard Brütsch for technical assistance, Margit Jäggi, Verena Grieder and Lieselotte Müller for the artwork, and Verena Zellweger for secretarial assistance. We are also indebted to the members of our laboratory for support, many good suggestions and critical reading of the manuscript. This work was supported by grants from Swiss National Science Foundation (3-26189.89), the US Public Health Service (GM 37803) and the Human Frontier Science Program Organization. M.H. was funded by a Long-term Fellowship from the European Molecular Biology Organization, and N.G.K. by a postdoctoral fellowship of the Human Frontier Science Program Organization.

References

Alpert,A.J. (1990) *J. Chromatogr.*, **499**, 177–196.
Baker,K.P. and Schatz,G. (1991) *Nature*, **349**, 205–208.
Bressan,G.M. and Stanley,K.K. (1987) *Nucleic Acids Res.*, **15**, 10056.
Cabral,F. and Schatz,G. (1978) *J. Biol. Chem.*, **253**, 4396–4401.
Daum,G., Böhni,P.C. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13028–13033.
Ey,P.L., Prowse,S.J. and Jenkins,C.R. (1978) *Biochemistry*, **15**, 429–436.
Fujiki,Y., Hubbard,A.L., Fowler,S. and Lazarow,P.B. (1982) *J. Cell. Biol.*, **93**, 97–102.
Glick,B., Wachter,C. and Schatz,G. (1991) *Trends Cell Biol.*, **1**, 99–103.
Haid,A. and Suissa,M. (1983) *Methods Enzymol.*, **96**, 192–205.
Hewick,R.M., Hunkapillar,M.W., Hood,L.E. and Dreyer,W.J. (1981) *J. Biol. Chem.*, **256**, 7990–7997.
Horst,M., Kronidou,N.G. and Schatz,G. (1993) *Curr. Biol.*, **3**, 175–177.
Hurt,E.C., Pesold-Hurt,B. and Schatz,G. (1984) *EMBO J.*, **3**, 3149–3156.
Hwang,S., Jascur,T., Vestweber,D., Pon,L. and Schatz,G. (1989) *J. Cell. Biol.*, **109**, 487–493.
Hwang,S., Wachter,C. and Schatz,G. (1991) *J. Biol. Chem.*, **266**, 21083–21089.
Jascur,T., Goldenberg,D.P., Vestweber,D. and Schatz,G. (1992) *J. Biol. Chem.*, **267**, 13636–13641.
Lewin,A., Hines,V. and Small,G.M. (1990) *Mol. Cell. Biol.*, **10**, 1399–1405.
Maarse,A.C., Blom,J., Grivell,L.A. and Meijer,M. (1992) *EMBO J.*, **11**, 3619–3628.
Manning-Krieg,U.C., Scherer,P.E. and Schatz,G. (1991) *EMBO J.*, **10**, 3273–3280.
O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.
Ohba,M. and Schatz,G. (1987) *EMBO J.*, **7**, 2117–2122.
Pfanner,N., Rassow,J., van der Klei,I.J. and Neupert,W. (1992) *Cell*, **68**, 999–1002.
Pon,L., Moll,T., Vestweber,D., Marshallsay,B. and Schatz,G. (1989) *J. Cell. Biol.*, **109**, 2603–2616.
Schatz,G. (1993) *Prot. Sci.*, **2**, 141–146.
Scherer,P.E., Manning-Krieg,U.C., Jenö,P., Schatz,G. and Horst,M. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11930–11934.
Segui-Real,B., Kispal,G., Lill,R. and Neupert,W. (1993) *EMBO J.*, **12**, 2211–2218.
Vestweber,D. and Schatz,G. (1988) *J. Cell. Biol.*, **107**, 2037–2043.

Received on March 29, 1993