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Identification of a 45-kDa protein at the protein import site of the yeast mitochondrial inner membrane

(crosslinking/translocation intermediate/submitochondrial vesicles)

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ABSTRACT **Import of proteins into mitochondria involves the cooperation of protein translocation systems in the outer and inner membranes. We have identified a 45-kDa protein at the protein import site of the yeast mitochondrial inner membrane. This 45-kDa protein could be crosslinked to a partly translocated precursor, which cannot be imported across the inner membrane when the matrix is depleted of ATP. In addition, an antibody against this protein strongly inhibited protein import into right-side-out inner-membrane vesicles. The 45-kDa protein accounts for only 0.1% of mitochondrial protein and appears peripherally attached to the outer face of the inner membrane. The properties of this protein suggest that it is a component of the protein import system of the mitochondrial inner membrane.**

Import of proteins from the cytoplasm into the mitochondrial interior requires the concerted action of two distinct protein translocation systems, one in the outer membrane and the other in the inner membrane (1–3). The system in the outer membrane consists of several receptor proteins together with a set of transmembrane proteins that presumably form a protein transport channel across the outer membrane (1, 4, 5). One subunit of this putative outer-membrane channel was identified by crosslinking it to a precursor protein that was stuck across the protein import channels of both membranes (6). This import-site protein 42 (ISP42) proved an integral outer-membrane protein essential for viability (7). The *Neurospora crassa* homologue (termed MOM38) was shown to be part of a complex that also contained one of the import receptors (8).

In contrast, no component of the inner-membrane translocation system has yet been identified. To characterize this machinery, we chose two complementary approaches. (*i*) We used a bifunctional crosslinker to identify proteins in the immediate vicinity of a precursor protein that had already been translocated across the outer membrane but had become stuck in the protein import channel of the inner membrane. (*ii*) We raised antisera against total inner-membrane proteins, selected those sera that inhibited protein import into isolated inner-membrane vesicles, and tracked down the protein antigen responsible for the inhibition.

Both of these independent approaches identified a 45-kDa inner-membrane protein that we term ISP45. We suggest that ISP45 is a component of the protein-translocation system associated with the mitochondrial inner membrane.

MATERIALS AND METHODS

Mitochondrial Precursors. The translocation intermediate used for crosslinking was generated with the fusion protein subunit 9 (Su9)–dihydrofolate reductase (DHFR) (9). This protein, which consists of the 69-residue presequence of ATPase Su9 from *N. crassa* fused at the N terminus to mouse DHFR, is imported into the matrix by energized mitochondria. The plasmid-borne gene was from W. Neupert (University of Munich, Germany). This gene was transcribed with SP6 polymerase, and the resulting mRNA was translated in the presence of [^{35}S]methionine, essentially as described (10). The inhibitory effect of antiserum on protein import into inner-membrane vesicles was assayed with purified, radiolabeled DV12 (variant of fusion-protein COXIV–DHFR, ref. 11).

Generation of Crosslinks. After translation, the Su9–DHFR-containing reticulocyte lysate was centrifuged for 20 min in an Airfuge (Beckman) at 30 psi (1 psi = 6.9 kPa) and depleted of ATP by incubation for 10 min at 30°C with apyrase (Sigma) at 50 units/ml. Mitochondria at 1 mg of protein per ml in import buffer (0.6 M sorbitol/50 mM Hepes·KOH, pH 7.4/25 mM KCl/10 mM $MgCl_2$/2 mM KP_i, pH 7.4/0.5 mM EDTA/fatty acid-depleted bovine serum albumin at 1 mg/ml) were depleted of ATP for 5 min at 30°C in the presence of apyrase at 10 units/ml, oligomycin at 12.5 μg/ml, and efrapeptin at 1 μg/ml. The mitochondria were then reenergized by incubation with 2 mM NADH for 3 min at 30°C. After 0.1 vol of ATP-depleted reticulocyte lysate was added, the mixture was incubated for 10 min at 30°C and then put on ice. The membrane potential was collapsed by addition of carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone to 25 μM. Surface-bound precursor was degraded with trypsin (100 μg/ml, 20 min at 0°C), followed by addition of soybean trypsin inhibitor to 1 mg/ml and phenylmethylsulfonyl fluoride to 1 mM. Mitochondria were reisolated by centrifugation (5 min at 15,000 × *g*) and resuspended in an equal volume of 0.6 M sorbitol/20 mM Hepes·KOH, pH 7.4. The crosslinking reaction was done by adding the cleavable, homobifunctional crosslinker dithiobis(succinimidyl propionate) to 400 μM from a 20 mM stock solution in dimethyl sulfoxide. After a 30-min incubation on ice, excess crosslinker was quenched by addition of lysine (pH 8.0) to 10 mM and addition of trypsin inhibitor to 1 mg/ml. After another 10-min incubation on ice, the reaction mixture was split into aliquots containing 200 μg of mitochondrial protein each; the mitochondria were reisolated by centrifugation, frozen in liquid nitrogen, and stored at −80°C until being analyzed.

Immune Depletion of Antiserum and Immunoprecipitation. The antiserum used for immunoprecipitating the 75-kDa crosslinked product (antiserum A) was raised by injecting rabbits with a preparation of unfixed submitochondrial particles from yeast; this antiserum was from Thomas Jascur

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Abbreviations: DHFR, dihydrofolate reductase; Su9, subunit 9; ISP42, import-site protein 42; FPLC, fast protein liquid chromatography.

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(our laboratory). To assay the ability of mitochondrial proteins to quench immunoprecipitation of the 75-kDa crosslinked product by antiserum A, 15 μl of antiserum A was incubated for 2 hr at room temperature together with the mitochondrial protein sample to be assayed in 250 μl of TNET (1% Triton X-100/150 mM NaCl/5 mM EDTA/20 mM Tris·HCl, pH 7.5). If the sample contained SDS, the final SDS concentration in the quenching reaction was kept <0.1%. In each case, a control reaction was included, in which the assay protein was replaced by an equal amount of buffer. The pretreated antiserum A was then added to 200 μg of mitochondria containing the crosslinked, radiolabeled translocation intermediate, which had been solubilized in 1 ml of TNET/1 mM phenylmethylsulfonyl fluoride/trypsin inhibitor at 1 mg/ml, incubated on ice for 10 min, and cleared from insoluble matter by centrifugation. After 50 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia) in TNET was added, the samples were incubated for 3 hr at 4°C, washed four times with 1-ml vol of TNET, heated to 95°C for 5 min in 100-μl sample buffer (2% SDS/150 mM Tris·HCl, pH 6.8/10% sucrose/2 mM EDTA), and analyzed by SDS/12% PAGE.

Purification of ISP45. Yeast mitochondria were purified by equilibrium centrifugation in a Nycodenz gradient (12). Purified mitochondria (40 mg) were suspended in 4 ml of 0.6 M sorbitol/20 mM Hepes·KOH, pH 7.4, precipitated by adding 4 ml of 80% (vol/vol) acetone/20% trichloroacetic acid, resuspended in 3-fold-concentrated sample buffer containing 100 mM dithiothreitol, heated for 5 min at 95°C, and electrophoresed in 12 preparative SDS/10% polyacrylamide gels (1.5 × 130 × 120 mm). Gels were stained for 5 min with Coomassie brilliant blue [0.125% in 50% (vol/vol) methanol/10% (vol/vol) acetic acid], destained for 5 min with 20% (vol/vol) methanol/7.5% (vol/vol) acetic acid, neutralized for 5 min with 1 M Tris base, and washed with water three times for 10 min. The 45-kDa region was excised, and the proteins were electroeluted in 0.1% SDS/100 mM NH_4HCO_3. The eluted proteins were collected in a small volume, precipitated for 14 hr at −20°C by adding 9 vol of ethanol, washed twice with ethanol, dried, resuspended in 200 μl of 8 M urea/0.1% trifluoroacetic acid, warmed briefly to 65°C, diluted to 6 M urea with 0.1% trifluoroacetic acid, and chromatographed at 40°C on a reverse-phase column [Vydac 214TP52 (2.1 mm × 250 mm) connected to a Hewlett–Packard model 1090 liquid chromatograph]. Buffer A was 0.1% aqueous trifluoroacetic acid; buffer B was 80% acetonitrile containing 0.09% trifluoroacetic acid. Adsorbed proteins were eluted at 150 μl per min with the following gradient program: 0 min, 25% buffer B; 15 min, 25% buffer B; 180 min, 80% buffer B. The column effluent was monitored at 214 nm. Peaks were collected manually. ISP45 eluted as a single peak (peak 4, see Fig. 3) after 120 min and was >90% pure as judged by one- and two-dimensional gel electrophoresis. Mitochondrial protein was measured by the BCA procedure (Pierce), and the amount of purified ISP45 was estimated by comparing intensities of silver-stained ISP45 bands with those of several reference proteins after SDS/PAGE.

Submitochondrial Localization of ISP45. Inner and outer mitochondrial membranes, soluble matrix and intermembrane space contents, and mitoplasts were isolated as described (12). For extraction at high pH (13), mitochondria were incubated at 1 mg/ml in 100 mM Na_2CO_3, pH 11.5/1 mM phenylmethylsulfonyl fluoride for 30 min on ice and reisolated by centrifugation for 30 min at 30 psi in an Airfuge. Proteins in the supernatant were precipitated with 5% trichloroacetic acid; the pellet was resuspended in an equal volume of 100 mM Na_2CO_3 and precipitated with 5% trichloroacetic acid; both precipitated protein fractions were solubilized in 20 μl of 1% SDS/100 mM Tris·HCl, pH 8.0, and tested for ability to inhibit immunoprecipitation of the 75-kDa crosslinked product.

Import into Inner Membrane Vesicles. Import of purified radiolabeled DV12 precursor into inner-membrane vesicles from yeast mitochondria was assayed as described (14). For antibody inhibition, 5 μg of inner-membrane vesicles was incubated on ice in 50 μl of import buffer with the antibody preparation to be tested; after 1 hr, 5 μl of variant DV12 (60 ng; ≈5 × 10^5 cpm/μg of protein) in 100 μl of fresh import buffer was added. After 5 min at 30°C, vesicles were reisolated and analyzed by SDS/12% PAGE and fluorography.

Affinity-Fractionation of Anti-Inner-Membrane Antiserum. Mitochondria were purified on a Nycodenz gradient (12) and suspended to 5 mg/ml in 200 ml of 0.6 M sorbitol/20 mM Hepes·KOH, pH 7.4/1 mM phenylmethylsulfonyl fluoride. Octyl poly(oxyethylene) was then added to 0.45%, and the mixture was incubated for 30 min on ice. Membranes were reisolated by centrifugation at 100,000 × *g* for 60 min, dissolved in solubilization buffer (20 mM Hepes·KOH, pH 7.0/2% octyl poly(oxyethylene)/1 mM phenylmethylsulfonyl fluoride) at 5 mg/ml, freed from insoluble material by centrifugation at 30,000 × *g* for 30 min and filtration through a 0.45-μm filter, and chromatographed on an anion-exchange column (25-ml bed vol; Superformance Fractogel EMD-TMAE-650(S), Merck no. 20286) with 120 ml of a linear 0–0.3 M NaCl gradient, followed by 20 ml of 1.0 M NaCl, both in solubilization buffer, at a flow rate of 2 ml/min. The resulting fractions were individually coupled to CNBr-activated Sepharose-4B (following manufacturer's instructions, Pharmacia), and the resulting affinity beads were incubated with 200-μl samples of the complex antiserum MH596. This rabbit antiserum had been raised against glutaraldehyde-fixed yeast mitochondrial inner-membrane vesicles. Bound immunoglobulins were then eluted with 100 mM glycine·HCl, pH 2.5, adjusted to pH 8.0 with 1 M Tris·HCl, dialyzed against the buffer used for assaying import into inner-membrane vesicles (14), and tested for ability to inhibit import into inner-membrane vesicles.

Miscellaneous. Published methods were used for isolating mitochondria (15), SDS/PAGE and fluorography (16), quantitative immunoblotting (17), and production of antisera (15). Efrapeptin was from Eli Lilly, acetonitrile was purchased from Baker, chromatography-grade H_2O was from Merck (Lichrosolv), and trifluoroacetic acid was from Sigma.

RESULTS

A Translocation Intermediate Spanning the Inner Membrane Can Be Crosslinked to a Discrete Set of Proteins. When matrix-targeted precursors are presented to mitochondria that maintain an electrochemical potential across the inner membrane but have a matrix depleted of ATP, the precursors are usually transported across the outer membrane but remain stuck across the inner membrane (2). The arrested precursors are productive translocation intermediates, as they are rapidly chased into the matrix by adding ATP, even when the potential across the inner membrane is abolished. We chose such a translocation intermediate for probing the molecular environment within the protein import system of the inner membrane.

In the experiment shown in Fig. 1, the inner-membrane translocation intermediate was generated with a radiolabeled fusion protein consisting of Su9 of the mitochondrial ATPase from *N. crassa* and mouse DHFR (Su9–DHFR). In fully energized mitochondria, this artificial precursor is transported to the matrix, where its presequence is cleaved off in two steps by the matrix processing protease (9). When the mitochondrial matrix was depleted of ATP, Su9–DHFR was transported across the outer membrane, and its presequence was inserted across the inner membrane. However, as translocation of the precursor chain across the inner membrane was arrested, only the first cleavage site of the presequence

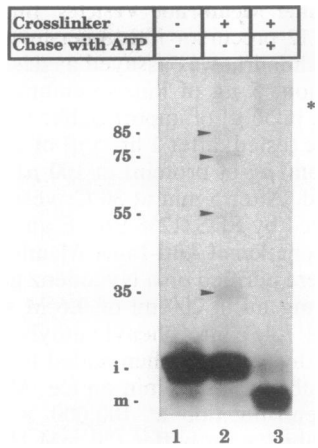


FIG. 1. An inner-membrane translocation intermediate can be crosslinked to a discrete set of proteins. Lanes: 1, once-cleaved translocation intermediate (i) of Su9–DHFR, generated by depleting mitochondrial matrix of ATP; 2, four major crosslinks generated by 400 μM dithiobis(succinimidyl propionate) (DSP)—the apparent molecular masses of these products (in kDa) are indicated at left; 3, same as lane 2, except that DSP was added after the translocation intermediate had been chased into the matrix by ATP addition. After generating the intermediate, all three samples were treated with carbonyl cyanide *p*-(trifluromethoxy)phenylhydrazone and incubated for 5 min at 30°C without (lanes 1 and 2) or with (lane 3) 0.75 mM ATP/3.8 mM creatine phosphate/creatine kinase at 40 μg/ml. m, Mature form generated by chasing intermediate into the matrix with ATP; asterisk at right marks unidentified crosslinked product formed with matrix-localized DHFR.

was accessible for cleavage by the matrix protease (Fig. 1, lane 1, i; see also ref. 10). When mitochondria containing this translocation intermediate were treated with the crosslinker dithiobis(succinimidyl propionate), four major crosslinked products of 35, 55, 75, and 85 kDa were generated (Fig. 1, lane 2, arrowheads). These crosslinks were not formed when the intermediate had first been chased into the matrix by adding ATP (lane 3) or when the precursor was presented to mitochondria lacking a potential across the inner membrane (data not shown).

Identification of Crosslinked Products. To identify the mitochondrial protein(s) crosslinked to the radiolabeled translocation intermediate, we tested whether the crosslinked products could be immunoprecipitated by antiserum against inner-membrane proteins. Fig. 2 shows that one of these antisera (antiserum A) precipitated the 75-kDa band. However, the antiserum recognized several dozen different mitochondrial proteins on an immunoblot, and the titer of the precipitating antibody was very low (data not shown). The target protein was, therefore, identified by a quenching assay. In this assay, total mitochondrial proteins were fractionated by column chromatography or by SDS/PAGE, and each fraction was tested for its ability to quench immunoprecipitation of the radioactive 75-kDa band by antiserum A. This assay detected as little as 20–40 ng of the quenching protein and allowed us to monitor purification of this protein.

To purify the mitochondrial protein contained in the 75-kDa crosslinked product, we first treated isolated mitochondria with trichloroacetic acid to inactivate proteases because the target protein was highly susceptible to proteolysis (data not shown). After preparative SDS/PAGE, final purification was achieved by reverse-phase chromatography (Fig. 3). Fig. 3 shows the elution profile from the HPLC column; Fig. 3 *Upper Inset* shows a silver-stained SDS/polyacrylamide gel of the eluted fractions, and *Lower Inset* shows the ability of HPLC fractions 3–8 to quench precipitation of the 75-kDa crosslinked band by antiserum A. Note that the absence of the 75-kDa band in the assay reflects the presence of the

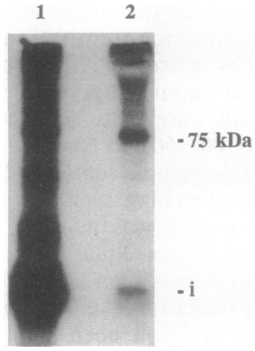


FIG. 2. Antiserum raised against inner-membrane proteins immunoprecipitates the 75-kDa crosslinked product. Lanes: 1, 5% of crosslinked mitochondrial sample used for generating immunoprecipitate of lane 2; 2, corresponding immunoprecipitate (15 μl of antiserum A was added to 0.2 mg of mitochondria). The 75-kDa crosslinked band is indicated at right. As the mitochondrial sample had been solubilized under nondenaturing conditions, some noncrosslinked translocation intermediate (i) was coimmunoprecipitated. This coimmunoprecipitation was not seen upon heating the sample in SDS before dilution with TNET.

target protein in the fraction tested. The target protein ISP45 eluted in fraction 4 (arrow) and migrated as a single 45-kDa band upon SDS/PAGE. This apparent molecular mass roughly agreed with the difference between the molecular masses of the intermediate-size form of Su9–DHFR (25 kDa) and that of the crosslinked product. ISP45 represented $\approx$0.1% of total mitochondrial protein, as judged from the fact that 20–40 μg of total mitochondrial protein had a similar quenching efficiency as 20 ng of purified ISP45 (data not shown).

ISP45 Is Exposed on the Outer Face of the Inner Membrane. Immunoprecipitation of the 75-kDa crosslinked band by antiserum A could be completely inhibited by solubilized inner membranes but was inhibited only a little by identical amounts of solubilized outer membranes and soluble proteins from the matrix and intermembrane space (Fig. 4 *Left*). Purity of submitochondrial fractions is indicated by the distribution of marker proteins given below the fluorogram. ISP45 is thus an inner-membrane protein; it may be a peripheral membrane protein, as it was quantitatively extracted from mitochondria by alkaline pH (Fig. 4 *Right*). However, ISP45 was not extracted from mitoplasts by high salt concentrations (data not shown). Because antiserum A was quenched by mitoplasts but was not quenched by intact mitochondria or by soluble intermembrane-space proteins (data not shown), ISP45 is bound to the outer face of the inner membrane.

Antibodies Against ISP45 Inhibit Protein Import Into Inner-Membrane Vesicles. Right-side-out mitochondrial inner-membrane vesicles import mitochondrial precursor proteins with very similar characteristics and efficiency as intact mitochondria (18). This import was inhibited by several antisera raised against native or glutaraldehyde-fixed inner-membrane vesicles (Fig. 5, *Top*, lane AS). One of these antisera (antiserum MH596) was selected for all further experiments. To identify the membrane antigens recognized by the inhibitory antibodies in serum MH596, we fractionated solubilized mitochondrial-membrane proteins by anion-exchange chromatography, coupled the proteins of each fraction to CNBr-activated Sepharose, used the immobilized mitochondrial proteins to affinity-purify antibodies from antiserum MH596, and tested the purified antibodies for ability to inhibit protein import into inner-membrane vesicles. Protein import into vesicles was assayed by potential-dependent generation of the mature form (m) of the artificial precursor protein DV12. In the experiment of Fig. 5, four inhibitory column peaks were detected: peak 1 at fraction 17, peak 2 at

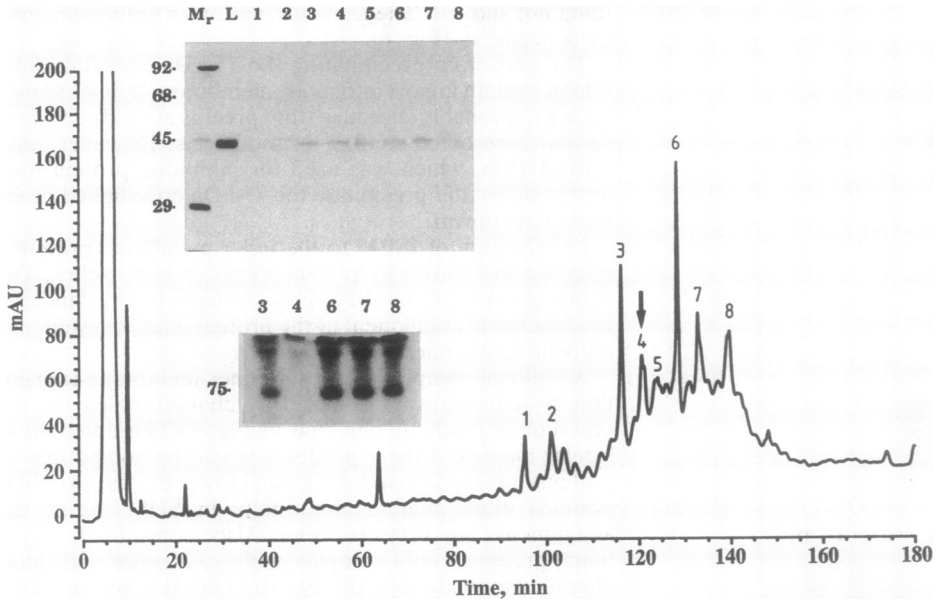
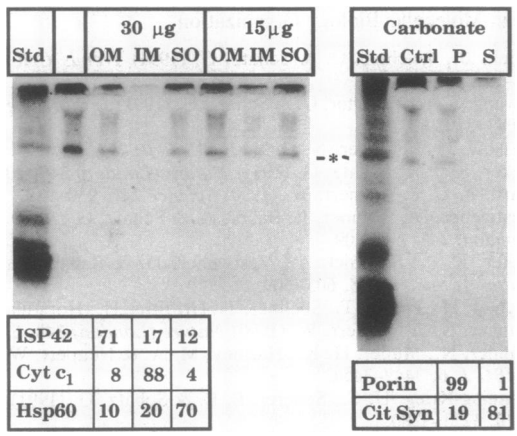


FIG. 3. Purification of ISP45. ISP45 was purified as described. Elution profile from a Vydac 214-TP52 column was monitored by measuring absorbance at 214 nm; the eight major protein-containing peaks are numbered, and the ISP45-containing peak 4 is marked by arrow. The ordinate shows absorbance in milliunits (mAU). (*Upper Inset*) Protein composition of eight peaks as displayed by SDS/10% PAGE and silver staining. M_r, molecular weight standards; L, mixture of proteins applied to HPLC column. (*Lower Inset*) Only column fraction 4 quenched immunoprecipitation of the 75-kDa crosslinked product by antiserum A.

fractions 26 and 27, peak 3 at fraction 31, and peak 4 representing the high-salt wash at fractions 38 and 39. The inhibitory activity of peak 1 was caused by antibodies against a 40-kDa inner-membrane protein (M.H., unpublished observations). The inhibitory activities in peaks 3 and 4 have not yet been characterized because these peaks contained many different proteins. As demonstrated below, the inhibitory activity in peak 2 was from ISP45.



ISP42	71	17	12
Cyt c_1	8	88	4
Hsp60	10	20	70

Porin	99	1
Cit Syn	19	81

FIG. 4. ISP45 is a peripheral inner-membrane protein. (*Left*) ISP45 is located in the inner membrane. Mitochondria were subfractionated, and 30 or 15 μg of protein of each fraction was tested for ability to quench immunoprecipitation of the 75-kDa crosslinked product (*) by antiserum A. Std, 1% of crosslinked mitochondria without immunoprecipitation; −, immunoprecipitation by unquenched antiserum A; OM, outer membrane; IM, inner membrane; SO, soluble protein of matrix and intermembrane space. Thirty micrograms of each fraction was also analyzed for the following marker proteins by quantitative immunoblotting: ISP42 (OM); cytochrome c_1 (Cyt c_1) (IM); heat shock protein 60 (Hsp60) (matrix). Distribution of these markers (expressed as percentage of total signal in all three fractions) is given below fluorogram. (*Right*) ISP45 is extracted from mitochondria by high pH. Sixty micrograms of mitochondrial protein was extracted at pH 11.5, and extract (S) and insoluble residue (P) were tested for ability to quench immunoprecipitation of the 75-kDa crosslinked product by antiserum A. Std, 1% of crosslinked mitochondria; Ctrl, immunoprecipitation without preabsorption of antibodies. Numbers below figure give distribution of typical integral-membrane protein (outer-membrane porin) and of typical soluble protein [matrix-located citrate synthase (Cit Syn)]; see *Left*.

(*i*) When the FPLC column fractions of Fig. 5 were tested for ability to quench immunoprecipitation of the 75-kDa crosslinked product by antiserum A, the highest quenching activity was found in peak 2 (data not shown). Analysis of the column fractions around peak 2 by SDS/PAGE revealed that four protein bands peaked together with quenching activity. These four bands were separated by SDS/PAGE, electroeluted, and assayed individually for ability to quench immunoprecipitation of the 75-kDa crosslink by antiserum A (Fig. 6 *Top* and *Middle*). Only the 45-kDa protein band quenched the antiserum. (*ii*) Each of the four electroeluted protein bands was incubated with an aliquot of the antibodies that had been affinity-purified against the immobilized proteins of peak 2; each aliquot was then tested for its ability to inhibit protein import into inner-membrane vesicles. As shown in Fig. 6 *Bottom*, only the electroeluted 45-kDa band quenched most of the inhibitory activity.

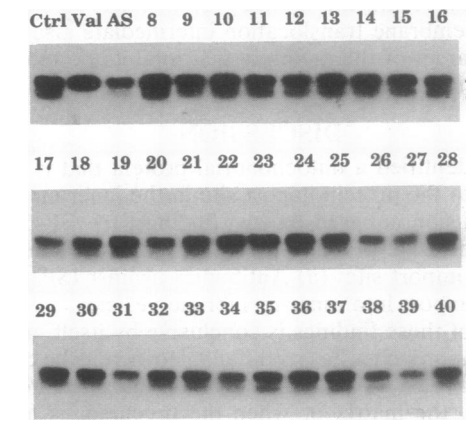


FIG. 5. Inhibition of protein import into inner-membrane vesicles with antibodies affinity-purified from antiserum MH596. Mitochondrial proteins were subfractionated by fast protein liquid chromatography (FPLC). The proteins of each fraction were immobilized on CNBr-Sepharose beads and used to affinity-purify immunoglobulins from antiserum MH596. The eluted affinity-purified immunoglobulins were tested for ability to inhibit precursor import into inner-membrane vesicles. Ctrl, import without antibody; Val, mitochondria were uncoupled by 1 μM valinomycin before adding precursor; AS, total IgGs from antiserum MH596. Numbers at top refer to fractions eluted from FPLC column. p and m, Precursor and mature (imported) form of variant DV12 precursor.

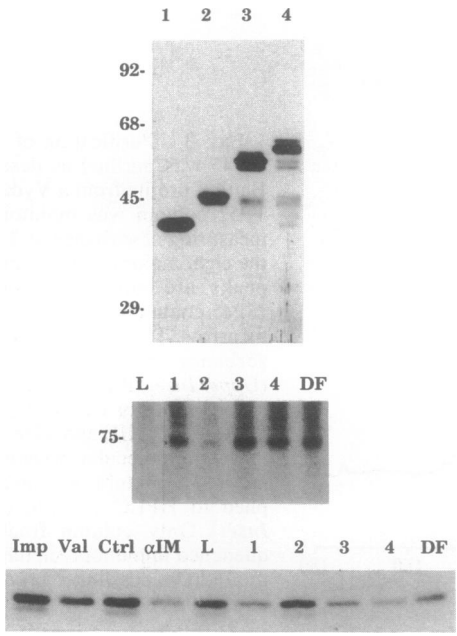


FIG. 6. Antibodies against ISP45 inhibit protein import into inner-membrane vesicles. (*Top*) Proteins peaking in FPLC fractions 26 and 27 (Fig. 5) were precipitated with 5% trichloroacetic acid, separated by SDS/PAGE, stained briefly with Coomassie brilliant blue, and electroeluted. To detect possible degradation products, the dye front was eluted separately. Eluted fractions were analyzed by SDS/12% PAGE and silver-staining. (*Middle*) Eluted proteins shown in *Top*, eluted dye front (DF), as well as an aliquot of pooled FPLC fractions 26 and 27 (L) were tested for ability to quench immunoprecipitation of the 75-kDa crosslinked product by antiserum A. (*Bottom*) Antibodies affinity-purified against proteins from pooled FPLC fractions 26 and 27 were pretreated with electroelution buffer (αIM), with an aliquot of fractions 26 and 27 (L), with one of the electroeluted protein bands 1–4 shown in *Top* or with the electroeluted dye front (DF), and then tested for ability to inhibit protein import into inner-membrane vesicles. Imp, import without antibodies; Val, vesicles had been uncoupled with 1 μM valinomycin before precursor addition; Ctrl, vesicles treated with IgGs against cytochrome c_1.

From these results the 45-kDa protein that is crosslinked to an inner-membrane translocation intermediate also appears to be the target of the antibody that inhibits protein import across the inner membrane.

DISCUSSION

We have identified a mitochondrial protein that is in close proximity to the protein import site in the inner membrane. This conclusion is based on two findings. (*i*) ISP45 can be crosslinked to a precursor that is stuck in the inner-membrane import site. (*ii*) Antibodies against ISP45 inhibit precursor import into inner-membrane vesicles.

Neither of these findings is conclusive by itself, and each of the two approaches has its pitfalls. However, the crosslink is not observed when the translocation intermediate is first chased into the matrix or when the precursor is added to uncoupled mitochondria. The low abundance of ISP45 further suggests that the crosslinking of this protein to the translocation intermediate is specific and indicative of close association. The low abundance of ISP45 also makes it unlikely that the inhibitory effect of anti-ISP45 antibodies on protein import across the inner membrane is caused by a nonspecific coating of this membrane by antibody molecules. Immunoglobulins affinity-purified against several column fractions shown in Fig. 5 reacted strongly against inner-membrane proteins and yet did not inhibit protein import (data not shown). The same is true for an antiserum against cytochrome c_1 (Fig. 6).

The antiserum precipitating the 75-kDa crosslink did not inhibit protein import into inner-membrane vesicles (data not shown), probably because the precipitating IgGs in this serum represented <0.01% of total IgGs. However, antiserum MH596, which was used for inhibiting protein import into vesicles, did precipitate the 75-kDa crosslinked product (data not shown).

Localization of ISP45 to the inner-membrane protein import site was shown by two independent approaches. These data suggest to us that ISP45 is not an innocent bystander, but is a functional component of the protein import system of the mitochondrial inner membrane.

Additional components of the inner-membrane translocation system might be revealed by characterizing the other crosslinked products depicted in Fig. 1. The apparent molecular masses of these products would be compatible with proteins of 10, 25, and 55 kDa. However, as crosslinked proteins often migrate abnormally in SDS/PAGE, these assignments are very tentative. Also, some of these additional proteins could complex with each other and thus be identified more directly through their interaction with ISP45. However, the previous successes of crosslinking methods in identifying components of membrane-associated protein translocation systems (6, 19–23) suggest that analysis of the other crosslinked products found here might be rewarding.

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