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Dynamic interaction of the protein translocation systems in the inner and outer membranes of yeast mitochondria

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Mitochondria contain two distinct protein import systems, one in the outer and the other in the inner membrane. These systems can act independently of one another in submitochondrial fractions or if a protein is transported to the outer membrane or to the intermembrane space. It has been proposed that the two systems associate reversibly when a protein is transported across both membranes, but this hypothesis has remained unproven. In order to address this question, we have checked whether antibodies against a subunit of one system can co-immunoprecipitate subunits of the other system. We find that the two systems associate stably if a matrix-targeted precursor is arrested during import; no association is seen in the absence of a stuck precursor. These experiments provide direct evidence that protein import into the mitochondrial matrix is mediated by the reversible interaction of the two translocation systems.

Key words: co-immunoprecipitation/protein import/*Saccharomyces cerevisiae*/translocation intermediate

Introduction

One of the most intriguing aspects of mitochondrial protein import is the fact that many proteins must be transported across two membrane barriers, the outer and the inner membrane. Some early evidence suggested that both membranes were spanned by a single, stable channel at sites where the two membranes are in close apposition. However, the 'single channel' model was difficult to reconcile with findings suggesting the existence of distinct protein translocation systems in the two mitochondrial membranes. Evidence for a system in the inner membrane came from the following four experiments. First, protein import into isolated yeast mitochondria that had been blocked by protease treatment, by antibodies against outer membrane proteins or by jamming the import sites with a chimeric precursor protein can be restored by disrupting the outer membrane (Ohba and Schatz, 1987a,b; Hwang *et al.*, 1989). These results suggest that the inner membrane contains cryptic import sites that are unmasked by disrupting the outer membrane barrier. Second, purified mitochondrial inner membrane vesicles import mitochondrial precursor proteins with the same efficiency and essentially the same characteristics as intact mitochondria (Hwang *et al.*, 1989). Third, an artificial precursor protein whose C-terminus had been cross-linked to a mutant bovine pancreatic trypsin inhibitor (Vestweber and Schatz, 1988) can first be transported across the outer and then across the inner membrane (Jascur *et al.*, 1992). Sequential transport across the two membranes into the matrix was also demonstrated for a fusion protein which consisted of a matrix-targeting signal fused to the N-terminus of cytochrome c heme lyase (Segui Real *et al.*, 1993). Authentic cytochrome c heme lyase is an intermembrane space enzyme whose import is completely independent of the inner membrane translocation system (Lill *et al.*, 1992).

Evidence for a protein transport system specific for the outer membrane came from the observations that isolated outer membrane vesicles can correctly insert the integral outer membrane protein porin (Gasser and Schatz, 1983; Mayer *et al.*, 1993; Smith *et al.*, 1994) and partly transport proteins destined for internal mitochondrial compartments (Mayer *et al.*, 1993, 1995).

While these experiments showed that each of the two mitochondrial membranes contains its own protein transport system, they did not tell us whether the two systems are stably associated with each other or whether they interact in a dynamic fashion. A dynamic interaction appears more likely (Glick *et al.*, 1991; Pfanner *et al.*, 1992), but has so far not been proven.

In order to investigate the association of the two transport systems in yeast mitochondria, we have tested whether antibodies against a subunit of one system can co-immunoprecipitate subunits of the other system. No such co-immunoprecipitation was seen. However, if the mitochondrial import sites were saturated with a chimeric precursor protein whose transport into the matrix was arrested by a C-terminal translocation-incompetent domain, the two systems were stably associated with each other. These results strongly support the earlier suggestion that the protein transport systems of the two mitochondrial membranes transiently interact with each other when transporting a protein into the matrix space.

Results

A translocation-arrested matrix-targeted precursor can be co-immunoprecipitated with antibodies against components of the protein translocation systems of both mitochondrial membranes

We tested for a dynamic association of the two protein transport systems in *Saccharomyces cerevisiae* mitochondria with the aid of a purified precursor protein that gets stuck across both mitochondrial membranes. This chimeric mitochondrial precursor consisted of the presequence of yeast cytochrome oxidase subunit IV (pCOXIV) fused to a variant of mouse dihydrofolate reductase (DHFR). The DHFR variant had a single C-terminal cysteine which was linked via the bifunctional

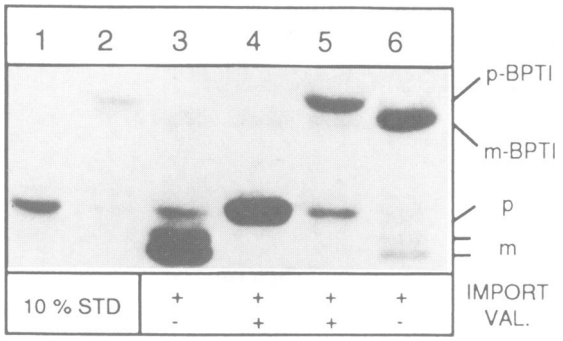

Fig. 1. Import characteristics of pCOXIV−DHFR and the translocation-arrested derivative pCOXIV−DHFR−BPTI. Lanes: 1, pCOXIV−DHFR standard (10% of the amount added to the import assays); 2, pCOXIV−DHFR−BPTI standard (10% of the amount added to the import assays); 3, pCOXIV−DHFR imported into energized mitochondria; 4, pCOXIV−DHFR mock-imported into mitochondria de-energized with 1 μM valinomycin; 5, pCOXIV−DHFR−BPTI mock-imported into mitochondria de-energized with 1 μM valinomycin; 6, pCOXIV−DHFR−BPTI imported into energized mitochondria. val, valinomycin; p and m, uncleaved and cleaved ('mature') forms of pCOXIV−DHFR; p-BPTI and m-BPTI, uncleaved and cleaved forms of pCOXIV−DHFR−BPTI. The underivatized pCOXIV−DHFR is processed at two sites (Vestweber and Schatz, 1988).

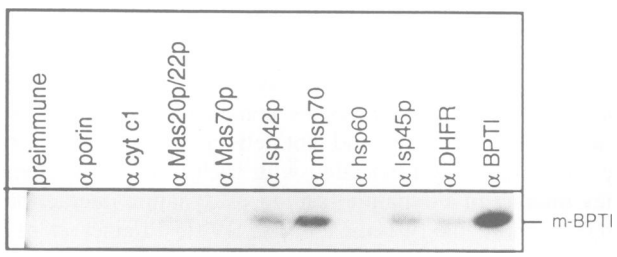

Fig. 2. Co-immunoprecipitation of translocation-arrested, processed COXIV−DHFR−BPTI with antibodies against components of the two mitochondrial protein translocation systems. Radiolabeled pCOXIV−DHFR−BPTI was imported into mitochondria. The mitochondria were then depleted of ATP (in order to preserve association between Isp45p and mhsp70, Kronidou *et al.*, 1994, Rassow *et al.*, 1994; Schneider *et al.*, 1994) and solubilized under non-denaturing conditions. The radiolabeled stuck precursor was immunoprecipitated by the indicated affinity-purified antibodies. The immunoprecipitated material was subjected to SDS−PAGE and the radiolabeled precursor detected by fluorography. Abbreviations as in Figure 1.

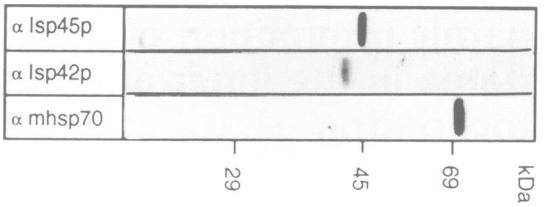

Fig. 3. The antibodies against Isp42p, Isp45p and mhsp70 are monospecific. One hundred micrograms (protein basis) of mitochondria were subjected to SDS−PAGE, blotted onto nitrocellulose filters and immunodecorated with affinity-purified antibodies against Isp42p, Isp45p or mhsp70. Immunodecorated bands were visualized with [^{125}I]protein A followed by autoradiography.

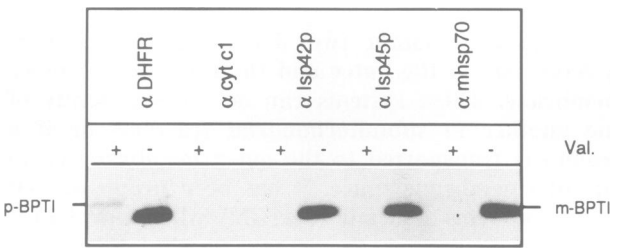

Fig. 4. Co-immunoprecipitation of radiolabeled COXIV−DHFR−BPTI with components of the translocation systems requires that the radiolabeled protein is stuck within the protein import site. Radiolabeled COXIV−DHFR−BPTI was presented to energized (−Val.) or de-energized (+Val.) mitochondria. The mitochondria were then depleted of ATP, solubilized with non-denaturing detergent and subjected to immunoprecipitation with the indicated affinity-purified IgGs. Immunoprecipitated proteins were analyzed by SDS−PAGE and fluorography. Abbreviations as in Figure 1.

crosslinker maleido-benzoyl-*N*-hydroxysuccinimide ester (MBS) to bovine pancreatic trypsin inhibitor (BPTI). BPTI cannot be translocated across mitochondrial membranes, because it is tightly folded and contains three internal disulfide bridges. The chimeric pCOXIV−DHFR−BPTI precursor thus gets stuck across both membranes (Vestweber and Schatz, 1988); the BPTI moiety remains outside the mitochondria and the N-terminal presequence is cleaved by the processing peptidase in the matrix (Figure 1, lane 6). As expected, the stuck precursor could be immunoprecipitated by antibodies against BPTI or against DHFR, regardless of whether the mitochondria had been solubilized with the denaturing detergent SDS (data not shown) or with the non-denaturing, non-ionic detergent Mega-8 (Figure 2).

After solubilizing the mitochondria with the non-ionic detergent, the stuck precursor was also co-immunoprecipitated by antibodies against Isp42p (also termed MOM38; a component of the outer membrane system), Isp45p (also termed MIM44; a component of the inner membrane system) or mhsp70 (associated with Isp45) (Figure 2). Co-immunoprecipitation with antisera against Isp42p, Isp45p or mhsp70 was not seen after solubilization with the denaturing detergent SDS; in SDS each antiserum only precipitated its cognate antigen (data not shown), demonstrating that each antiserum was monospecific. Monospecificity of the three antisera was further confirmed by testing each of them by immunoblotting against total mitochondrial proteins (Figure 3). Antibodies against other mitochondrial proteins, such as hsp60, cytochrome c_1 or porin, also failed to co-immunoprecipitate the stuck precursor (Figure 2). Co-immunoprecipitations by antisera against Isp42p, Isp45p and mhsp70 thus reflected genuine association of the stuck precursor with these proteins.

Unexpectedly, antibodies against other components of the outer membrane system, such as Mas70p or Mas20p, failed to co-immunoprecipitate the stuck precursor (Figure 2). These proteins may thus not be core components of the translocation channel or may dissociate from the other channel subunits during solubilization or immunoprecipitation.

Co-immunoprecipitation of the two translocation systems was only seen when the precursor was stuck across both membranes and not when it had been arrested at an earlier import stage, i.e. bound to the surface receptors of uncoupled mitochondria (Figure 4). This result further documents the specificity of the co-immunoprecipitation approach used here.

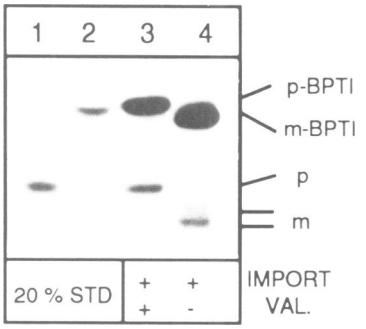

Fig. 5. Import of saturating amounts of unlabeled purified pCOXIV–DHFR–BPTI. Lanes: 1, pCOXIV–DHFR standard (20% of the amount added to the import assays, ~10 pmol); 2, pCOXIV–DHFR–BPTI standard (20% of the amount added to the import assays, ~10 pmol); 3, pCOXIV–DHFR–BPTI mock-imported into mitochondria de-energized with 1 μM valinomycin; 4, pCOXIV–DHFR–BPTI imported into energized mitochondria. Samples were analyzed by SDS–PAGE, blotting onto nitrocellulose filters and immunodecoration with antibodies against DHFR. Immunodecorated bands were visualized with [^{125}I]protein A followed by autoradiography. Abbreviations as in the legends to Figures 1 and 2.

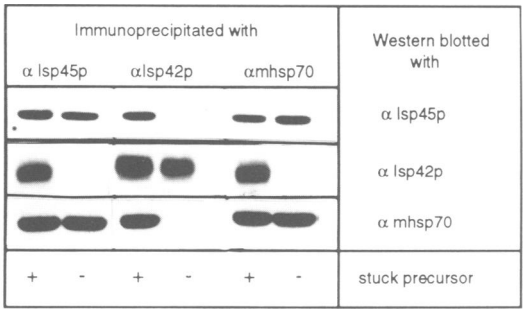

Fig. 6. Stable association of the two yeast mitochondrial protein transport systems requires the presence of the translocation-arrested precursor. Microgram amounts of pCOXIV–DHFR were isolated and derivatized with BPTI as described (Vestweber and Schatz, 1988). After import of the chimeric precursor construct into mitochondria, the mitochondria were solubilized under non-denaturing conditions and subjected to immunoprecipitation with affinity-purified antibodies against mhsp70, Isp42p or Isp45p (+ stuck precursor). Parallel samples (– stuck precursor) were treated identically except that no chimeric precursor was added. The immunoprecipitates were collected on protein A–Sepharose beads and analyzed by SDS–PAGE, blotting onto nitrocellulose filters and immunodecoration with affinity-purified antibodies against Isp45p, Isp42p and mhsp70. Immunodecorated bands were visualized with [^{125}I]protein A followed by autoradiography and were then quantified by laser densitometry. The intensities of the bands (from left to right) were as follows: lane 1, 106 92 91 0 75 96; lane 2, 46 0 48 68 51 0; lane 3, 32 33 28 0 29 29. These values represent percentages of antigen immunoprecipitated with the indicated IgG. One hundred percent represents the amount immunoprecipitated from the same amount of mitochondria under denaturing conditions. The relatively low values for mhsp70 reflect the fact that only 1/4–1/3 of that protein is associated with the mitochondrial inner membrane. Abbreviations as in Figure 1.

Stable association of the two protein translocation systems depends on the presence of a stuck import intermediate

As co-immunoprecipitation of the stuck precursor in the experiments of Figure 2 was not quantitative, the results showed only that stuck precursor molecules could associate with the transport system of the outer membrane as well as with that of the inner membrane. In order to prove that the stuck precursor was *simultaneously* bound to *both* systems, we saturated the mitochondrial import sites with purified, unlabeled pCOXIV–DHFR–BPTI, immunoprecipitated the stuck precursor with antibodies against a component of one translocation system and tested the immunoprecipitate for components of the other system by immunoblotting. First we carried out the control experiment shown in Figure 5 to demonstrate that import of purified, unlabeled pCOXIV–DHFR, as well as its BPTI derivative, resembled that seen with trace amounts of the radiolabeled proteins synthesized *in vitro*; import required a potential across the inner membrane and was accompanied by proteolytic removal of the presequence. The actual experiment (Figure 6) showed that in the presence of the stuck precursor, the immunoprecipitates invariably contained mhsp70, Isp42p and Isp45p, regardless of which antibodies had been used for immunoprecipitation; antibodies against the outer membrane component Isp42p co-immunoprecipitated the inner membrane components Isp45p and mhsp70 and antibodies against the inner membrane components mhsp70 and Isp45p co-immunoprecipitated the outer membrane component Isp42p. Thus the import channels in the outer and inner membrane were stably associated with each other.

No such association was detected in the absence of a stuck precursor (Figure 6); antiserum against the outer membrane protein Isp42p precipitated only Isp42p, but not the inner membrane components Isp45p or mhsp70 and antisera against mhsp70 or Isp45p precipitated only mhsp70 or Isp45p, but not Isp42p. In the absence of the stuck precursor, the import channels of the two membranes were thus apparently disengaged from each other, but the interaction between the inner membrane components mhsp70 and Isp45p remained intact (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994).

In order to verify that the immunodecorated bands in the co-immunoprecipitates represented major protein bands, we saturated mitochondria with stuck precursor, solubilized them under non-denaturating conditions and incubated the extract with anti-mhsp70 or anti-Isp45p IgGs that had been covalently coupled to protein A–Sepharose beads. Mitochondrial proteins that had bound to the immobilized IgGs were then eluted with SDS, subjected to SDS–PAGE and stained with silver. Both immunoprecipitates contained only four major bands, representing (in order of decreasing size) mhsp70, Isp45p, Isp42p and COXIV–DHFR–BPTI (Figure 7). The unequal intensities of the bands probably reflect the well-known fact that different proteins vary greatly in their reaction towards the silver stain.

Discussion

The experimental approach

In this study we have used co-immunoprecipitation in the presence of non-ionic detergents to probe the interaction between the protein transport systems in the two mitochondrial membranes. This technique has proven very useful in studying specific interactions between membrane proteins, but it is not without its pitfalls. First, it critically hinges upon the purity of the antibodies used. Second, a negative result is inconclusive, since the protein–protein interactions under study may be disrupted during solubilization and immunoprecipitation. Third, false

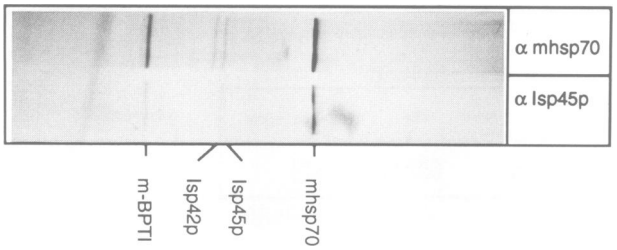

Fig. 7. Protein pattern of co-immunoprecipitates from mitochondria containing saturating amounts of stuck COXIV−DHFR−BPTI. Mitochondria were allowed to accumulate saturating amounts of non-radioactive COXIV−DHFR−BPTI. They were then solubilized under non-denaturing conditions and subjected to immunoprecipitation with IgG against either mhsp70 or Isp45p. The IgGs had been covalently coupled to protein A−Sepharose beads to avoid the presence of IgG chains in the samples analyzed by SDS−PAGE. The immunobound proteins were eluted with SDS and analyzed by SDS−PAGE and silver staining.

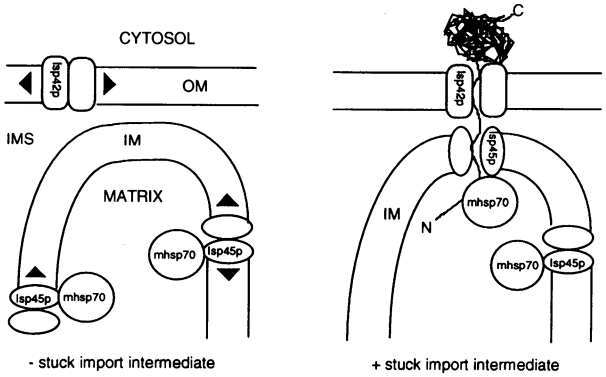

Fig. 8. Model for the transient interaction of the two mitochondrial protein translocation machineries during import of a matrix-targeted precursor protein. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; C, C-terminus of the stuck precursor; N, N-terminus of the stuck precursor.

positive results may be caused by artefactual interactions of proteins after solubilization. The experiments reported here include several controls that render such artefacts unlikely. We have used affinity-purified IgGs and shown them to be monospecific for their cognate antigen. Also, the co-immunoprecipitations were absolutely dependent on the presence of a translocation-arrested precursor protein. Finally, we failed to detect co-immunoprecipitation of inner or outer membrane proteins unrelated to the two protein translocation systems.

Two dynamically interacting protein transport systems

Reversible association between the protein transport systems of the two mitochondrial membranes had previously been inferred from the observations that precursors arrested during transport into the matrix by a stably folded C-terminal domain span both membranes (Schwaiger *et al.*, 1987; Vestweber and Schatz, 1988) and that translocation intermediates can be cross-linked to subunits of both translocation systems (Vestweber *et al.*, 1989; Scherer *et al.*, 1992). However, the cross-linking efficiencies in these studies were too low to prove that a stuck precursor molecule was simultaneously lodged within both translocation channels. Also, it remained open whether the suggested association of the two protein transport channels was reversible. The fact that isolated outer and inner membranes can transport proteins was a further hint at a reversible association of the two systems. However, it remained to be shown that in intact mitochondria the two systems could link up in response to a matrix-targeted precursor protein.

Here we tested for a dynamic association of the two protein translocation machineries by saturating the mitochondrial import system with a purified precursor protein that is targeted to the matrix but gets stuck across both mitochondrial membranes. Our results indicate that the resulting translocation intermediate causes a stable association of the two translocation systems, but that the two systems disengage in the absence of such a precursor (Figure 8).

For transporting a protein into the matrix, there is no obvious advantage in having two distinct, dynamically interacting import channels, as the two channels appear to separate only slowly from each other (Hwang *et al.*, 1991; Glick *et al.*, 1992). Also, normal import of matrix proteins does not appear to proceed via translocation intermediates in the intermembrane space (Hwang *et al.*, 1991). Import of matrix proteins is thus probably mediated by the two systems operating in tandem. However, reversible interaction of the two channels can readily explain how some proteins are sorted to the intermembrane space (Glick *et al.*, 1992; Lill *et al.*, 1992) or directly into the inner membrane (Pfanner *et al.*, 1992), whereas others are directly transported to the matrix.

The nature of mitochondrial membrane 'contact sites'

Our results also bear on the long-standing question of whether the zones of adhesion between the two mitochondrial membranes (Kellems *et al.*, 1974; Schleyer and Neupert, 1985; Pon *et al.*, 1989) are caused by an interaction of the protein transport systems in the two membranes. These membrane adhesions are readily observed in isolated mitochondria in the absence of any protein import (Hackenbrock, 1968), whereas we now show that association between the two transport systems is seen only in the presence of a translocating precursor chain. This finding strengthens our earlier proposal (Glick *et al.*, 1991; Horst *et al.*, 1993) that the membrane adhesions are formed by as yet unidentified proteins and that the protein import systems of the two membranes may reversibly diffuse into these adhesion zones for efficient protein import across both membranes.

Materials and methods

Solubilization of yeast mitochondria and co-immunoprecipitation of the stuck precursor

Radiolabeled pCOXIV−DHFR was isolated and derivatized with BPTI as described (Vestweber and Schatz, 1988.) After import of 40 pmol chimeric precursor into 200 μg mitochondria for 20 min at 30°C, the mitochondria were treated with 5 μg/ml oligomycin, 2 μg/ml efrapeptin and 20 U/ml apyrase (grade VIII; Sigma) for 5 min at 30°C to deplete ATP levels. Mitochondria were then washed twice with 1 ml breaking buffer (1.2 M sorbitol, 20 mM HEPES−KOH, pH 7.4, 1 mM phenylmethylsulfonyl fluoride) and solubilized at 5 mg/ml in digitonin buffer [0.5% digitonin (Boehringer Mannheim), 20 mM HEPES−KOH, pH 7.4, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 0.25 μg/ml elastinal and

5 μg/ml pepstatin]. After a 10 min incubation on ice, the samples were pelleted and resuspended at a protein concentration of 8.75 mg/ml in Mega-8 (octanoyl-*N*-methylglucamid; Boehringer Mannheim) buffer (4% Mega-8, 250 mM NaCl, 50 mM HEPES−KOH, pH 7.4, 1 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 0.25 μg/ml elastinal and 5 μg/ml pepstatin) and incubated for 10 min on ice. Insoluble material was removed by centrifugation. The supernatant was diluted with Mega-8 buffer to 1 ml and mixed with 50 μl of a 50% (v/v) slurry of Sepharose CL-4B (Pharmacia LKB). After incubation for 1 h at 4°C, the beads were removed by centrifugation. The supernatants were mixed with 50 μl of a 50% (v/v) slurry of protein A−Sepharose (Pharmacia LKB) and with 20 μl of the desired affinity-purified antibody. After incubation for 2 h at 4°C, the protein A−Sepharose beads were washed three times with Mega-8 buffer, followed by elution of the bound immune complexes with SDS−PAGE sample buffer. Eluted proteins were separated on 10% SDS−PAGE and the radioactive precursor was visualized by fluorography.

Saturation of mitochondrial import sites with a translocation-arrested chimeric precursor

Microgram amounts of pCOXIV−DHFR were isolated and derivatized with BPTI as described (Vestweber and Schatz, 1988). After import of 50 pmol chimeric precursor construct into 0.5 mg mitochondria for 20 min at 30°C, the mitochondria were solubilized under non-denaturing conditions (see above) and subjected to immunoprecipitation. Parallel samples were treated identically except that no chimeric precursor was added. The immunoprecipitates were collected on protein A−Sepharose beads and analyzed by SDS−PAGE, blotting onto nitrocellulose filters and immunodecoration with the appropriate antibodies. Immunodecorated bands were visualized with [^{125}I]protein A followed by autoradiography.

Miscellaneous

Published methods were used for the isolation of mitochondria (Daum *et al.*, 1982), import into mitochondria (Manning Krieg *et al.*, 1991), SDS−PAGE and fluorography (Hurt *et al.*, 1984), immunoblotting (Haid and Suissa, 1983) and generation and use of affinity resins (Ey *et al.*, 1978). Efrapeptin was a generous gift from Eli Lilly & Co.

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