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# Protein import into yeast mitochondria is inhibited by antibodies raised against 45-kd proteins of the outer membrane

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**Import of several precursor proteins into isolated yeast mitochondria is inhibited by rabbit antiserum raised against the total mitochondrial outer membrane or against electrophoretically purified 45-kd outer membrane proteins. Antisera against other outer membrane proteins are only marginally active or inactive. Inhibition by the antiserum against 45-kd proteins is only weak with untreated mitochondria, but reaches 80–90% with mitochondria that had been pretreated with 0.1 mg/ml trypsin. This trypsin pretreatment by itself inhibits precursor import only slightly (30–50%). Selective inhibition of import does not correlate with binding of the various IgGs to the mitochondrial surface and is also observed with the corresponding Fab fragments. Inhibition by antibodies against 45-kd outer membrane proteins strongly suggests the existence of a mitochondrial surface protein mediating protein import and offers a means of isolating this protein.**

*Key words:* protein import/yeast mitochondria/antibody/outer membrane

## Introduction

How do proteins destined to be transported from the cytosol into mitochondria recognize the mitochondrial surface? Studies on protein translocation across the endoplasmic reticulum have shown that specific recognition in that system is mediated by a 72-kd receptor protein that is anchored within the endoplasmic reticulum, but largely exposed in the cytosol (Meyer *et al.*, 1982; Gilmore *et al.*, 1982). This protein specifically binds a complex consisting of ribosome, nascent secretory protein and a cytosolic ribonucleoprotein complex (the 'signal recognition particle') and thereby initiates translocation of the secretory protein into the endoplasmic reticulum (Walter *et al.*, 1984). This makes it tempting to speculate that protein translocation into mitochondria, too, is mediated by a receptor protein on the mitochondrial surface.

Efforts to isolate such a 'mitochondrial import receptor' have so far been unsuccessful. However, the existence of such a protein has been inferred from the observation that treatment of the mitochondrial surface with different proteases abolished protein import without disrupting the outer membrane (Riezman *et al.*, 1983a; Zwizinski *et al.*, 1983, 1984; Argan *et al.*, 1983). While this result is intriguing, it could simply reflect non-specific perturbation of the mitochondrial surface by hydrophobic peptides generated during proteolysis. Also, this approach does not readily lend itself to isolating the hypothetical receptor protein.

As an alternative approach, we have raised antisera against proteins of the mitochondrial outer membrane and checked whether any of these would inhibit mitochondrial protein import. Although this approach, too, is not without pitfalls, it is relatively non-perturbing and could, in principle, offer a means of isolating either a mitochondrial protein antigen or its corresponding gene.

Here we show that IgGs directed against a selected subpopulation of mitochondrial outer membrane proteins inhibit protein import into isolated mitochondria.

## Results

### *Protein import into yeast mitochondria is inhibited by Fab against 45-kd outer membrane proteins*

Preliminary experiments suggested that import of a radiolabelled, purified precursor protein into isolated yeast mitochondria was partly inhibited by antisera raised against total mitochondrial outer membrane or against 45-kd outer membrane proteins that had been eluted as a single band from SDS–polyacrylamide gels of outer membranes. Antisera against other outer membrane proteins inhibited slightly or not at all (not shown). Since inhibitory effects of crude antisera are difficult to interpret, we repeated the experiments with the corresponding monovalent Fab fragments. Use of these fragments eliminates artefacts resulting from cross-linking of membrane antigens, from non-specific interactions of Fc moieties with membrane surfaces, or from other components of the crude serum. As shown in Figure 1 (left half), Fab fragments from sera raised against total outer membrane (OM) or against

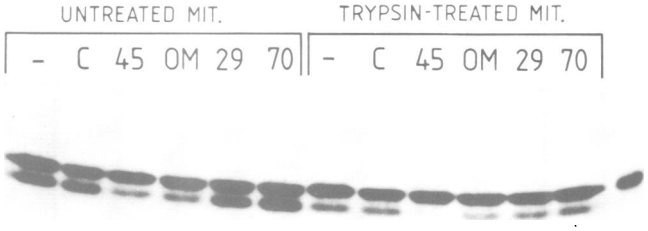


**Fig. 1.** Fab fragments directed against total outer membrane or against 45-kd outer membrane proteins inhibit protein import into mitochondria. Yeast mitochondria (100 μg protein; either untreated or pretreated with 0.1 mg trypsin/ml) were incubated at 0°C for 30 min in a final volume of 50 μl with 1 mg Fab fragments prepared from the IgG fractions listed below. Import of $^{35}$S-labelled, purified pCOXIV–DHFR fusion protein ($1.4 \times 10^5$ c.p.m.) were then assayed for 10 min at 30°C. Control experiments (not shown) verified that under these conditions cleavage of the fusion protein was completely abolished by uncouplers of oxidative phosphorylation and, thus, a result of mitochondrial import; also, the amount of cleaved fusion protein was proportional to time and amount of mitochondria. After the 10-min incubation with fusion protein, the mitochondria were re-isolated and analyzed by SDS–12% polyacrylamide gel electrophoresis and fluorography. The last lane on the right was loaded with 10% of the amount of fusion protein that had been presented to each of the mitochondrial samples. Other lanes contained mitochondrial samples that had been treated as follows: –, no Fab (control); C, 45, OM, 29, 70, pre-incubated with Fab from non-immune IgG or IgGs directed against the 45-kd outer membrane proteins, total outer membrane, 29- or 70-kd outer membrane protein respectively. The upper band is the uncleaved fusion protein (see last lane on the right) and the lower band is the cleaved, imported protein.

**Table I.** Quantification of the inhibitory effects of the various Fab fragments on mitochondrial protein import

| Fab added to mitochondria | Relative import rate (%) | | | |
|---|---|---|---|---|
| | Untreated mitochondria | | Mitochondria treated with 0.1 mg trypsin/ml | |
| | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| None | (100) | (100) | (100) | (100) |
| Non-immune | 100 | 91 | 100 | 79 |
| Anti-outer membrane | 63 | 40 | 70 | 58 |
| Anti-45-kd protein | 59 | 57 | 15 | 21 |
| Anti-29-kd protein | 94 | 105 | 100 | 89 |
| Anti-70-kd protein | 100 | 120 | 105 | 94 |

The bands shown in Figure 1 were quantified by densitometry. Control experiments (documented in Ohba and Schatz, 1987) established that, under these conditions, the amount of cleaved, imported fusion protein was proportional to time and the amount of mitochondria.

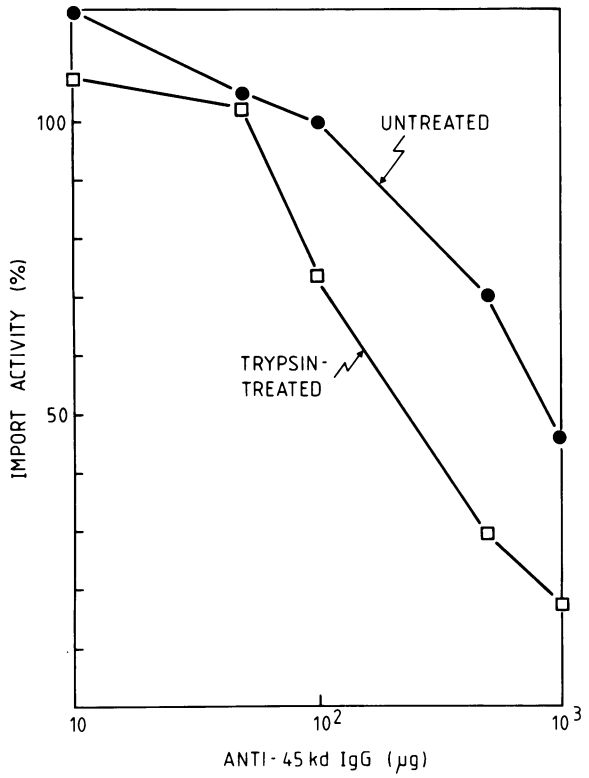


**Fig. 2.** Pretreatment of mitochondria with trypsin renders mitochondrial protein import more sensitive to anti-45-kd IgGs. Yeast mitochondria (100 μg protein) were left untreated or treated with 0.1 mg/ml trypsin for 30 min at 0°C and then incubated for 30 min at 0°C with the indicated amounts of IgG from rabbit antiserum raised against the 45-kd proteins of the mitochondrial outer membrane. Import of the radiolabelled purified pCOXIV−DHFR fusion protein was then assayed as described in Figure 1, Table I and Materials and methods. The extent of import in the absence of added IgG was taken as 100%. As shown in the accompanying paper (Ohba and Schatz, 1987), this 100% value is 30−50% lower for mitochondria that had been pretreated wth 0.1 mg/ml trypsin.

45-kd outer membrane proteins (45) partially inhibited mitochondrial protein import; no inhibition was observed with Fab fragments directed against two other major outer membrane proteins: the 29-kd pore-forming protein, and the cytosolically exposed 70-kd protein. None of the antibodies inhibited binding of the precursor to mitochondria. However, with this precursor (as with the others used in this study) most of the 'binding' appeared to be non-specific as the bound forms could not be chased into mitochondria (not shown).

*Partial proteolysis of the mitochondrial surface by trypsin enhances inhibition by anti-45-kd Fab*

Inhibition of protein import by antibodies against outer membrane or the 45-kd proteins was only partial, even though large amounts of Fab fragments had been employed. One reason for this could be that the 45-kd antigen recognized by the Fab fragments was not a rate-limiting component of the mitochondrial import machinery. In that case, partial removal of these proteins should accentuate the inhibition by subsequently added Fab. Indeed, when the mitochondria were pretreated with 0.1 mg trypsin/ml at 0°C for 30 min, inhibition by the anti-45-kd Fab fragments was now essentially complete (Figure 1, right half; Table I). As shown in the accompanying paper (Ohba and Schatz, 1987), this trypsin-treatment did not impair either the outer membrane barrier or mitochondrial energy coupling and inhibited import of the radiolabelled precursor (the pCOXIV−DHFR fusion protein) by only 30−50%. In agreement with earlier work (Zwizinski *et al.*, 1983, 1984; Riezman *et al.*, 1983b), exhaustive treatment of mitochondria with trypsin blocked protein import even in the absence of added antibodies (Ohba and Schatz, 1987).

Pretreatment of the mitochondria with 0.1 mg/ml trypsin also enhanced inhibition by the corresponding IgG fraction; 50% inhibition for untreated and trypsin-treated mitochondria was observed with 0.87 and 0.23 mg IgG respectively (Figure 2). IgGs against other outer membrane proteins inhibited only slightly or not at all (cf. below). All subsequent experiments were conducted with purified IgG fractions.

*Anti-45-kd IgGs inhibit import of different precursors*

The anti-45-kd IgGs also inhibited import of the authentic cytochrome oxidase subunit IV precursor (Figure 3A) or that of a fusion protein which contained the presequence of yeast alcohol dehydrogenase III (an imported matrix enzyme) fused to mouse dihydrofolate reductase (Figure 3B). Inhibition of import by the anti-45-kd IgGs is thus not limited to the pCOXIV−DHFR fusion protein.

*Preabsorption of anti-45-kd IgGs with mitochondrial outer membranes abolishes inhibition*

Inhibitory effects of antisera, and even Fab fragments, are sometimes caused by traces of proteases that are difficult to remove. Such an artefact was made unlikely by the demonstration that pre-incubation of anti-45-kd IgG fraction with purified yeast mitochondrial outer membranes abolished the inhibitory effect of these IgGs (Figure 4).

*Inhibition of mitochondrial protein import by anti-45-kd IgGs is not merely a consequence of IgGs covering the mitochondrial surface*

Selective inhibition of protein import by IgGs against 45-kd outer membrane proteins might result from the fact that mitochondria bind more of these IgGs than any of the other IgG fractions tested here. In that case, the inhibitory effect of anti-45-kd IgGs might be caused by non-specific events rather than by specific binding to a component of the mitochondrial import machinery. However, Figure 5 shows that non-inhibitory IgGs bind to mitochondria as well, or better, than the anti-45-kd IgGs. In this particular experiment, anti-45-kd IgGs inhibited protein import only with trypsin-treated, but not with untreated, mitochondria; this could have resulted from the fact that, in contrast to the previously men-

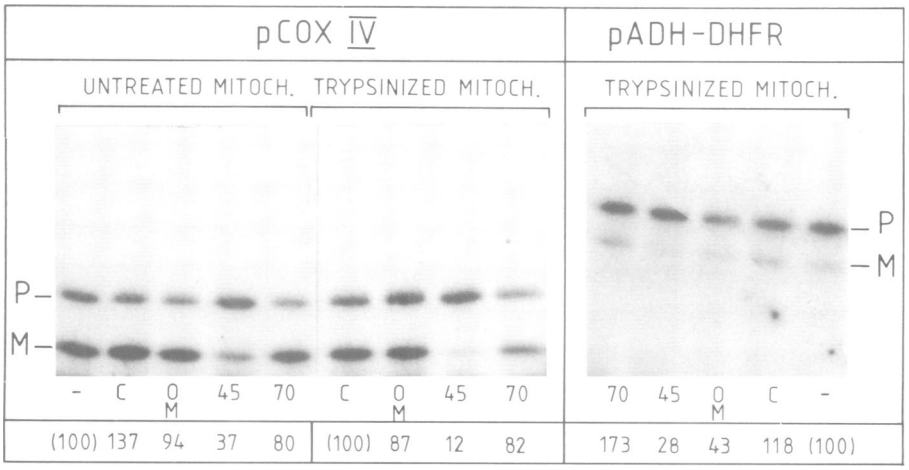


**Fig. 3.** The anti-45-kd antiserum also inhibits mitochondrial import of the authentic cytochrome oxidase subunit IV precursor and of DHFR linked to the presequence of alcohol dehydrogenase III. The authentic subunit IV precursor (pCOXIV) and the pADH−DHFR fusion protein (van Loon *et al.*, 1986) were synthesized by cell-free transcription/translation in a reticulocyte lysate in the presence of [$^{35}$S]methionine. They were then added to untreated or trypsin-treated yeast mitochondria which had been pre-incubated for 30 min at 0°C without IgG (−) or with 0.5 mg aliquots of either non-immune IgG (C) or IgGs raised against total outer membrane (OM) or the 45- or 70-kd outer membrane proteins. Import was assayed as described by van Loon *et al.* (1986). P and M, precursor and mature forms respectively. The relative intensity of the band attributable to imported, mature protein (M) was measured by densitometry and is given in arbitrary numbers (no serum = 100) below each lane.

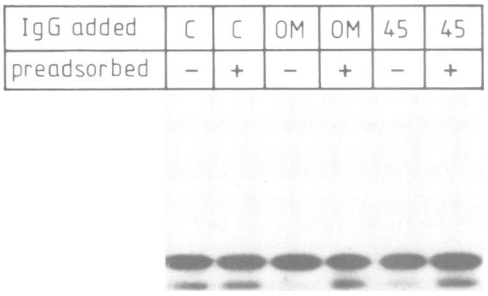


**Fig. 4.** The inhibitory activity of IgGs directed against total outer membrane is eliminated by pre-incubation with mitochondrial outer membrane vesicles. Pre-immune-IgG (0.91 mg), anti-(45-kd protein) IgG (0.36 mg) or anti-(outer membrane) IgG (0.94 mg) were pre-incubated without additions or with 100 μg of yeast mitochondrial outer membrane vesicles for 60 min at 0°C in 40 μl 'mannitol buffer'. The vesicles were then removed by centrifugation in a Beckman Airfuge (20 p.s.i., 10 min) and the supernatant was incubated for 30 min at 0°C with untreated yeast mitochondria (100 μg; final volume 50 μl). The mitochondria were then assayed for import of the purified pCOXIV−DHFR fusion protein as in Figure 1. −, no IgG; C, non-immune IgG; OM, 45, IgGs directed against total outer membrane or the 45-kd outer membrane proteins respectively.

tioned experiments, mitochondria had been washed by sedimentation through a sucrose cushion immediately before the import assay. The data of Figure 5 indicate that the anti-45-kd IgGs inhibit mitochondrial protein import by binding to the protein import machinery or a component closely associated with this machinery.

*The anti-45-kd IgGs react with several 45-kd proteins of the outer membrane*

The specificity of some of the rabbit antisera was checked by immune blotting, against electrophoretically resolved isolated outer membranes or whole mitochondria (Figure 6). As reported before (Riezman *et al.*, 1983b), the antiserum raised against the entire outer membrane reacted mainly with the 29-kd 'porin' and 45-, 63- and 70-kd proteins; the antiserum against the 29-kd 'porin' was essentially monospecific; that against the 33-kd protein was monospecific for that protein if tested against outer membranes, but also reacted with a 28-kd protein of whole mitochondria. Most importantly, the antiserum raised against 45-kd proteins was monospecific for a protein band of that size if tested against whole mitochondria; if tested against the outer membrane, however, it also decorated a 36-kd protein.

We suspected that the 36-kd protein might be a proteolysis product which had been generated from a 45-kd protein during the lengthy procedure for isolating outer membranes. This was strongly suggested by the following three-step experiment. First, a large excess of antiserum against total outer membrane was allowed to react with electrophoretically resolved outer membranes. Second, antibodies bound to the 45-, 42- and 36-kd protein bands were separated from each other by excising the appropriate regions from the immune blots. Third, each of these antibodies was eluted and retested against electrophoretically resolved total outer membrane (Figure 7). Antibody eluted from the 45-kd region of the immune blot redecorated both 45- and 36-kd proteins; conversely, antibody eluted from the 36-kd region redecorated mainly 45-kd proteins. (Because of the small amount of antibody eluted from the 36-kd region and because of the low level of 36-kd antigen in outer membranes, the very weak 36-kd signal is not visible in this photograph of the redecoration experiment.) Antibody eluted from the 42-kd region of the immune blot redecorated mainly 42-kd proteins and, to a lesser extent, adjacent bands of ~40 and 45 kd.

This proves that the 45-kd and 36-kd outer membrane proteins cross-react immunologically and strongly suggests that the latter protein is a breakdown product of the former. Indeed, when the anti-45-kd IgGs were tested against electrophoretically resolved total yeast proteins which had been quickly extracted by alkali from whole yeast cells (Yaffe and Schatz, 1984), they decorated only a 45-kd band (not shown). Indirect evidence from our laboratory had previously indicated that the anti-45-kd serum recog-

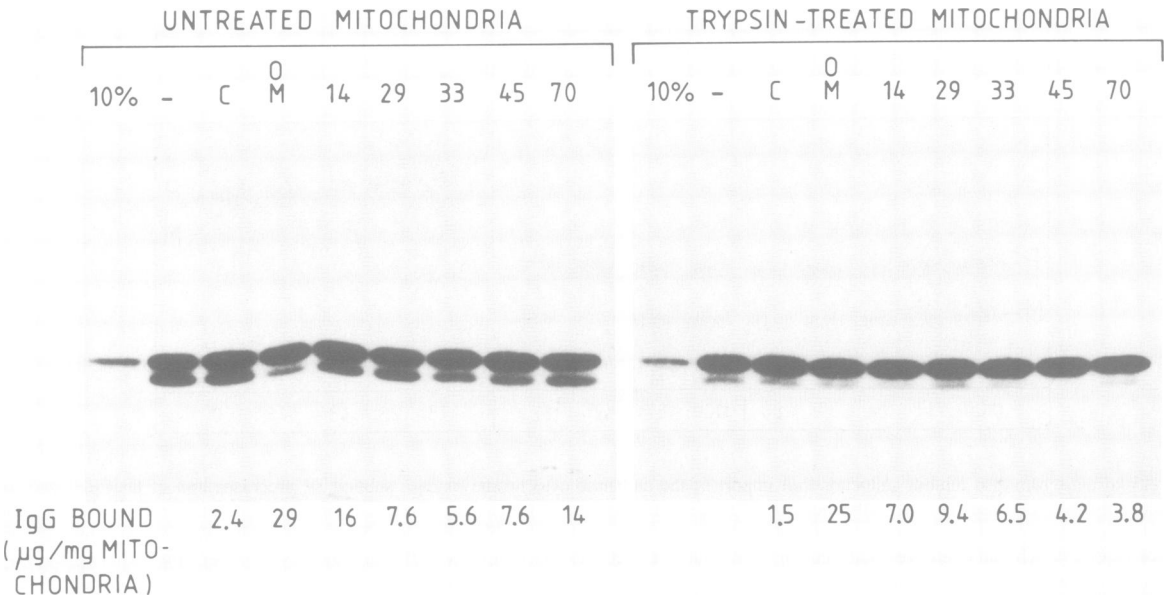


**Fig. 5.** Non-inhibitory IgGs bind to mitochondria more extensively than the inhibitory anti-45-kd IgGs. Mitochondria (100 μg; either untreated or pretreated with 0.1 mg trypsin/ml) were pre-incubated in 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4 in a final volume of 75 μl without IgG (–) or with 750-μg aliquots of IgG from the following rabbit sera: C, non-immune serum; OM, antiserum against total outer membrane; 14, 29, 33, 45, 70, antisera against electrophoretically purified outer membrane proteins of the indicated mol. wts. After 30 min at 0°C, mitochondria were re-isolated by centrifugation through a 0.6 ml cushion of 0.6 M sucrose, 20 mM Hepes–KOH pH 7.4, resuspended in 75 μl of 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4, and assayed for import of the purified pCOXIV–DFHR fusion protein. They were then re-isolated by centrifugation through a 0.6 ml cushion of 0.6 M sucrose, 20 mM Hepes–KOH pH 7.4, 1 mM phenyl methyl sulfonyl fluoride, 5 mM EDTA, resuspended in 0.15 ml of the 'cushion solution' containing 2% SDS and one 100-μg aliquot was analyzed for imported fusion protein by SDS–12% polyacrylamide gel electrophoresis and fluorography. A second aliquot (50 μg) was analyzed for bound IgGs by SDS–10% polyacrylamide gel electrophoresis and immune blotting using radioiodinated goat antiserum against rabbit IgG. For quantitation, graded amounts of pure rabbit IgG were electrophoresed on separate lanes of the same SDS–polyacrylamide gels and carried through the immune blotting procedure. The radioiodinated immune comlexes on the blots were quantified by fluorography and densitometry. The amounts of IgG bound to mitochondria are given below each lane.

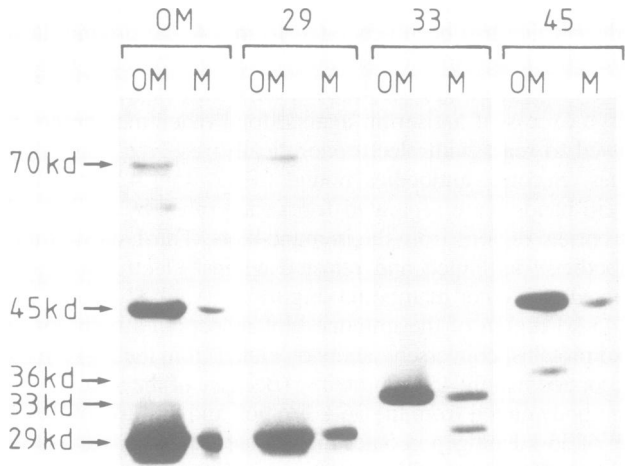


**Fig. 6.** Specificity of antisera. Rabbit antisera raised against total mitochondrial outer membrane (OM) or against the major outer membrane proteins of apparent mol. wt 29, 33 and 45 kd were tested by immune blotting against electrophoretically resolved polypeptides of yeast mitochondria (M; 500 μg protein/lane) or of yeast mitochondrial outer membrane (OM; 75 μg protein/lane). Immune complexes were visualized by decoration with radioiodinated protein A and autoradiography.

nizes at least two different 45-kd outer membrane proteins; in intact mitochondria, one of these is accessible to externally added proteases whereas the other one is inaccessible (Riezman *et al.*, 1983a). To obtain direct evidence for more than one 45-kd outer membrane protein, the proteins of isolated outer membranes were first resolved by isoelectric focusing and, in a second dimension, by SDS–polyacrylamide gel electrophoresis (Figure 8). On being stained for protein, the two-dimensional map (Figure 8A) reveals the major outer membrane proteins at 70, 57 and 29 kd (the 29-kd protein forms a streak close to the basic end of the first dimension) as well as several spots around 45, 36 and 33 kd. When the gel was subjected to immune blotting with anti-45-kd IgGs, major signals were detected at a 45-kd streak at the basic end, and with spots at 45, 44 and 36 kd (Figure 8B). These spots correspond to the arrowed spots in the gel stained for protein (Figure 8A). The immune-reactive spots at 36 kd are much more prominent compared with the 45-kd spots than in the experiments shown in Figure 6 in which the outer membrane antigens had been directly subjected to SDS–polyacrylamide gel electrophoresis. This agrees with the suggestion (Figure 7) that the 36-kd band is a proteolytic artefact since the lengthy isoelectric focusing step in two-dimensional analysis should yield more proteolyic artefacts than a single SDS–polyacrylamide gel electrophoresis.

These results show that the inhibitory anti-45-kd IgGs react with several outer membrane proteins in the 45-kd range. Most, if not all of the cross-reacting 36-kd proteins appear to be proteolytic breakdown products of these 45-kd proteins. While the anti-45-kd IgGs are, thus, not monospecific, they mainly react with only a few outer membrane proteins.

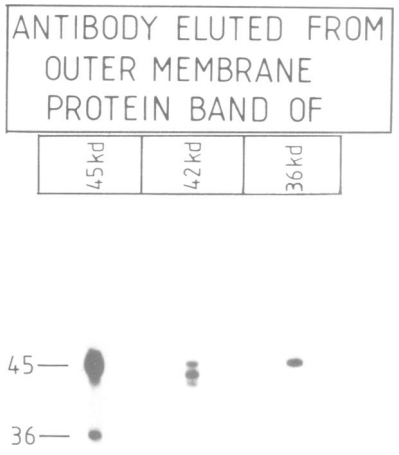


**Fig. 7.** The 36-kd outer membrane protein(s) cross-reacts with antibody against the 45-kd outer membrane proteins. Isolated yeast mitochondrial outer membrane (832 μg protein) was subjected to electrophoresis in two 10 × 10 cm SDS−10% polyacrylamide gel slabs and the resolved protein bands were transferred to two nitrocellulose sheets. After blocking excess protein-binding sites with a 2% solution of defatted milk proteins, the sheets were incubated for 12 h at 23°C with 30 ml of 2% milk proteins in 0.14 M NaCl, 50 mM $NaP_i$ pH 7.4 (PBS) containing 3 ml of rabbit antiserum raised against total outer membrane proteins. The sheets were washed three times for 20 min each at 23°C with 30 ml aliquots of 2% milk−PBS, washed three times with PBS, and a strip cut from one edge was decorated with radioiodinated protein A. With the superimposed fluorogram as a guide, immune complexes present in regions corresponding to 45, 42 and 36 kd were excised from the sheets and the bound immune globulins were eluted by incubating the strips for 2 min at 23°C with 50 mM glycine−HCl, pH 2.2, 0.5 M NaCl, 1% bovine serum albumin. The extraction was repeated once. Each eluate was quickly neutralized with 2 M Tris−base and the neutralized eluates were pooled. Aliquots of the affinity purified immune globulins were then retested by immune blotting against total outer membrane. Mol. wts (in kd) are indicated on the right.

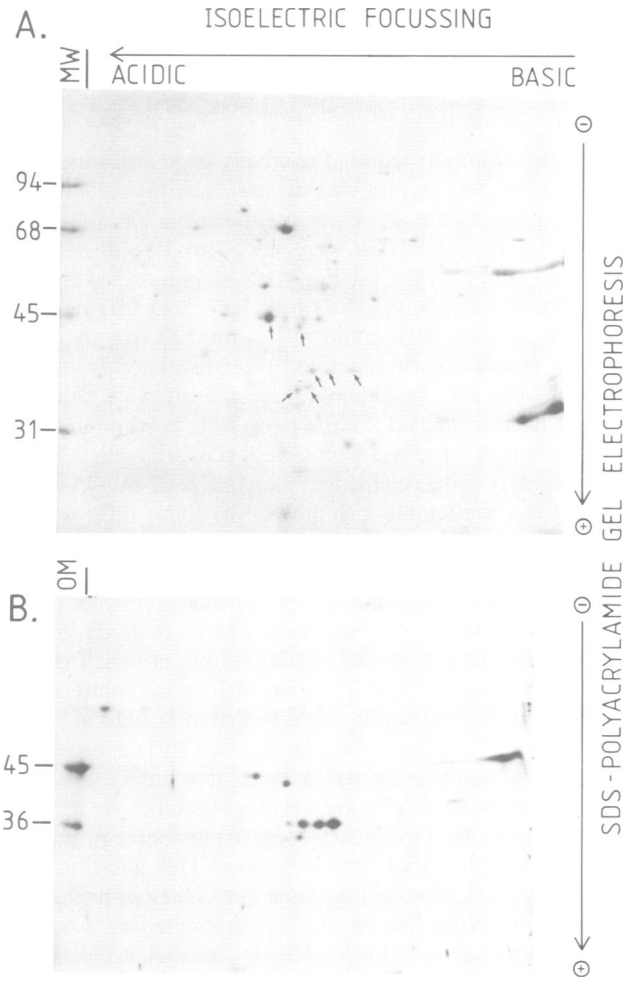


**Fig. 8.** The antiserum raised aginst 45-kd proteins of the outer membrane reacts with several 45-kd proteins. Purified outer membrane from yeast mitochondria (200 μg) was subjected to isoelectric focusing followed by SDS−polyacrylamide gel electrophoresis. **A** The resulting two-dimensional gel was stained with Coomassie Brilliant Blue. **B** The gel was subsequently analyzed by immune blotting with rabbit antiserum against the 45-kd outer membrane proteins (50 μl antiserum diluted with 40 ml of 2% milk protein, PBS per nitrocellulose replica of the gel). Immune complexes were visualized with radioiodinated protein A followed by autoradiography. The vertical lane on the left contained 50 μg outer membrane (OM) that had been solubilized in the non-ionic detergent used for isoelectric focusing. Mol. wts (in kd) are indicated on the left.

## Discussion

Antibodies inhibiting specific biological functions have proved to be powerful tools for identifying and isolating molecules mediating these functions. If such antibodies are used to screen gene libraries in suitable expression vectors, they can also provide rapid access to the corresponding genes.

Here we show, for the first time, that protein import into isolated yeast mitochondria is strongly inhibited by antibodies recognizing a small subset of mitochondrial outer membrane proteins. This strongly suggests that the mitochondrial surface contains one or more components mediating protein import. Since treatment of the mitochondrial surface with relatively high levels of proteases blocks protein import even in the absence of any antibodies (Ohba and Schatz, 1987) and as limited proteolysis of the mitochondrial surface enhances inhibition by anti-45-kd antibodies, the surface component(s) appear(s) to be protein(s).

Inhibition of complex biological processes by antibodies must be interpreted with caution. A large number of potential artefacts must be ruled out before one can conclude that inhibition by an antibody reflects specific binding of the antibody to its antigen. Accordingly, a major part of the present work was devoted to proving this point. First, treatment was usually studied in a simple system containing only isolated mitochondria, an ATP-regenerating system and a purified precursor protein. This eliminated possible problems arising from the use of complex reticulocyte lysates. Second, only one of the several antibodies raised against different outer membrane proteins was shown to be inhibitory. Third, inhibition by this, and only this, antibody was also reproduced with the corresponding Fab fragments. Fourth, inhibition by this antibody, and lack of inhibition by others, did not correlate with the binding of these antibodies to the mitochondrial surface. On the contrary, some of the non-inhibitory IgGs bound much more extensively than the inhibitory anti-45-kd IgGs. Fifth, binding of the various IgGs to the mitochondrial surface roughly agreed with the mitochondrial content of the corresponding antigens. Upon pretreating the mitochondria with 0.1 mg trypsin/ml, IgG binding decreased for protein antigens susceptible to this treatment (e.g. the 70- and

45-kd proteins), but remained unchanged for resistant antigens (e.g. the 29-kd 'porin'). Sixth, inhibition by anti-45-kd IgGs was abolished by pre-absorption with purified outer membranes. Finally, the IgGs inhibited import of several different precursor proteins.

Inhibition of import required relatively large amounts of IgGs. Titration experiments (not shown) indicated that inhibition decreased if less IgG was added and became insignificant if $<100-200$ μg of anti-45-kd IgGs were added to 100 μg of isolated mitochondria. From the binding data of Figure 5 we estimate that only a small fraction (1%) of the IgG molecules recognized the native antigen. This could reflect the fact that the antisera had been raised against denatured proteins eluted from SDS–polyacrylamide gels. In addition, these antisera were rather weak and may have contained a large excess of 'pre-immune' IgGs.

It is striking that pretreatment of mitochondria with 0.1 mg/ml trypsin strongly enhanced the inhibitory effect of anti-45-kd IgGs. Without such pretreatment, inhibition by these IgGs was often marginal. In contrast, inhibition by IgGs raised against the total outer membrane was usually stronger with untreated mitochondria. This remains unexplained. The enhancing effect of pretreatment with trypsin could mean that effective antibody inhibition requires the target antigen to be rate-limiting, or to be freed from other proteins that shield it from externally added antibody molecules. Another explanation would be that there exist at least two import pathways, one mediated by a 45-kd surface protein, and the other by some different protease-sensitive surface component.

Since the inhibitory IgGs are not monospecific, we cannot tell which, if any, of the cross-reacting outer membrane proteins participate in mitochondrial protein import. This question is now being investigated. Several functional mitochondrial presequences can form amphiphilic helices whose hydrophilic face is positively charged (Roise *et al.*, 1986; Allison and Schatz, 1986; von Heijne, 1986). We suggested that this structural feature, rather than a specific sequence motif, is recognized by the mitochondria import machinery. This suggestion does not, of course, negate the operation of a mitochondrial surface protein in mitochondrial protein import. The exposed amphiphilic presequences could initially insert into the bilayer of the outer membrane with low affinity, move laterally to the import machinery and productively bind to that machinery with higher affinity. Alternatively, the import machinery itself might contain a binding site for positively charged amphiphilic helices; it is well established that some proteins interact strongly with amphiphilic peptides (e.g. Cox *et al.*, 1985). These questions will only be answered once the mitochondrial import machinery has been isolated. The inhibitory antibodies described here should help in this task.

## Materials and methods

*Preparation and trypsin treatment of mitochondria*

Mitochondria were isolated from the wild-type *Saccharomyces cerevisiae* strain D-273-10B (ATCC 25657) grown on semi-synthetic medium containing 2% lactate (Daum *et al.*, 1982). For trypsin treatment, mitochondria were suspended to 5 mg/ml in 0.6 M mannitol, 20 mM Hepes–KOH (pH 7.4) containing 100 μg/ml trypsin (from bovine pancreas; pretreated with *N*-tosyl-L-phenylalanine chloromethyl ketone; 3.5 IU/mg if assayed with *N*-alpha-benzoyl-L-arginine-4-nitroanilide at 25°C; Merck Co., FRG). They were then incubated at 0°C for 30 min. The reaction was stopped by adding soybean trypsin inhibitor to a final concentration of 1 mg/ml and incubating for another 10 min at 0°C. The mitochondria were sedimented by centrifugation through 0.6 M sucrose, 20 mM Hepes–KOH (pH 7.4), 100 μg/ml trypsin inhibitor and resuspended in 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4 to a concentration of ~10 mg/ml. For 'mock' treatment (untreated mitochondria), mitochondria were treated in the same way as above except that trypsin was omitted in the first incubation.

*Antibody treatment of mitochondria*

One hundred micrograms of mitochondrial protein (untreated or trypsin-treated) were incubated for 30 min at 0°C in 50 μl of 'mannitol buffer' containing 1 mM phenyl methyl sulfonyl fluoride and 100–1000 μg of IgG or Fab fragments prepared from each antiserum. The 'import assay mixture' described below was then added in order to measure the import activity of mitochondria. Where indicated, mitochondria were re-isolated after the incubation with antibodies, before the import assay, by sedimentation through 0.6 M sucrose, 20 mM Hepes–KOH (pH 7.4), 100 μg trypsin inhibitor/ml.

*Mitochondrial import experiments*

The 'import assay mixture' (total volume 150 μl) usually contained purified precursor ($^{35}$S-labelled pCOXIV–DHFR; $1.5 \times 10^5$ c.p.m., 40 ng) an ATP-generating system (6.7 mM phosphoenol pyruvate, 1.3 mM ATP, 0.6 unit pyruvate kinase) 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4, 1.3 mM $MgCl_2$, 6.7 mM GTP, 3.3 mg/ml bovine serum albumin, 134 μg/ml trypsin inhibitor, and 0.1 mM phenyl methyl sulfonyl fluoride. The import reaction was started by adding 50 μl suspension containing 100 μg mitochondria and antibodies. After 10 min incubation at 30°C, the mitochondria were re-isolated by centrifugation through 0.6 M sucrose, 20 mM Hepes–KOH pH 7.4, 2 mM EDTA for 10 min at 12 000 *g*, solubilized in SDS (3 min at 95°C) and analyzed by SDS–12% polyacrylamide gel electrophoresis followed by fluorography. Fluorographs were quantified by densitometric scanning.

*Two-dimensional gel electrophoresis of outer membrane proteins*

Outer membrane vesicles (200 μg protein) were solubilized with 2% SDS at 95°C for 3 min and applied to a cylindrical isoelectric focusing gel (diameter 2.5 mm, length 14 cm) in the presence of 8 M urea, 2% NP-40, 2% Ampholine (pH 3.5–10) essentially as described (O'Farrell, 1975). After isoelectric focusing for 14 h at room temperature, the gel rod was layered onto an SDS–12% polyacrylamide gel slab (14 × 10 cm × 1.5 mm). After electrophoresis in the second dimension, protein spots were stained with Coomassie Brilliant Blue R-250 or transferred onto a nitrocellulose sheet for subsequent antibody decoration.

*Preparation of IgG and Fab fragments from antisera*

Rabbit antisera against total outer membrane or against individual outer membrane proteins was raised as described (Riezman *et al.*, 1983a). IgG fractions were prepared from each antiserum by protein A–Sepharose column chromatography as recommended by the manufacturer (Pharmacia): about 2 ml of antiserum were slowly passed through a 0.5 ml protein A–Sepharose column, and bound IgG was eluted with 1 M acetic acid (pH ~2). The eluate was neutralized with 2 M Tris–base, dialyzed against distilled water and concentrated by lyophilization. About 10 mg of purified IgG was obtained from each antiserum; it was stored at −70°C.

Fab fragments were prepared from IgG fractions according to Mage (1981). Briefly, IgG fractions (10 mg/ml) were digested with papain (crystallized, 0.1 mg/ml) in the presence of 25 mM beta-mercaptoethanol for 1 h at 37°C; digestion was stopped by adding iodoacetate to 30 mM and the mixture was dialyzed against water. Fab and Fc fragments were separated from each other by CM–cellulose column chromatography; the Fab fragments were then passed through a small protein A–Sepharose column to remove residual Fc fragments, dialyzed against distilled water, concentrated by lyophilization and stored at −70°C.

*Miscellaneous*

Published methods were used for immune blotting (Haid and Suissa, 1983), purification of radiolabelled pCOXIV–DHFR fusion protein (Eilers and Schatz, 1986) and for the preparation of yeast mitochondrial outer membrane (Riezman *et al.*, 1983). The antibodies used in this study have been described earlier (Daum *et al.*, 1982a; Riezman *et al.*, 1983a,b).

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