The EMBO Journal vol.9 no.10 pp.3191 - 3200, 1990

Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70

Victoria Hines[1], Anders Brandt[1,3], Gareth Griffiths[2], Heinz Horstmann[2], Hildegard Brütsch[1] and Gottfried Schatz[1]

[1]Biocenter, University of Basel, CH-4056 Basel, Switzerland and [2]European Molecular Biology Laboratory, D-6900 Heidelberg, FRG

[3]Permanent address: Department of Physiology, Carlsberg Laboratory, DK-2500 Copenhagen, Denmark

Communicated by G.Schatz

The yeast mitochondrial outer membrane contains a major 70 kd protein with an amino-terminal hydrophobic membrane anchor and a hydrophilic 60 kd domain exposed to the cytosol. We now show that this protein (which we term MAS70) accelerates the mitochondrial import of many (but not all) precursor proteins. Anti-MAS70 IgGs or removal of MAS70 from the mitochondria by either mild trypsin treatment or by disrupting the nuclear *MAS70* gene inhibits import of the F_1-ATPase β-subunit, the ADP/ATP translocator, and of several other precursors into isolated mitochondria by up to 75%, but has little effect on the import of porin. Intact cells of a *mas70* null mutant import the F_1-ATPase α-subunit and β-subunits, cytochrome c_1 and other precursors at least several fold more slowly than wild-type cells. Removal of MAS70 from wild-type mitochondria inhibits binding of the ADP/ATP translocator to the mitochondrial surface, indicating that MAS70 mediates one of the earliest import steps. Several precursors are thus imported by a pathway in which MAS70 functions as a receptor-like component. MAS70 is not essential for import of these precursors, but only accelerates this process.

Key words: MAS70/membrane proteins/mitochondria/protein import/yeast

Introduction

Transport of a protein from the cytoplasm into mitochondria requires energy, one or more targeting signals on the transported protein, and a machinery that decodes these signals and moves the protein to its correct intramitochondrial location (Verner and Schatz, 1988; Hartl and Neupert, 1990). While the energy sources for transport and some of the targeting signals have been identified, relatively little is known about the transport machinery. The first components to be identified were a matrix-localized processing protease which cleaves 'matrix-targeting' sequences from imported precursor proteins (Böhni *et al.*, 1980; Hawlitschek *et al.*, 1988; Yang *et al.*, 1988; Ou *et al.*, 1989), and stress proteins in the cytosol and the matrix that maintain precursors in the correct conformation for translocation and refolding in the matrix (Deshaies *et al.*, 1988; Murakami *et al.*, 1988; Cheng *et al.*, 1989). These proteins are all soluble. However, mitochondrial protein import also requires proteins associated with the mitochondrial membranes, because import of precursors by isolated mitochondria is inhibited by treating the mitochondrial surface with proteases (Riezman *et al.*, 1983b; Zwizinski *et al.*, 1984) or Fab fragments against outer membrane proteins (Ohba and Schatz, 1987). Attempts to trace these effects to distinct outer membrane proteins have recently led to the identification of two such proteins: one (termed Import Site Protein 42 or ISP42) is a 42 kd outer membrane protein from yeast mitochondria that appears to be a component of the translocation channel (Vestweber *et al.*, 1989). The ISP42 gene has been cloned and its protein product shown to be essential for viability of the yeast cells (K.Baker and A.Schaniel, in preparation). The second protein (termed MOM19) was identified in outer membranes of *Neurospora* mitochondria; it mediates binding of most (but not all) precursor proteins to the mitochondrial surface (Söllner *et al.*, 1989) and may thus function as a receptor. Whether this protein, too, is essential for viability is not known.

Here we describe yet another component of the mitochondrial protein import machinery. Several years ago we showed that the yeast mitochondrial outer membrane contains a major 70 kd protein composed of two functionally distinct regions. The first region encompasses the amino-terminal 41 residues; it targets and anchors the protein to the outer membrane. The second region represents the protein's 60 kd carboxy-terminal part; it protrudes into the cytoplasm and is released as a soluble fragment by low trypsin levels (Riezman *et al.*, 1983a; Hase *et al.*, 1983, 1984). Haploid cells in which the gene for this protein has been disrupted are viable, but show a long lag in forming colonies on synthetic media and barely grow on non-fermentable carbon sources at 37°C (Riezman *et al.*, 1983c). The ill-defined phenotype of this null mutant made it difficult to identify the function of the 70 kd outer membrane protein.

In this report we show that IgGs monospecific for this protein inhibit import of several precursors into isolated yeast mitochondria. A similar inhibition is seen upon removing the protein from mitochondria by gentle trypsinization or by genetic means. Deleting the protein from yeast cells also retards the mitochondrial import of several precursors *in vivo*. We propose that the 70 kd protein functions as a receptor-like component which enhances import efficiency and suggest that it be termed MAS70 (for Mitochondrial Assembly).

Results

Anti-MAS70 IgGs inhibit import of several precursors into isolated yeast mitochondria

Incubation of yeast mitochondria with IgGs monospecific for MAS70 strongly inhibits import of the F_1-ATPase β-subunit and of the ADP/ATP translocator. Maximal inhibition for these precursors is usually between 50 and

70%, depending on the mitochondrial preparation, and is reproducibly observed at an IgG/mitochondria weight ratio of 1. Import of other authentic mitochondrial precursor proteins, such as alcohol dehydrogenase III and cytochrome b_2 (Figure 1), as well as import of the COXIV−DHFR fusion protein (Figure 2, WT) are inhibited weakly whereas import of porin is not significantly affected (COXIV, subunit IV of yeast cytochrome oxidase; COXIV-DHFR, fusion protein containing the COXIV presequence attached to mouse dihydrofolate reductase). IgGs against porin, the most abundant outer membrane protein, do not inhibit import of any precursor tested (not shown). This control, and the results mentioned below, make it unlikely that the inhibition by anti-MAS70 IgGs is merely caused by non-specific coating of the mitochondrial surface.

In the experiments illustrated in Figures 1 and 2, mitochondria were allowed to import precursors for a single period of time only and were then assayed for the amount of imported precursor. Even though these time periods were within the range during which import is a linear function of time and the amount of mitochondria (Materials and methods and not shown), we checked directly whether anti-MAS70 IgGs decrease the rate, and not merely the final extent, of import. The time course experiments depicted in Figure 3 show clearly that anti-MAS70 IgGs affect the rate of import. In addition, the inhibition pattern for the three precursors tested agrees with the patterns revealed by Figures 1 and 2.

Mitochondria from the mas70 null mutant import several precursors more slowly than wild-type mitochondria

The results mentioned above suggest that MAS70 mediates the import of a subset of precursors into isolated mitochondria. Precursors whose import is strongly inhibited by anti-MAS70 IgGs should thus be imported more slowly by mitochondria from a *mas70* null mutant; moreover, import into these MAS70 deficient mitochondria should be insensitive to anti-MAS70 IgGs. This is indeed the case. Mitochondria from the *mas70* null mutant import the ADP/ATP translocator only half as fast as mitochondria from isogenic wild-type cells and this import is not significantly inhibited by anti-MAS70 IgGs (Figure 2, upper part). The slight inhibition by very high IgG levels probably reflects

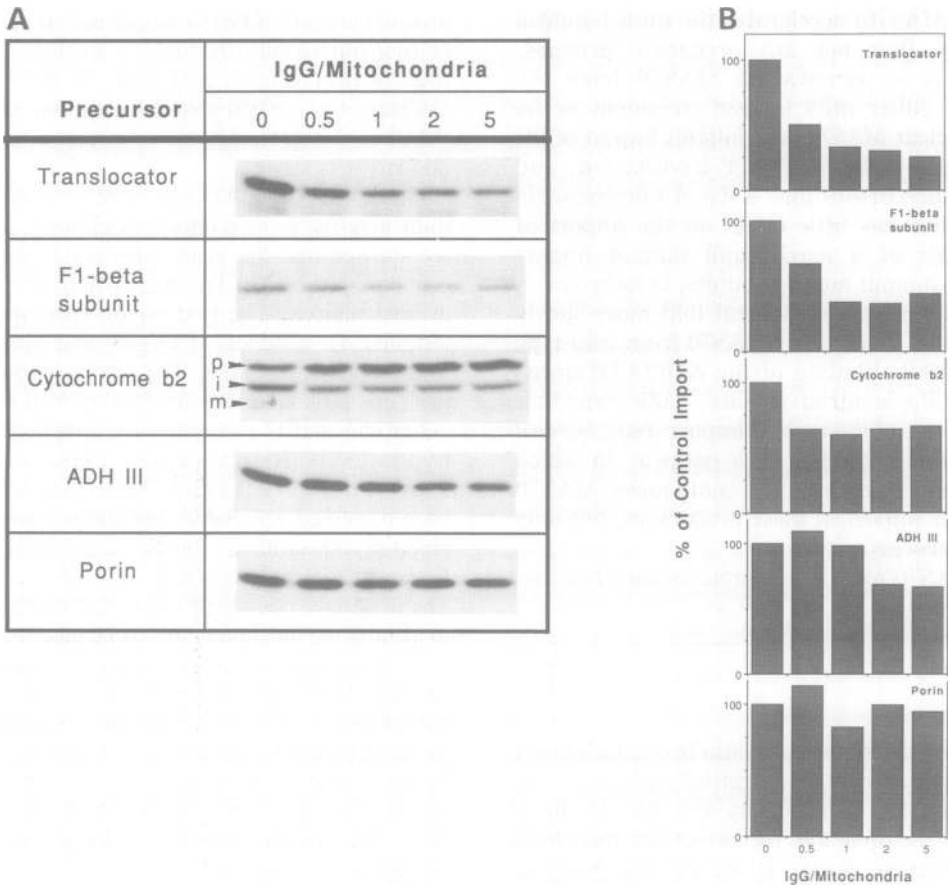

Fig. 1. Anti-MAS70 IgGs inhibit the import of several authentic precursor proteins into isolated yeast mitochondria. **(A)** Wild-type mitochondria (15 μg protein) were incubated for 40 min at 0°C with or without the amounts of anti-MAS70 IgGs indicated in the figure panels. The incubation medium contained 0.6 M sorbitol, 20 mM HEPES−KOH, pH 7.4, and 2 mg bovine serum albumin/ml; the total volume was 30 μl. Each mitochondrial aliquot was then tested for its ability to import the radiolabeled precursors to the mitochondrial proteins indicated in the figure. Import was stopped by the addition of 0.1 μg valinomycin/ml, non-imported precursor was digested with proteinase K, and imported radiolabeled proteins were analyzed by SDS−PAGE and fluorography. **(B)** The protease-inaccessible radiolabeled bands shown in (A) were quantified by densitometry of the fluorograms; 100% import represents import in the absence of added IgG (B). To assess all import stages of cytochrome b_2 (including a protease-accessible, membrane spanning translocation intermediate), the mitochondria were not treated with protease before electrophoretic analysis and import was quantified by summing all intermediates and the mature form. In all figures, the amount of IgGs added is given as the weight ratio IgG/mitochondrial protein. Translocator, ADP/ATP translocator; ADH III, the mitochondrial isozyme of yeast alcohol dehydrogenase; porin, major 29 kd pore forming protein of the outer membrane, p, i and m, precursor, intermediate and mature forms, respectively.

non-specific effects. Similar results are obtained for the import of the F_1-ATPase β-subunit (not shown, but see also Figure 4).

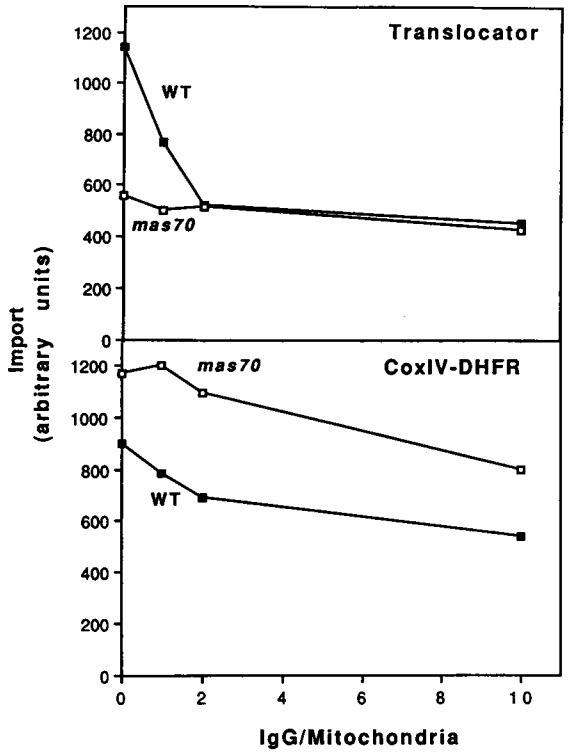

Fig. 2. The *mas70* null mutation affects import of the ADP/ATP translocator, but not that of COXIV−DHFR. Mitochondria from a *mas70* null mutant (*mas70*) or its wild-type parent (WT) were incubated with the indicated amounts of anti-MAS70 IgGs and tested for import of the ADP/ATP translocator ('Translocator') or the COXIV−DHFR fusion protein as outlined in Figure 1. Black squares: wild-type mitochondria; open squares: mitochondria from the *mas70* null mutant. Import was quantified by denistometric scanning of the fluorograms.

Two observations indicate that the lower import rates of *mas70* mitochondria do not simply reflect a preferential damage of these mitochondria during isolation. First, *mas70* mitochondria have the same respiratory rate, and the same respiratory control ratio, as mitochondria from the wild-type parent (not shown). Second, *mas70* mitochondria import the COXIV−DHFR fusion protein as rapidly as wild-type mitochondria (Figure 2, lower part and Figure 4). Import of this artificial precursor into wild-type mitochondria is only weakly inhibited by anti-MAS70 IgGs (Figure 2, lower part), in agreement with our earlier data (Ohba and Schatz, 1987). With this precursor, any small difference in import rates between wild-type and mutant mitochondria is probably obscured by the unavoidable variability between different mitochondrial preparations. Import into *mas70* mitochondria is unaffected by an IgG/mitochondria ratio of 2 which maximally inhibits import of the ADP/ATP translocator by wild-type mitochondria (Figures 1 and 2, upper part). As the antiserum is monospecific for MAS70 and does not react with total proteins from *mas70* mitochondria (Materials and methods), the combined results suggest that anti-MAS70 IgGs decrease the rate of mitochondrial protein import by specifically binding to MAS70 on the mitochondrial surface.

Removal of MAS70 from the mitochondrial surface by low trypsin levels mimics the effects of anti-MAS70 IgGs

MAS70 is the most protease sensitive major outer membrane protein; exposure of intact mitochondria or isolated outer membrane vesicles to low (1−10 μg/ml) trypsin levels releases a 60 kd MAS70 fragment from the mitochondrial surface (Riezman *et al.*, 1983a). Figure 4B confirms that wild-type mitochondria treated with 10 μg trypsin/ml have completely lost MAS70. The 60 kd MAS70 fragment is quantitatively recovered in the supernatant (not shown). Under these conditions (and even at 10-fold higher trypsin

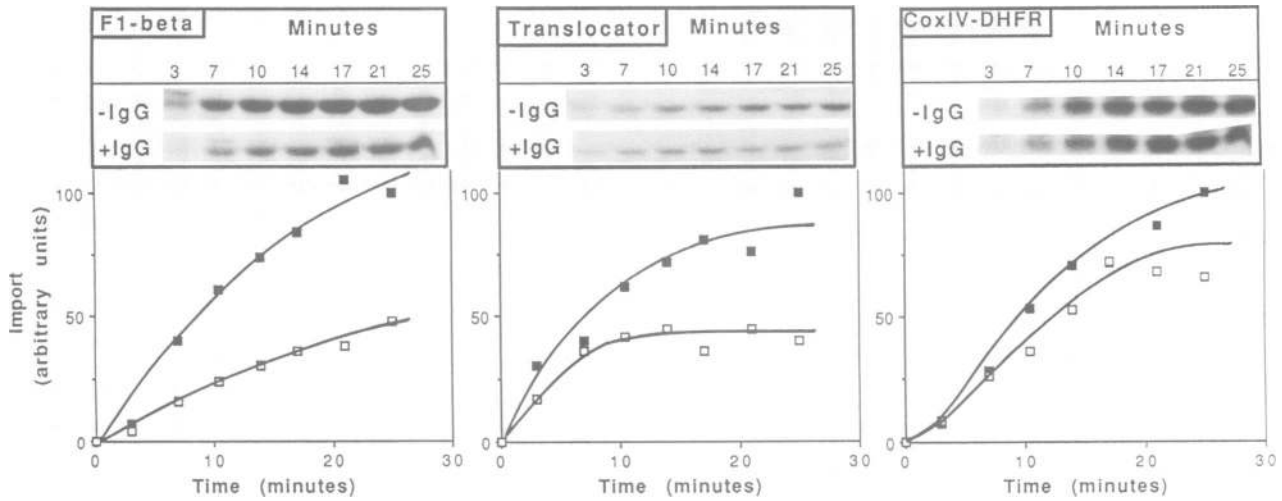

Fig. 3. Anti-MAS70 IgGs decrease the import rate of several precursors. Wild-type mitochondria were incubated without (−IgG) or with a 2.5-fold weight excess of anti-MAS70 IgGs over mitochondrial protein (+IgG) as described in Figure 1. After 40 min at 0°C, the kinetics of precursor import were measured essentially as in Figure 1 with two exceptions: (i) samples were assayed after the indicated times; (ii) import of the F_1-ATPase β-subunit ('F1-beta') and of the COXIV−DHFR fusion protein were assayed at 25°C whereas import of the ADP/ATP translocator ('Translocator') was measured at 15°C. Import was stopped by adding 0.1 μg valinomycin/ml, samples were treated with proteinase K, and the amount of protease-inaccessible radiolabeled ADP/ATP translocator or of protease-inaccessible radiolabeled mature F_1-ATPase β-subunit ($F_1\beta$) or COXIV−DHFR fusion protein was determined as described in Figure 1. Photographs of the fluorograms are shown in the upper parts. The lower parts show quantification of the fluorograms in arbitrary scanner units. Open squares represent IgG-treated mitochondria, black squares untreated mitochondria. For each precursor, import after 25 min in the absence of IgGs was taken as 100%.

concentrations), the intermembrane space marker cytochrome b_2 is not degraded, indicating that trypsin treatment does not abolish the integrity of the outer membrane barrier. Similarly, ISP42 of the outer membrane remains intact even after treatment with 100 μg trypsin/ml.

The antibody inhibition data mentioned above indicate that import of the F_1-ATPase β-subunit and of the ADP/ATP translocator involves MAS70. Consistent with this, removal of MAS70 from wild-type mitochondria by treatment with 10 μg trypsin/ml strongly inhibits import of these two precursor proteins; import of alcohol dehydrogenase III or the COXIV–DHFR fusion protein (which is only slightly affected by anti-MAS70 IgGs) is only marginally decreased by these low trypsin levels (Figure 4A). Import of the F_1-ATPase β-subunit and of the ADP/ATP translocator by mitochondria from the *mas70* null mutant is much slower than by untreated wild-type mitochondria and is unaffected by low trypsin levels. Thus, inhibition of wild-type mitochondria by mild trypsinization only reflects removal of MAS70. The slower import of the F_1-ATPase β-subunit and the ADP/ATP translocator by *mas70* mitochondria is a specific effect as these mitochondria import alcohol dehydrogenase III or the COXIV–DHFR fusion protein as rapidly as wild-type mitochondria.

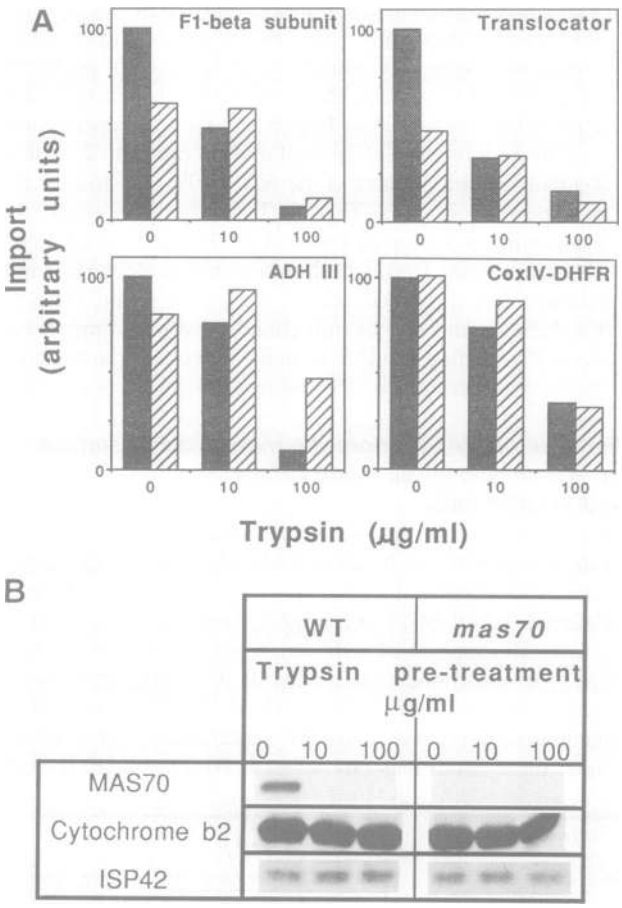

Fig. 4. Removal of MAS70 from wild-type mitochondria by low trypsin levels inhibits import of several precursor proteins into wild-type mitochondria, but not into MAS70-deficient mitochondria. (**A**) Freshly isolated mitochondria from the wild-type (filled bars) or the corresponding *mas70* null mutants (hatched bars) were suspended in 0.6 M sorbitol, 20 mM HEPES–KOH, pH 7.4, to 5 mg protein/ml and incubated for 30 min at 0°C without trypsin or with either 10 or 100 μg trypsin/ml. Soybean trypsin inhibitor was then added to 1 mg/ml; after 5 min at 0°C, the mitochondria were re-isolated by centrifugation and resuspended in 0.6 M sorbitol, 20 mM HEPES–KOH, pH 7.4, 1 mg trypsin inhibitor/ml. Aliquots (20 μg) of each mitochondrial sample were then tested for import of the F_1-ATPase β-subunit, the ADP/ATP translocator ('Translocator'), the mitochondrial isozyme III of alcohol dehydrogenase (ADH III) or the COXIV–DHFR fusion protein (see Figure 1). The bars represent arbitrary densitometric units corresponding to protease-inaccessible radiolabeled ADP/ATP translocator or to protease-inaccessible mature forms of the other three radiolabeled precursor proteins. In all cases, the signal obtained with untreated wild-type mitochondria was taken as 100%. Numbers below the bars indicate the concentration of trypsin (in μg/ml) at which the mitochondria had been pretreated. (**B**) Aliquots (50 μg) of the untreated or trypsin-treated mitochondrial samples assayed in the experiments depicted in (A) were analyzed for MAS70, cytochrome b_2 and ISP42 by SDS–PAGE and immune blotting. A photograph of the fluorogram is shown. WT and *mas70*, mitochondria from wild-type or *mas70* null mutant, respectively.

The residual import of the F_1-ATPase β-subunit or the ADP/ATP translocator by *mas70* mitochondria or mildly trypsinized wild-type mitochondria can be further reduced by treatment with 100 μg trypsin/ml. These higher trypsin levels also inhibit import of alcohol dehydrogenase III or the COXIV–DHFR fusion protein (Figure 4A) even though they do not affect ISP42 or the integrity of the outer membrane barrier (Figure 4B). This additional inhibition suggests that the F_1-ATPase β-subunit and the ADP/ATP translocator can be imported also by a MAS70 independent pathway that requires some as yet unknown mitochondrial proteins.

We conclude that inhibition of wild-type mitochondria by low trypsin levels only reflects removal of MAS70 and that MAS70 accelerates import of several precursors into mitochondria.

MAS70 mediates precursor binding to the mitochondrial surface

Import of precursor proteins into mitochondria involves several distinct steps in which the precursor may interact successively with soluble proteins in the cytosol, with 'receptors' on the mitochondrial surface, with a transmembrane transport channel, and with components inside the mitochondria (Verner and Schatz, 1988; Hartl and Neupert, 1990). Its location on the mitochondrial surface suggests that MAS70 mediates an early import step. To test this, we studied the function of MAS70 in the import of the ADP/ATP translocator because the initial import steps of this protein have been well characterized (Pfanner and Neupert, 1987; Pfanner *et al.*, 1987; Smagula and Douglas, 1988). In the absence of ATP and at elevated temperature, the ADP/ATP translocator binds to mitochondria (Figure 5A, lane 1), but remains sensitive to moderate (50 μg/ml) proteinase K concentrations (Figure 5A, lane 2). Upon addition of ATP, 46% of this import intermediate is chased to the fully inserted form which is resistant to proteinase K (Figure 5A, lanes 3 and 4) as well as to extraction by high pH (Figure 5A, lanes 4–6). Formation of this protease sensitive intermediate by wild-type mitochondria is strongly inhibited by anti-MAS70 IgGs or by removal of MAS70 with low trypsin levels (Figure 5B). The average inhibition by these two treatments is 70% (Figure 5B, BINDING).

To determine whether the protease sensitive form of the ADP/ATP translocator is a *bona fide* import intermediate, aliquots of the samples shown in Figure 5B (BINDING) were also chased by adding ATP. In untreated samples, or samples treated with pre-immune IgGs, 70% of the bound precursor

(lanes 1 and 2) was chased to a protease-protected form (lanes 7 and 8). In samples treated with anti-MAS70 IgGs (lane 4), 50% of the bound form was chased (lane 10); however, since the unchased sample already contained less bound precursor, the total amount of chaseable precursor was only 30% of that seen with the control samples shown in lanes 7 and 8. Trypsin-treated wild-type mitochondria, or *mas70* mitochondria, accumulated virtually no chaseable intermediate (compare lanes 3, 5 and 6 with lanes 9, 11 and 12). Thus, most of the residual binding to these samples was non-productive.

In the presence of ATP and in the absence of a membrane

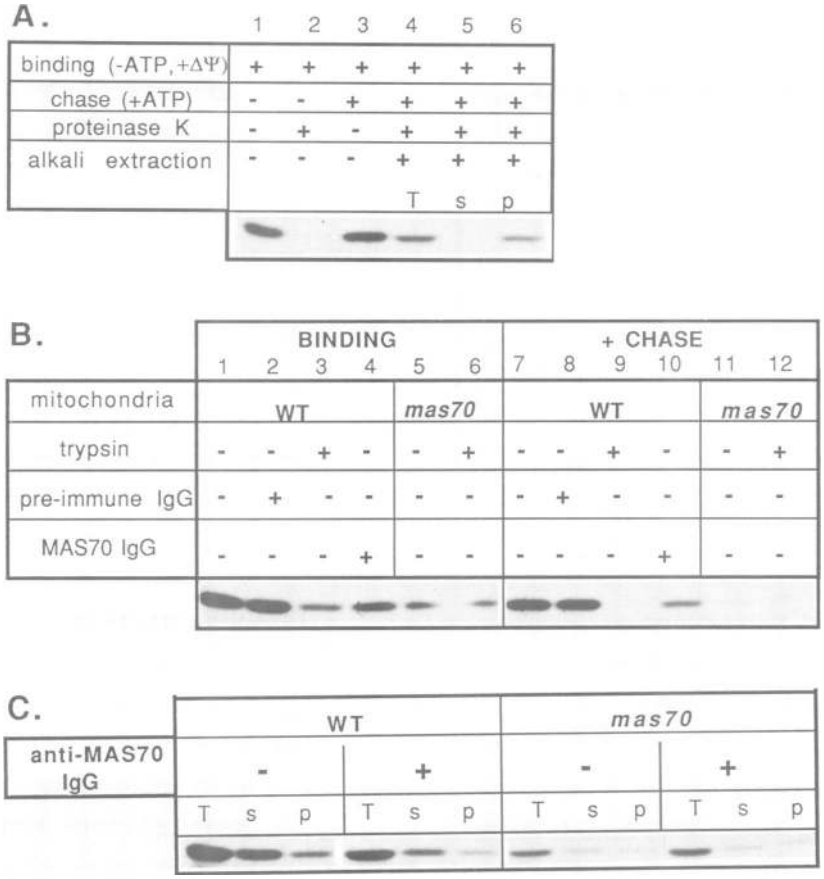

Fig. 5. MAS70 promotes binding of the ADP/ATP translocator precursor to the mitochondrial surface. (**A**) Reticulocyte lysate (45 μl) containing radiolabeled ADP/ATP translocator and isolated yeast mitochondria (120 μg protein) were depleted of ATP by apyrase (with lysate: 2.25 units for 10 min at 25°C; with mitochondria: 1.5 units for 10 min at 4°C in a final volume of 0.55 ml) and incubated together for 15 min at 25°C in a final volume of 0.6 ml of binding buffer (the translocator import buffer described in Materials and methods, but lacking creatine kinase, creatine phosphate and ATP). The mitochondria were then re-isolated by centrifugation, suspended in 0.6 ml of binding buffer and processed as follows. Lane 1, mitochondria were again sedimented and directly dissociated in SDS−sample buffer. Lane 2: mitochondria were incubated for 30 min at 0°C with 50 μg proteinase K/ml; the protease was stopped with 1 mM PMSF, the mitochondria were sedimented and dissociated in SDS−sample buffer. Lane 3: bound precursor was chased for 15 min at 25°C in the presence of 60 μg creatine kinase/ml, 4.5 mM creatine phosphate, 1 mM ATP, the mitochondria were re-isolated, and dissociated in SDS−sample buffer. Lane 4: mitochondria were chased (see lane 3), treated wtih proteinase K (see lane 2), re-isolated, mixed with 100 μg of carrier mitochondria, incubated for 20 min at 0°C in 200 μl of 0.1 M Na_2CO_3, pH 11.5, and divided into two equal aliquots. One aliquot was directly precipitated with 5% trichloroacetic acid (T = total); the other aliquot was centrifuged through a 50 μl cushion of 0.2 M sucrose, 0.1 M Na_2CO_3, pH 11.5 (+4°C; 15 min at 30 p.s.i.; Beckman Airfuge) to separate solubilized from integral membrane proteins. Proteins were then precipitated from the supernatant (s) with 5% trichloroacetic acid and dissociated into SDS−sample buffer (lane 5). The pellet (p) was directly dissociated in SDS−sample buffer (lane 6). All samples were analyzed by SDS−PAGE and fluorography. (**B**) Mitochondria from the *mas70* null mutant (*mas70*) or the wild-type parent (WT) were incubated for 30 min at 0°C without (−) or with (+) 10 μg trypsin/ml as described in Figure 4. After addition of soybean trypsin inhibitor to 1 mg/ml, mitochondria were sedimented and suspended to 5 mg/ml in 0.6 M sorbitol, 20 mM HEPES−KOH, pH 7.4. Mitochondria that had not been trypsin treated were then incubated for 40 min at 0°C with a 2.5-fold weight excess of either pre-immune IgGs or anti-MAS70 IgGs (see Figure 1). Trypsin-treated mitochondria were incubated identically, but without any IgGs. Mitochondria were then depleted of ATP with apyrase (see A) and 30 μg of ATP-depleted mitochondria were incubated for 15 min at 25°C with 15 μl of ATP-depleted reticulocyte lysate containing radiolabeled ADP/ATP translocator in a final volume of 200 μl binding buffer. Each sample was then divided into two equal aliquots and each aliquot was centrifuged to sediment the mitochondria. BINDING (lanes 1−6): the sedimented mitochondria were directly dissociated in SDS−sample buffer. +CHASE (lanes 7−12): the sedimented mitochondria were suspended in 200 μl translocator import buffer, chased for 15 min at 25°C as described in (A), treated with proteinase K (see A), re-isolated, and dissociated in SDS−sample buffer. (**C**) Mitochondria from the *mas70* null mutant (*mas70*) or its wild-type parent (WT) were incubated without (−) or with (+) a 2.5-fold weight excess of anti-MAS70 IgGs as in Figure 1. They were then suspended to 200 μg/ml in 200 μl of translocator import buffer, and incubated first for 5 min at 25°C in the presence of 0.1 μg valinomycin/ml and 12.5 μg oligomycin/ml and then for 15 min at 25°C with 20 μl of translation mixture containing radiolabeled ADP/ATP translocator. Finally, all samples were treated with proteinase K, extracted with Na_2CO_3, pH 11.5, and analyzed by precipitation with 5% trichloroacetic acid, SDS−PAGE and fluorography (see A). T, total protease-resistant radiolabeled ADP/ATP translocator; s and p, alkali-extractable and alkalki-nonextractable fraction of T.

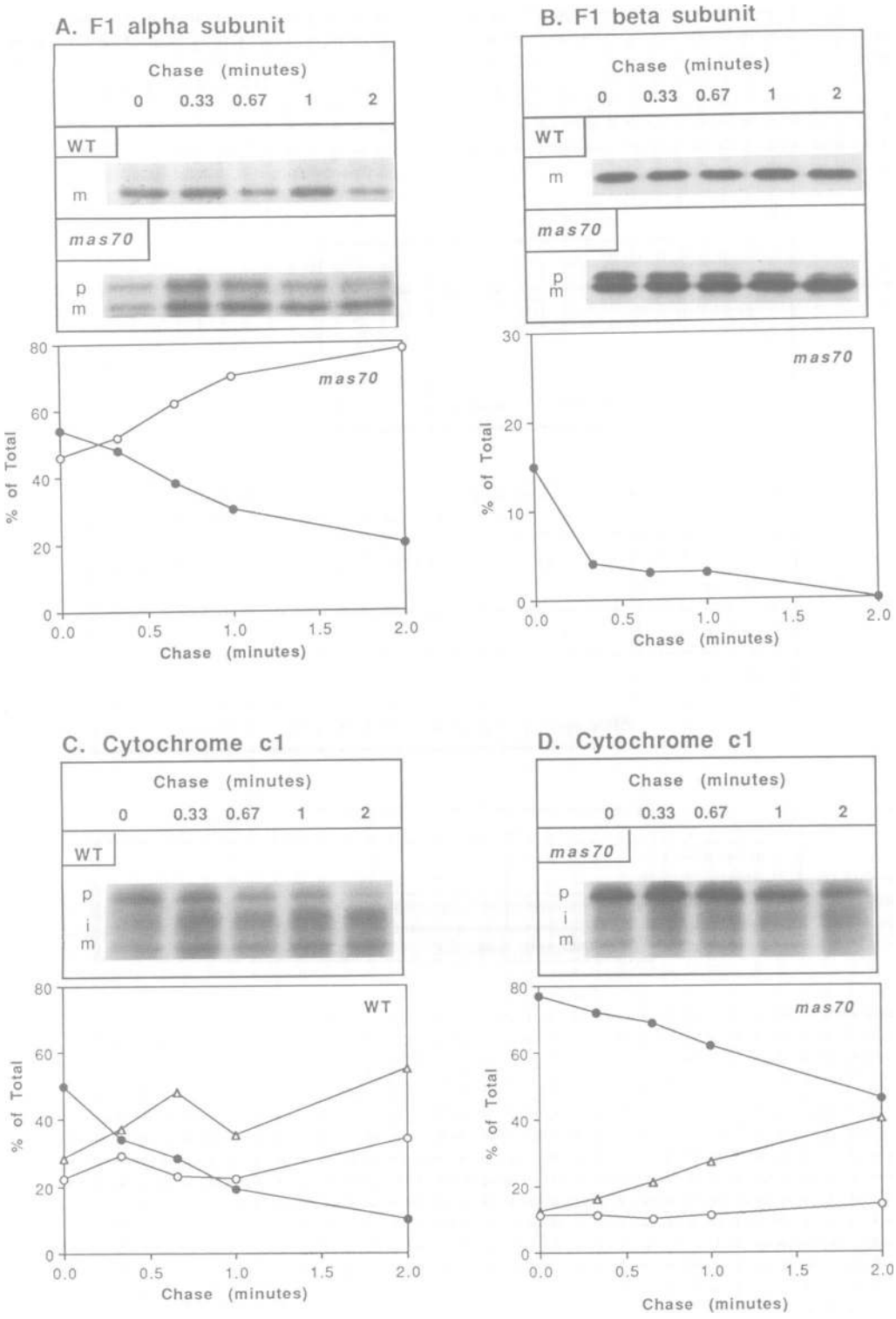

Fig. 6. MAS70 accelerates mitochondrial protein import *in vivo*. The *mas70* null mutant and its wild-type parent were grown in semisynthetic medium (Daum *et al.*, 1982) containing 2% glucose to an OD_{600} of 1.5, collected by centrifugation, resuspended in 40 mM KP_i, 1% glucose, pH 5.5, to an OD_{600} of 10, and pulse-labeled with [^{35}S]methionine (100 Ci/mmol; 0.6 mCi/ml) for 2.5 min at 30°C. Labeling was stopped by adding 10 mM unlabeled L-methionine and 0.1 mg cycloheximide/ml and incubation at 30°C was continued. At the times indicated in the figure, aliquots were removed, the cells were collected by centrifugation, and total cell proteins were extracted (Yaffe and Schatz, 1984) and subjected to immunoprecipitation with rabbit antisera against the proteins indicated in the figure. Immunoprecipitates were collected onto protein A–Sepharose beads and analyzed by SDS–PAGE and fluorography. The amounts of radioactivity in mature and precursor forms were quantified by scanning the fluorograms and are expressed as percentage of the total radioactivity recovered in mature and precursor forms. (**A**) F_1-ATPase α-subunit; (**B**) F_1-ATPase β-subunit; (**C**) and (**D**), cytochrome c_1 (C, wild-type; D, *mas70* mutant). Upper parts: fluorograms; p, i and m, precursor, intermediate and mature forms respectively. Lower parts: quantification of fluorograms. Filled circles, precursors; open triangles, intermediate form; open circles, mature form.

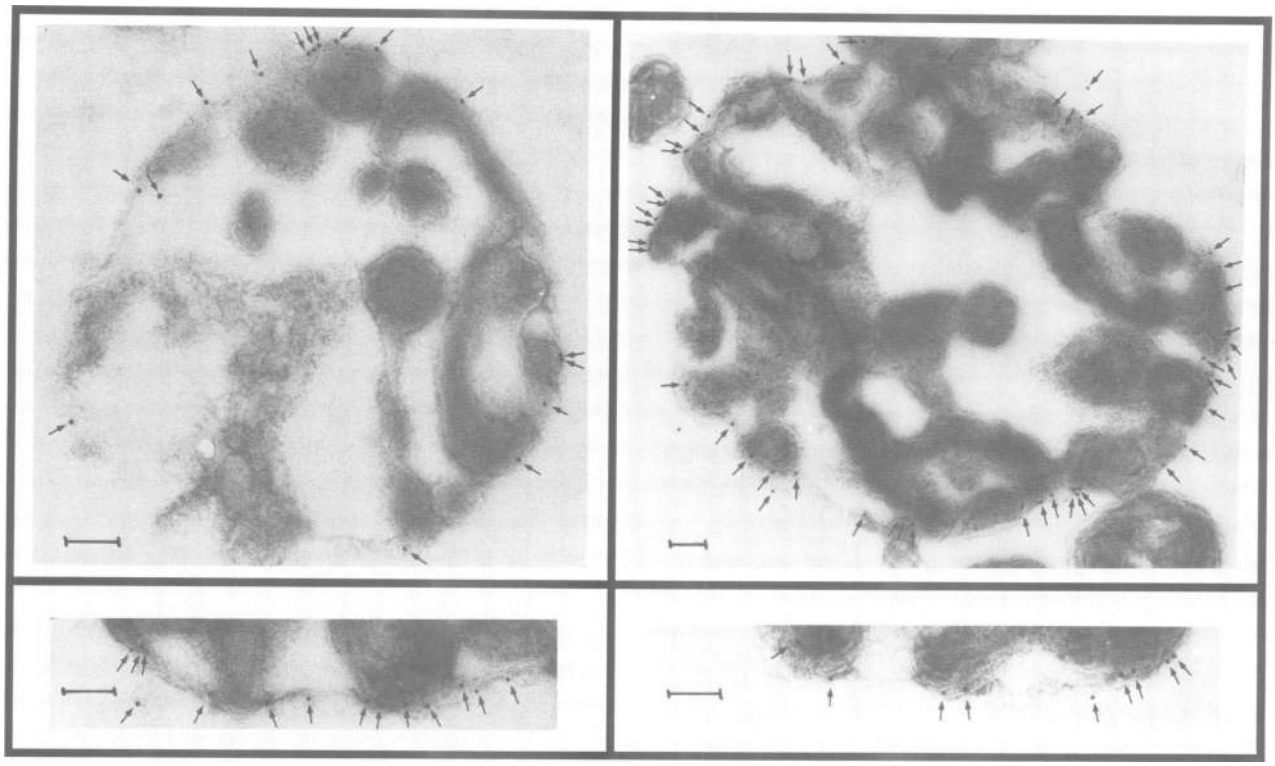

Fig. 7. Visualization of MAS70 in wild-type mitochondria by immune electron microscopy. Isolated wild-type mitochondria were analyzed by cryosectioning, treatment with affinity-purified anti-MAS70 IgG (Materials and methods) and protein A linked to 9 nm gold particles, and contrasting wtih 1% ammonium molybdate/0.02% methyl cellulose essentially as described (Griffiths *et al.*, 1983, 1984, 1990). Bars: 0.1 μm.

potential, import of the ADP/ATP translocator is interrupted at a later stage (Figure 5C). This 'step 3 intermediate' (Pfanner and Neupert, 1987) is resistant to moderate concentrations of proteinase K and can be solubilized from the membranes by high pH. The subsequent stages are only initiated if the inner membrane is energized; they involve insertion into the inner membrane and assembly to the dimeric mature translocator in the inner membrane. This fully inserted stage 5 intermediate is resistant to even high proteinase K concentrations and to extraction by high pH.

When the radiolabeled ADP/ATP translocator was presented to de-energized mitochondria, anti-MAS70 IgGs strongly inhibited formation of the 'stage 3' intermediate as tested by resistance to moderate proteinase K concentrations (Figure 5C, WT, lanes T) and extractability at high pH (WT, lanes s). De-energized mitochondria of the *mas70* null mutant accumulated significantly less of this intermediate, and accumulation was essentially unaffected by anti-MAS70 IgGs.

MAS70-free mitochondria are thus qualitatively different from MAS70-containing wild-type mitochondria: while they can import the ADP/ATP translocator to its fully assembled stage 5 form (data not shown), they cannot form a productive import intermediate in the absence of ATP. They also show a diminished capacity to form a later (stage 3) intermediate in the absence of a membrane potential and the presence of ATP.

While the precise role of MAS70 in the import pathway remains to be determined, the present data suggest that MAS70 mediates one of the earliest import steps before the potential- and ATP-dependent insertion of the precursor into the mitochondrial membranes.

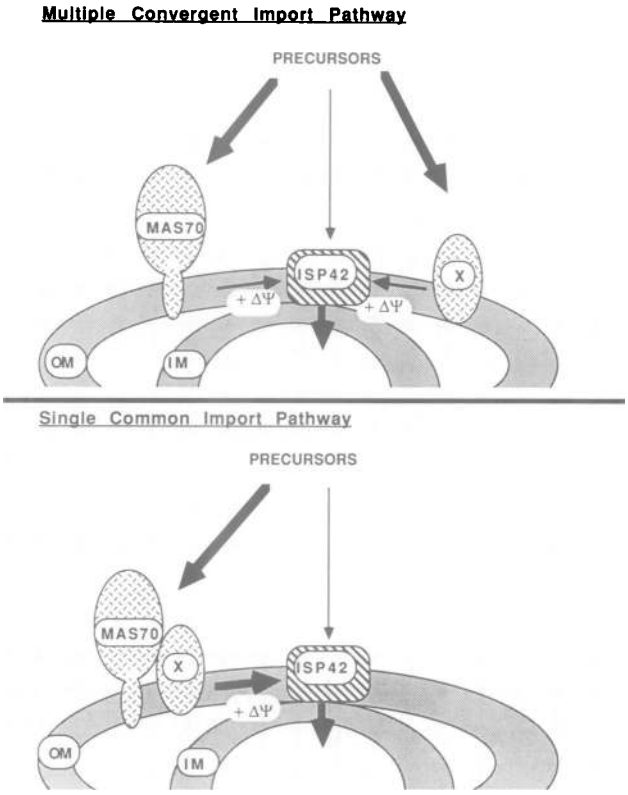

Fig. 8. Possible functions of MAS70 in mitochondrial protein import in yeast. OM and IM, outer and inner membrane, respectively; X, receptor-like protein functioning in addition to MAS70; ISP42, outer membrane protein mediating a later stage of import (Vestweber *et al.*, 1989). Thick arrows signify main pathway, thin arrows alternative, slower pathways. See text for further details.

MAS70 mediates mitochondrial protein import in vivo

All of the data mentioned so far were obtained by studying import of precursors into isolated mitochondria. Although *in vitro* import systems have proved very useful in the past, they may not always reflect the kinetics and rate-limiting steps of mitochondrial protein import *in vivo*. This is an important consideration when studying a component that is not essential for protein import, but only increases its rate.

The availability of a *mas70* null mutant allowed us to test the role of MAS70 in mitochondrial protein import *in vivo*. We had shown earlier that pulse-labeling of intact wild-type yeast cells for a few minutes leads to the transient appearance of the precursor form of the F_1-ATPase β-subunit and that this precursor is quickly chased to the mature form (Reid and Schatz, 1982; Yaffe and Schatz, 1984). Generation of the mature form during the chase is blocked by uncouplers of oxidative phosphorylation and thus reflects translocation into the mitochondria (Yaffe and Schatz, 1984).

After a 2.5 min pulse with labeled methionine, wild-type cells have already imported and processed all of the labeled F_1-ATPase α-subunit (Figure 6A); under the same conditions, the *mas70* mutant accumulates 54% of this protein as precursor and converts it to the mature form with a half-time of $\sim$1.6 min. This implies that mitochondrial import of this precursor is at least 2-fold slower in the MAS70-free cells. With the precursor to cytochrome c_1, import is slowed down by a factor of 3 (Figure 6C, D; half-times for processing: 0.75 min for the wild-type and 2.5 min for the *mas70* null mutant). Similar delays in processing are seen with the precursor of the F_1-ATPase β-subunit (Figure 6B) as well as with the precursors of the matrix-located 60 kd heatshock protein and cytochrome oxidase subunit IV (not shown). Thus, deletion of MAS70 from the cells slows, but does not completely block import of these precursors into mitochondria *in vivo*. This agrees with our *in vitro* data and the observation by Riezman *et al.* (1983c) that maturation of the F_1-ATPase β-subunit is 2-fold slower in the *mas70* null mutant than in wild-type cells. As mitochondria isolated from the *mas70* null mutant have the same coupling efficiency as the corresponding wild-type mitochondria (see above), this retardation of import is probably not an indirect effect, but caused by an alteration of the mitochondrial protein import machinery.

Distribution of MAS70 on the mitochondrial surface

MAS70 is a marker protein for the yeast mitochondrial outer membrane (Riezman *et al.*, 1983a; Hase *et al.*, 1984). In order to learn more about its distribution on the mitochondrial surface, we visualized MAS70 in mitochondria from wild-type cells and the *mas70* null mutant by immune electron microscopy. As expected, wild-type mitochondria are strongly labeled (Figure 7) whereas mitochondria of the *mas70* null mutant reveal only about one gold particle per mitochondrial profile (not shown). In wild-type mitochondria, gold particles are almost exclusively found in association with the outer membrane (Figure 7). While they are clearly present in those regions of outer membrane that are not in contact with the inner one (see the high magnification insets in the lower part of the figure), they are enriched in the contact region. However, the degree of enrichment cannot be quantified accurately because most of the yeast mitochondrial outer membrane is closely apposed to the inner membrane.

Discussion

The function of MAS70 in mitochondrial protein import

Four observations suggest that MAS70 is a component of the mitochondrial protein import machinery. First, IgGs monospecific for MAS70 inhibit import of several precursor proteins into isolated yeast mitochondria. This inhibition affects different precursors differently and is not observed with MAS70-free mitochondria, suggesting that the IgGs do not affect mitochondrial integrity or import efficiency non-specifically. Also, anti-porin IgGs do not inhibit even though they bind strongly to the mitochondrial surface (Ohba and Schatz, 1987); this makes it unlikely that the observed inhibitions are merely caused by a general coating of the mitochondria with IgGs. Second, a similar inhibition is seen upon removing MAS70 from the mitochondria by mild trypsinization. As this procedure does not significantly inhibit import of alcohol dehydrogenase III or the COXIV–DHFR fusion protein, it probably does not degrade other components involved in mitochondrial protein import. Third, mitochondria from a *mas70* null mutant import several precursors more slowly than wild-type mitochondria. This is probably not an isolation artefact, as *mas70* mitochondria have the same respiratory control ratio, and import at least two precursors as rapidly as wild-type mitochondria. Fourth, disruption of the *MAS70* gene strongly decreases the rate of mitochondrial protein import *in vivo*.

While each of these arguments may be challenged, the strength of the present evidence derives from the diversity of experimental approaches that all yield the same answer.

Some of the results reported here offer some insight into how MAS70 stimulates protein import into mitochondria. With at least one precursor, it stimulates an early import step before the ATP- and potential-dependent insertion reaction(s) (Figure 5) and it accelerates import of several precursors *in vitro* and *in vivo* (Figures 3 and 6). We have also found that a complex between bound ADP/ATP translocator precursor and MAS70 can be solubilized from

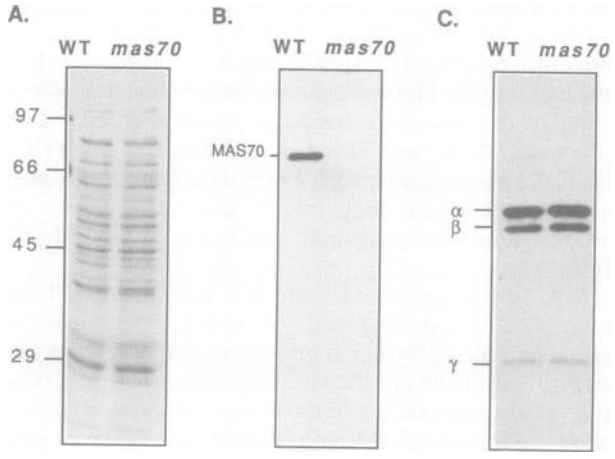

Fig. 9. The antiserum to MAS70 is monospecific. (**A**) Mitochondria from the *mas70* null mutant (*mas70*) or is wild-type parent (WT) (30 μg protein/lane) were analyzed by SDS–PAGE and staining with Coomassie Blue. (**B**) and (**C**) Unstained duplicates of the gel shown in (A) were subjected to immune blotting with rabbit antiserum against MAS70 (B) or rabbit antiserum against the α, β and γ-subunits of F_1-ATPase β-subunit (C).

mitochondria with Triton X-100 (V.Hines, unpublished). MAS70 is only linked to the outer membrane by a short uncharged amino-terminal domain; this makes it unlikely that it forms a transmembrane import channel by itself, although it could contribute to such a channel in combination with partner proteins. A search for such partner proteins is currently under way. We favor the view that MAS70 accelerates protein import by collecting precursors on the mitochondrial surface and delivering them to the transmembrane import machinery. Such a receptor-like role is also compatible with the fact that MAS70 is not uniquely present in sites of contact between the two mitochondrial membranes. Our data are compatible with two different models which need not be mutually exclusive (Figure 8). According to the first model, MAS70 is one of several different mitochondrial surface receptors with partly overlapping ligand specificities which bind different precursors and deliver them to the mitochondrial import channel (multiple convergent pathways).

The 19 kd outer membrane protein of *Neurospora crassa* mitochondria (Söllner *et al.*, 1989) could be such an additional receptor (represented by X in Figure 8). According to the second model, the different precursors are all imported by the same linear series of steps, one of which is accelerated by MAS70 (single common pathway). According to this second model, MAS70 could function in association with one or more additional surface proteins (represented by X in the figure) in facilitating early import steps. Without MAS70, one of these partner proteins would function at reduced efficiency. A variant of the single common pathway model (which is not shown in the figure) would be that MAS70 functions without being bound to one of the subsequent components and that precursors can also interact directly, albeit more slowly, with one of the subsequent components (X) in the linear pathway. In either case, the alternative import pathway observed with MAS70-free mitochondria involves a component sensitive to 100 μg (but not to 10 μg) trypsin/ml. This component is different from ISP42 because ISP42 is unaffected by either of these trypsin levels. This conclusion is supported by the observation that import into MAS70-free mitochondria can be inhibited by anti-outer membrane IgGs which do not significantly recognize ISP42 (V.Hines and U.Krieg, unpublished). The models shown in Figure 8 also propose that MAS70 acts before ISP42 since ISP42 can only be crosslinked to a precursor protein that has already undergone potential-dependent insertion across both mitochondrial membranes (Vestweber *et al.*, 1989). Most likely, ISP42 is a component of the transport channel.

What are the common features of precursors which are recognized by MAS70? These features could reside in the targeting sequence or any other region of the precursor molecule. Work with *Neurospora* mitochondria has suggested that the precursor of the ADP/ATP translocator (which lacks a cleavable presequence) utilizes an 'import receptor' that differs from that used by precursors with cleavable presequences (Zwizinski *et al.*, 1984; Pfanner and Neupert, 1987). This implies that the putative receptor for the ADP/ATP translocator in *Neurospora* mitochondria recognizes a unique feature of that precursor. In contrast, the present evidence with yeast shows that MAS70 facilitates import of the ADP/ATP translocator as well as that of precursors with cleavable presequences, both *in vivo* and *in vitro*. The features recognized by MAS70 are thus unknown. It is even possible that MAS70 does not interact with the precursors themselves, but with a cytosolic protein which binds to precursors and targets them to the pathway which is accelerated by MAS70. Gene fusion experiments should help to answer some of these questions.

Other functions of MAS70?

MAS70 belongs to a protein family characterized by several 34-residue repeats. This tetratrico peptide repeat family includes proteins involved in mitosis, transcription control and neurogenesis (Sikorski *et al.*, 1990; M.S.Buguski, personal communication). At present it is difficult to identify a common function of these diverse proteins. Of course, there may be no common function, the repeat motif merely attesting to a common ancestor protein. Alternatively, the known role of many of these proteins (particularly those controlling cell division) could be rationalized by postulating an interaction with the cytoskeleton. The abundance of MAS70, and its disposition on the surface of the outer membrane, make it an attractive candidate for a link between mitochondria and the cytoskeleton. The 34-residue repeats of MAS70 are entirely contained within its cytosolically exposed 60 kd domain; deletions within this domain invariably interfere with MAS70 function (Hase *et al.*, 1984). Guiding proteins into mitochondria may thus not be the only role of MAS70 in the yeast cell.

Materials and methods

Yeast strains

Unless noted otherwise, mitochondria were isolated from the wild-type *Saccharomyces cerevisiae* strain D273-10B (*MATα*; ATCC 25657). The *mas70* disruption mutant (*MAS70*::*URA3*; termed HR-1) and its wild-type parent SF747 (*MATα gal2 his4 leu2 ura3*) have been described (Riezman *et al.*, 1983c).

Cell growth and isolation of mitochondria

Unless specified otherwise, cells were grown to the early stationary phase in semisynthetic medium containing 2% Na-lactate and 0.1% glucose (initial pH = 5.5) (Daum *et al.*, 1982). Mitochondria were isolated from spheroplasts (Daum *et al.*, 1982).

Import assays

Either freshly prepared mitochondria, or mitochondria which had been stored at −70°C (Kozlowski and Zagorski, 1988) were used. However, the experiments shown in Figures 2 and 4 were carried out with freshly isolated mitochondria in order to facilitate comparison between wild-type and *mas70* mitochondria. The import buffer was varied for the different precursors analyzed. For the F_1-ATPase β-subunit, ADH III, cytochrome b_2, or COXIV−DHFR, it contained 0.6 M sorbitol, 10 mM HEPES−KOH pH 7.4, 2.5 mM unlabeled L-methionine, 40 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM KP_i, 1 mM dithiothreitol, 2 mg fatty acid-free bovine serum albumin/ml, 60 μg/ml creatine kinase, 4.5 mM creatine phosphate and 1 mM ATP (buffer A). For import of porin, the serum albumin concentration in buffer A was raised to 20 mg/ml. For import of the ADP/ATP translocator (translocator import buffer), the concentrations of KCl and serum albumin in buffer A were raised to 150 mM and 20 mg/ml respectively. Each import assay usually had 15 μg of mitochondrial protein and a final volume of 0.1 ml. The radiolabeled precursors were purified COXIV−DHFR (Eilers and Schatz, 1986) or authentic precursors synthesized in a nuclease-pretreated reticulocyte lysate by transcription/translation (Hurt *et al.*, 1984) from the corresponding plasmid-borne genes (see Acknowledgements) cloned behind either the phage T5 promoter (Stüber *et al.*, 1984) or the phage SP6 promoter (Melton *et al.*, 1984); they were added to 90 μl of the import assay in 10 μl aliquots. For each precursor, conditions were adjusted so that import was linear with time and the amount of mitochondria, unless noted otherwise. These conditions were as follows: for ADP/ATP translocator: 15 min at 10°C; for the F_1-ATPase β-subunit: 10 min at 30°C; for COXIV−DHFR: 10 min at 30°C; for ADH III: 10 min at 15°C; for cytochrome b_2 and porin: 10 min at 10°C. Import was stopped by adding valinomycin to 0.1 μg/ml. With the exception of cytochrome b_2 (see below), non-imported

precursors were then digested with proteinase K under the following conditions: ADP/ATP translocator and porin: 100 μg/ml, 30 min at 0°C; for all other precursors: 50 μg/ml, 30 min at 0°C. Proteinase K was inactivated by adding phenylmethylsulfonyl fluoride (PMSF) to 1 mM and incubation for 5 min at 0°C. The mitochondria were then re-isolated by centrifugation, and imported (i.e. protease-inaccessible) radiolabeled protein was assayed by SDS–PAGE, fluorography of the dried gels, and densitometry of the fluorograms (Daum *et al.*, 1982; Hurt *et al.*, 1984). For assaying import of cytochrome b_2, protease treatment of the mitochondria was omitted (see legend to Figure 1).

Miscellaneous

The preparation of rabbit antiserum against MAS70 has been described (Riezman *et al.*, 1983a). The antiserum recognizes only MAS70 in wild-type mitochondria and does not recognize any proteins in mitochondria from a *mas70* disruption strain (Figure 9). For immune electron microscopy, IgGs against MAS70 were affinity-purified against MAS70 immobilized on nitrocellulose sheets. Bound IgGs were eluted from the sheets with 0.1 M glycine, pH 2.5, and the solution was neutralized with Tris base and concentrated with a Centricon 30 concentrator (Amicon Corp., USA). Published procedures were used for SDS–PAGE (Daum *et al.*, 1982), immune blotting (Haid and Suissa, 1983), isolation of IgG by chromatography on protein A–Sepharose (Ey *et al.*, 1978), and measurement of protein (BCA procedure; brochure distributed by Pierce Chemical Co.).

Acknowledgements

We are grateful to Dr M.G.Douglas (University of North Carolina, Chapel Hill, USA) for the *AAC1* gene, to Dr G.A.Reid (University of Edinburgh, UK) for the genes encoding the precursors of cytochrome b_2 and the F_1-ATPase β-subunit, to Dr K.Mihara (Kumamoto University, Japan) for the porin gene, to B.Marshallsay, W.Oppliger and G.Walker for excellent technical assistance, to B.Tschopp for typing the manuscript and to L.Müller and M.Jäggi for the artwork. This study was supported by grants from the Swiss National Science Foundation (3-26189,89), from the US Public Health Service (2 RO1 GM 37803) and from the Human Frontier Science Program Organization, as well as by postdoctoral fellowships (to V.H.) from the Jane Coffin Childs Memorial Fund for Medical Research and the US National Science Foundation.

References

Böhni,P., Gasser,S., Leaver,C. and Schatz,G. (1980) In Kroon,A.M. and Saccone,C. (eds), *The Organization and Expression of the Mitochondrial Genome*. Elsevier/North Holland, pp. 423–433.

Cheng,M.Y., Hartl,F.-U., Martin,J., Pollock,R.A., Kalousek,F. and Neupert,W. (1989) *Nature*, **337**, 620–625.

Daum,G., Böhni,P.C. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13028–13033.

Deshaies,R.J., Koch,B.D., Werner-Washbourne,M., Craig,E.A. and Schekman,R. (1988) *Nature*, **332**, 800–804.

Eilers,M. and Schatz,G. (1986) *Nature*, **322**, 228–232.

Ey,P.L., Prowse,S.J. and Jenkins,C.R. (1978) *Biochemistry*, **15**, 429–436.

Griffiths,G., Simons,K., Warren,G. and Tokuyasu,K. (1983) *Methods Enzymol.*, **96**, 466–485.

Griffiths,G., McDowall,A., Back,R. and Dubochet,J. (1984) *J. Ultrastruct. Res.*, **89**, 65–78.

Griffiths,G., Matteoni,R., Back,R. and Hoflack,B. (1990) *J. Cell Sci.*, **95**, 441–461.

Haid,A. and Suissa,M. (1983) *Methods Enzymol.*, **96**, 192–205.

Hartl,F.-U. and Neupert,W. (1990) *Science*, **247**, 930–938.

Hase,T., Riezman,H., Suda,K. and Schatz,G. (1983) *EMBO J.*, **2**, 2169–2172.

Hase,T., Müller,U., Riezman,H. and Schatz,G. (1984) *EMBO J.*, **3**, 3157–3164.

Hawlitschek,G., Schneider,H., Schmidt,B., Tropschug,M., Hartl,F.-U. and Neupert,W. (1988) *Cell*, **53**, 795–806.

Hurt,E.C., Pesold-Hurt,B. and Schatz,G. (1984) *EMBO J.*, **3**, 3149–3156.

Kozlowski,M. and Zagorski,W. (1988) *Anal. Biochem.*, **172**, 382–391.

Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.

Murakami,M., Pain,D. and Blobel,G. (1988) *J. Cell Biol.*, **107**, 2051–2058.

Ohba,M. and Schatz,G. (1987) *EMBO J.*, **6**, 2109–2115.

Ou,W., Ito,A. and Omura,T. (1989) *EMBO J.*, **8**, 2605–2612.

Pfanner,N. and Neupert,W. (1987) *J. Biol. Chem.*, **262**, 7528–7536.

Pfanner,N., Tropschug,M. and Neupert,W. (1987) *Cell*, **49**, 815–823.

Reid,G.A. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13062–13067.

Riezman,H., Hay,R., Gasser,S., Daum,G., Witte,C. and Schatz,G. (1983a) *EMBO J.*, **2**, 1105–1111.

Riezman,H., Hay,R., Witte,C., Nelson,N. and Schatz,G. (1983b) *EMBO J.*, **2**, 1113–1118.

Riezman,H., Hase,T., van Loon,A.P.G.M., Grivell,L.A., Suda,K. and Schatz,G. (1983c) *EMBO J.*, **2**, 2161–2168.

Sikorski,R.S., Buguski,M.S., Goeble,M. and Hieter,P. (1990) *Cell*, **60**, 307–317.

Smagula,C. and Douglas,M.G. (1988) *J. Biol. Chem.*, **263**, 6783–6790.

Söllner,T., Griffiths,G., Pfaller,R. Pfanner,N. and Neupert,W. (1989) *Cell*, **59**, 1061–1070.

Stüber,D., Ibrahimi,I., Cutler,D., Dobberstein,B. and Bujard,H. (1984) *EMBO J.*, **3**, 3143–3148.

Verner,K. and Schatz,G. (1988) *Science*, **241**, 1307–1313.

Vestweber,D., Brunner,J., Baker,A. and Schatz,G. (1989) *Nature*, **341**, 205–209.

Yaffe,M.P. and Schatz,G. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4819–4823.

Yang,M., Jansen,R.E., Yaffe,M.P. and Schatz,G. (1988) *EMBO J.*, **7**, 3857–3862.

Zwizinski,C., Schleyer,M. and Neupert,W. (1984) *J. Biol. Chem.*, **259**, 7950–7956.

Received on June 5, 1990; revised on June 29, 1990