Mas37p, a Novel Receptor Subunit for Protein Import into Mitochondria

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Abstract. By screening a collection of Saccharomyces cerevisiae mutants temperature sensitive for growth on a nonfermentable carbon source, we have isolated a gene (termed MAS37) which encodes a novel receptor for protein import into mitochondria. Mas37p is a 37-kD outer membrane protein with two putative membrane-spanning regions. Inactivation of the MAS37 gene renders cells temperature-sensitive for respiration-driven growth, inhibits import of precursors into isolated mitochondria, and is synthetically lethal with a deletion of one of the genes encoding the import receptors Mas70p or Mas20p. Inactivation of Mas37p with specific antibodies inhibits import of different precursors to different extents; the precursor specificity of Mas37p resembles that of the previously described import receptor Mas70p. Mas70p and Mas37p form a 1:1 complex in detergent extracts of mitochondria and overexpression of one protein enhances that of the other. We suggest that the Mas37p/Mas70p heterodimer functions as a receptor for protein import into yeast mitochondria and that the mitochondrial receptor system consists of hetero-oligomeric subcomplexes with distinct binding activities, but overlapping precursor specificities.

I MPORT of precursor proteins from the cytosol into mitochondria is effected by a set of cytosolic and mitochondrial proteins that together constitute the mitochondrial protein import machinery (Schatz, 1993). The initial binding of precursors to the mitochondrial surface is mediated by outer membrane proteins termed "import receptors." These receptors are distributed over the entire mitochondrial surface, and deliver the bound precursors to "import sites" where the import channels in the two mitochondrial membranes are closely apposed. The existence of import receptors was first inferred from the observation that treatment of intact mitochondria with low levels of protease abolished binding and import of precursors (Riezman et al., 1983a; Zwinzinski et al., 1984). Studies with Saccharomyces cerevisiae and Neurospora crassa have identified two mitochondrial protein import receptors: a 20-kD protein (termed Mas20p in yeast and MOM19 in N. crassa), and a 70-kD protein (termed Mas70p in yeast and MOM72 in N. crassa) (Söllner et al., 1989, 1990; Hines et al., 1990; Ramage et al., 1993). The two receptors interact preferentially with different sets of precursors. Mas70p promotes import of the F1-ATPase β subunit, the ADP/ATP translocator and cytochrome c, but not that of artificial precursors containing dihydrofolate reductase (DHFR) as a passenger protein; Mas20p interacts with most precursors containing a cleavage presequence, including DHFR-containing fusion proteins (Hines et al., 1990; Becker et al., 1992; Hines and Schatz, 1993; Ramage et al., 1993; Moczko et al., 1994). However, the specificities of the two receptors appear to overlap to such an extent that neither by itself essential for mitochondrial protein import or cell viability (Riezman et al., 1983b; Ramage et al., 1993; Moczko et al., 1994). Deletion of both Mas20p and Mas70p in yeast is lethal (Ramage et al., 1993). Synthetic lethality is usually taken as evidence for physical or functional interaction between two proteins, supporting the common role of Mas70p and Mas20p in the import process.

We have now identified another yeast mitochondrial outer membrane protein encoded by a gene whose deletion is synthetically lethal with a deletion of MAS70. The protein (termed Mas37p) was found serendipitously, while screening yeast mutants which are defective in mitochondrial membrane composition. While a direct involvement of Mas37p in lipid metabolism or lipid import could not be defined, genetic data suggested a physical or functional interaction with the known mitochondrial protein import receptors Mas70p and Mas20p. Biochemical analysis showed that Mas37p is a novel receptor for mitochondrial protein import.

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whose precursor specificity resembles that of Mas70p. Mas37p and Mas70p can each support import to a limited extent by themselves, but the fully functional receptor appears to be a 1:1 complex of the two proteins. This complex can be isolated from detergent extracts of mitochondria.

Materials and Methods

Yeast Mutant Screening

288 temperature-sensitive pet mutants (Burk et al., 1976) were screened for defective phospholipid synthesis at restrictive temperature as follows: strains were grown in 5 ml YPGal for 16 h at 23°C and 37°C, harvested, and resuspended in 1 ml YPGal at a cell density of 10 mg dry cells/ml. After preincubation for 4 h at the respective temperature, cells were labeled for 1 h with 1 µCi 3P-labeled phosphate. Cells were sedimented by centrifugation, washed with ice-cold distilled water, resuspended in 50 µl MgCl₂ (0.034%) and rapidly frozen in liquid nitrogen. Lipids were extracted with 250 µl chloroform/methanol 2:1 (vol/vol), after homogenizing cells with glass beads. Aliquots of the lipid extracts containing 2.5 × 10⁷ dpm were subjected to two-dimensional thin-layer chromatography on silica gel H60 plates (Zinser et al., 1991). Chromatograms were autoradiographed and mutants with altered phospholipid labeling pattern at 37°C as compared to 23°C or to wild type were re-screened on a four times larger scale. Quantitative phospholipid analysis was as published (Broekhuyse, 1965).

Cloning of the MAS37 Gene and Construction of a Null Mutant

Yeast strain SG2-9C was derived from several back-crosses of the originally isolated pet3027+ mutant with wild-type strain w-303. The wild-type MAS37 gene was isolated by functional complementation of the ts pet phenotype of strain SG2-9C with a yeast genomic library in the shuttle vector YEpI3 (Nasmyth and Reed, 1980). Transformation was performed by the spheroplast method (Beggs, 1978) and Leu+-transformants capable of growing at 37°C on plates containing lactate as the sole carbon source were selected. Mitotic instability of the plasmid was analyzed by growing transformants in nonselective media for more than 10 generations before replicating to selective media.

Disruption of the gene was performed by inserting the yeast HIS4 gene into the HincII site of the coding region of the MAS37 gene, terminating the coding sequence after 354 nt. To prepare the Δmas37 strain (YSG1, MATα, rps2, leu2, his4, lys2, mas37::HIS4) the linear EcoRI/HindIII fragment containing the disrupted gene was transformed (Ito et al., 1983) into strain YTT84 (Lithgow et al., 1994a) and His+ transformants were selected. YTT84 is isogenic with the parental strain YTJB4 (Lithgow et al., 1994a) and His+ transformants were selected. Mitotic instability of the plasmid was analyzed by growing transformants in nonselective media for more than 10 generations before replicating to selective media.

Coimmunoprecipitation of Mas37p and Mas70p

IgGs were coupled to protein A-Sepharose with dimethyliminidate according to the manufacturer's instructions (Pierce Chem. Co., Rockford, IL). Samples of mitochondria (500 µg protein) were diluted with TX-100 buffer at a final concentration of 5 mg/ml protein. Solubilized mitochondrial proteins were separated by centrifugation at 55,000 rpm for 3 h (Optima TLX tabletop ultracentrifuge, Beckman) and allowed to diffuse at 4°C for at least 6 h before use. Mitochondrial pellets (500 µg protein) were resuspended in TX-100 buffer at a final concentration of 5 µg/ml protein. Solubilized mitochondrial proteins were separated by centrifugation at 55,000 rpm for 3 h (Optima TLX tabletop ultracentrifuge, Beckman). Fractions of 200 µl each were collected for immunoprecipitation or precipitation with 20% trichloroacetic acid in 80% acetone.

Miscellaneous

Published methods were used for SDS-PAGE, immunoblotting, measuring mitochondrial protein concentration by absorbance at 280 nm (Glick et al., 1992) and for extraction of membrane vesicles with Triton X-114 (Boutlier, 1981) or sodium carbonate (Fujiki et al., 1982). Scanning densitometry was used to quantify the results of the immunoblotting experiments.

Results

Isolation of the MAS37 Gene

In an attempt to isolate genes controlling mitochondrial

Phospholipid metabolism in yeast, we screened a collection of yeast mutants temperature sensitive for growth on nonfermentable carbon sources (ts pet mutants) for incorporation of 32P into cellular lipids at the restrictive temperature. The collection comprised 288 complementation groups (Burkl et al., 1976). One of the mutants, pet3027ts, was highly temperature sensitive for incorporation of 32P into cardiolipin and had lowered levels of cardiolipin and phosphatidylethanolamine if grown at the restrictive temperature.

Phospholipid metabolism in yeast, we screened a collection of yeast mutants temperature sensitive for growth on nonfermentable carbon sources (ts pet mutants) for incorporation of 32P into cellular lipids at the restrictive temperature. As cardiolipin is specific for mitochondria, and as cardiolipin and phosphatidylethanolamine are the only phospholipids made by yeast mitochondria, the pet3027ts mutation thus appeared to have a pleiotropic effect on mitochondrial function. The properties of this mutant will be described in detail elsewhere (S. Gratzer and S. Kohlwein, manuscript in preparation).

The wild-type allele of pet3027ts was isolated by transforming the mutant with a yeast genomic DNA library and selecting for transformant that could grow on nonfermentable carbon sources at 37°C. The complementing plasmid was characterized, its DNA insert was isolated and sub-cloned, and the complementing activity was localized to a 2.2-kb EcoRI/EcoRV fragment that potentially encoded a 32-residue protein of molecular weight 37,469 (Fig. 1). Because of its role in mitochondrial/assembly (see below), the gene was termed MAS37.

Hybridization of a labeled MAS37 fragment to total yeast genomic DNA and to DNA from individual yeast chromosomes (data not shown) indicated that MAS37 is a single-copy gene on chromosome XIII. It is flanked by RNAS14 (upstream; inverse orientation; Minivielle-Sebastian et al., 1991) and by FET3 (downstream; same orientation; Askwith et al., 1994).

**Phenotype of Cells Lacking a Functional MAS37 Gene**

Yeast cells in which more than 90% of the MAS37 open reading frame had been replaced by the yeast URA3 gene were viable and grew at wild-type rates on any carbon source at 23°C. At 37°C, they grew poorly on glucose and not at all.

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on nonfermentable carbon sources (data not shown). *MAS37* is thus not essential for viability, but essential for mitochondrial function at elevated temperature.

The phenotype of the *Mas37p*-deficient mutant resembled that of mutants lacking *Mas70p*, a receptor for protein import into mitochondria (Riezman et al., 1983b; Steger et al., 1990). To further study the role of *Mas37p*, we constructed yeast mutants which lacked not only *Mas37p*, but also one of the two known protein import receptors, *Mas70p* or *Mas20p*. Neither of these receptors is by itself essential for protein import or cell viability (Riezman et al., 1983b; Ramage et al., 1993; Moczko et al., 1994). In contrast, spores lacking *Mas37p* and either *Mas70p* or *Mas20p* germinated and formed microcolonies, but stopped growth as unbudded cells after 8–10 divisions. The doubly disrupted cells remained viable for several days as judged by staining with acridine orange and their mitochondria retained the ability to accumulate the mitochondria-specific styryl dye DASPMI; however, the mitochondria were highly vesiculated and the cytoplasm contained many vacuole-like structures (data not shown). The synthetic lethality caused by deleting *Mas37p* together with one of the known import receptors *Mas20p* and *Mas70p*, as well as the effect of these pairwise deletions on mitochondrial morphology, strongly suggested a role for *Mas37p* in mitochondrial protein import.

### *Mas37p* Is a Mitochondrial Outer Membrane Protein Exposed to the Cytosol

The NH₂-terminal region of *Mas37p* can potentially form a positively charged amphiphilic helix resembling a mitochondrial-targeting signal (Fig. 2 A). This region is followed by a putative transmembrane region; a second putative transmembrane region is found between residues 245 and 265 (Fig. 2 B). The import receptors characterized to date have a sequence motif FxKALxF that is present in the COOH-terminal turn of tetratricopeptide repeat helices: once in *Mas20p* and seven times in *Mas70p* (Ramage et al., 1993). The *Mas37p* sequence FSKLLYFtsl conforms to this motif. Apart from this motif, *Mas37p* shows no striking similarities to any known protein.

To determine the intracellular location of *Mas37p*, we fused the 209 COOH-terminal amino acids of *Mas37p* to *E. coli* trpE, expressed the fusion protein in *E. coli* and raised antibodies against the purified fusion protein. The antibodies detected a single 37-kD band in an extract of total proteins from wild-type yeast; this band was not seen with an extract from the *Δmas37* null mutant (Fig. 3 A, lane 10). When this antiserum was used to test different subcellular fractions by immunoblotting, *Mas37p* proved to be highly enriched in mitochondrial outer membranes (Fig. 3 A).

To determine the topology of *Mas37p* in the outer membrane, intact mitochondria were treated with trypsin. Like *Mas70p*, *Mas37p* was completely degraded by 3.5 μg/ml trypsin. This low level of trypsin does not disrupt the outer membrane: the adenine nucleotide carrier, a protein of the inner membrane partially exposed to intermembrane space, was specifically fragmented by trypsin in mitoplasts, but was not significantly affected in intact mitochondria (Fig. 3 B). *Mas37p* is thus exposed on the mitochondrial surface.

### Inactivation or Deletion of *Mas37p* Inhibits Import of Some Precursor Proteins into Isolated Yeast Mitochondria

Pretreatment of mitochondria with antibodies monospecific for *Mas37p* inhibited the in vitro import of the adenine nucleotide carrier and of cytochrome *c* by 70% and 30%, respectively, but did not affect import of the HSP60 precursor (Fig. 4, wild-type). This inhibition pattern closely resembles those seen upon inhibition or deletion of the *Mas70p*/*MOM72* receptor (Hines et al., 1990; Steger et al., 1990; Hines and Schatz, 1993). Inhibition by the anti-*Mas37p* antiserum was specific as no inhibition was seen with mitochondria from a yeast mutant whose *MAS37* gene had been disrupted (Fig. 4, *Δmas37*).

Similarly, *Δmas37* mitochondria were defective in the import of the adenine nucleotide carrier (AAC), but imported the precursors of HSP60 and of the mitochondrial alcohol dehydrogenase isozyme (ADHIII) at wild-type rates (Fig. 5 A). Thus, inhibition or deletion of *Mas37p* inhibits import of selected precursor proteins in vitro.

Import of the unadenatured ADHIII precursor is not measurably accelerated by *Mas70p*, in contrast to import of the urea-denatured precursor. Import of DHFR-containing fusion proteins is *Mas70p*-independent regardless of protein conformation (Hines and Schatz, 1993; Wachter et al., 1994). Very similar results were obtained for *Mas37p*: *Δmas37* mitochondria are barely able to import the urea-denatured ADHIII precursor, but imported the urea-denat-
Figure 3. Mas37p is tightly bound to the mitochondrial surface. (A) Subcellular localization of Mas37p. 30 μg protein of each subcellular fraction was analyzed by SDS-PAGE and immunoblotting for porin (outer mitochondrial membrane), the adenine nucleotide carrier (AAC, inner mitochondrial membrane) and Mas37p. Lane 1, cytosol; lane 2, plasma membrane; lane 3, microsomes sedimented at 30,000 g; lane 4, microsomes sedimented at 40,000 g; lane 5, microsomes sedimented at 100,000 g; lane 6, total mitochondria; lane 7, mitochondrial outer membrane; lane 8, mitochondrial "contact sites" (Pon et al., 1989); lane 9, mitochondrial inner membrane; lane 10, mitochondria prepared from Δmas37 cells; lane 11, mitochondria prepared from cells harboring the pet3027 mutation. (B) Topology of Mas37p in the mitochondrial outer membrane. Isolated mitochondria or mitoplasts (100 μg protein in a final volume of 100 μl) were treated with the indicated amounts of trypsin for 30 min on ice. Soybean trypsin inhibitor (1 μg) and PMSF (20 μg) were added and the samples analyzed by SDS-PAGE and immunoblotting with antisera against Mas70p (outer membrane marker), Mas37p and AAC (inner membrane marker).

Figure 4. Affinity-purified antibodies against Mas37p inhibit mitochondrial protein import. Mitochondria (10 μg protein) from wild-type cells (A) or Δmas37 cells (B) were pretreated with buffer (filled bars) or with 100 ng antibodies against porin (hatched bars), or with 100 ng antibodies against Mas37p (open bars) in a final volume of 200 μl for 30 min on ice. Import of the 60-kD heat-shock protein (HSP60) was assayed at 10°C for 3 min, that of the adenine nucleotide carrier (AAC) and of cytochrome c (cyt c1) at 14°C for 3 min. Under these conditions, import was linear with time and with the amount of mitochondria. The protease-protected forms of the imported proteins are shown; in the case of cytochrome c they include the intermediate and the mature form. The fluorograms (upper panels) were quantified by densitometry (lower panels). The amount of each precursor imported into mitochondria pretreated only with buffer was taken as 100%. This value corresponds to ~5% of the precursor added to each import reaction.

Mas37p and Mas70p Enhance Each Other's Overexpression

Because loss of Mas37p activity has a similar effect on mitochondrial protein import as loss of Mas70p activity, we investigated the possibility that the two proteins are partner subunits of an oligomeric receptor. We first checked whether Mas37p and Mas70p are present in a defined stoichiometry. Yeast cells transformed with a multicopy plasmid carrying either the MAS37 gene or the MASTO gene overexpressed the corresponding protein product two- to threefold (Fig. 6 A). However, when the cells were cotransformed with both genes, each protein was overexpressed seven- to eightfold.
Figure 5. Mitochondria lacking Mas37p have specific defects in protein import. (A) Mitochondria (20 μg protein in a final volume of 100 μl) from wild-type or Δmas37 cells were assayed for the import of the precursor to the 60-kD heat-shock protein (HSP60) at 10°C, for import of the mitochondrial isoform of alcohol dehydrogenase (ADHIII) at 12°C or for import of the adenine nucleotide carrier (AAC) at 14°C. At the indicated time points (min), import was stopped with carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) and the mitochondria were treated with proteinase K (50 μg/ml). After addition of PMSF to 200 μg/ml, the mitochondria were reisolated and analyzed by SDS-PAGE and fluorography. (B) The precursors of ADHIII and the fusion protein between subunit 9 of the F1F0-ATPase and dihydrofolate reductase (Su9-DHFR, Pfanner et al., 1987) were pretreated with 8 M urea (+ urea) or left untreated (− urea) and were then presented to either wild-type (■) or Δmas37 (Δ) mitochondria. Import was assayed at 15°C for 10 min and analyzed by SDS-PAGE and fluorography. The fluorographs were analyzed by densitometry, and the results are presented as a percentage of the total amount of each precursor in the assay. (C) Import of the adenine nucleotide carrier was assayed as described above. For the “bind and chase” analysis, the adenine nucleotide carrier was prebound to mitochondria (20 μg protein in a final volume of 100 μl, deenergized with CCCP) from wild-type (■) or Δmas37 (Δ) cells. After 5 min on ice, the mitochondria were isolated by centrifugation, washed in import buffer containing 20 mg/ml bovine serum albumin, 1 mM DTT, 1 mM ATP, and 2 mM NADH, and then resuspended in 100 μl of the same buffer. After incubation at 25°C for 20 min, the mitochondria were resolated, treated with 100 μg/ml proteinase K for 20 min on ice, washed in import buffer containing 1 mM PMSF and analyzed by SDS-PAGE and fluorography. The import and productive binding of Su9-DHFR was determined as described above, except that the import buffer was diluted twofold with 0.6 M sorbitol. To restore the membrane potential and initiate the chase reaction, the mitochondria were resolated and resuspended in the same buffer containing 2 mM NADH, 1 mM ATP, and 10 mM DTT instead of CCCP. All values are expressed as a percentage of the total amount of precursor added to the reaction.

In all cases, overexpression was measured relative to the level of two other outer membrane proteins, the import site protein Isp42p (Vestweber et al., 1989), and the import receptor Mas20p (Ramage et al., 1993); neither of these proteins was affected in any of the transformants. Also, overexpression of the import receptor Mas20p did not enhance overexpression of either Mas70p or Mas37p (data not shown). Simultaneous overexpression of Mas37p and Mas70p thus caused a specific and stoichiometrical increase in the level of both proteins, consistent with the idea that the two proteins are subunits of an oligomeric complex.

Overexpression of Both Mas37p and Mas70p Accelerates Import of Urea-denatured ADHIII into Isolated Mitochondria

Interaction with Mas37p and Mas70p is a rate-limiting step in the import of urea-denatured ADHIII. To test whether overexpressed Mas37p and Mas70p were functional, mitochondria from cells overexpressing both proteins were assayed for their ability to import urea-denatured ADHIII. At low temperature and at high dilution, import of the urea-denatured ADHIII precursor was relatively slow, but overexpression of both Mas37p and Mas70p accelerated import three- to fourfold (Fig. 6 B). Just as import of urea-denatured DHFR fusion proteins was not affected by the loss of Mas70p (Hines and Schatz, 1993) or Mas37p (Fig. 5 B), overexpression of Mas37p and Mas70p had little effect on the rate of import of Su9-DHFR (Fig. 6 B). Thus, increasing the level of both Mas37p and Mas70p in mitochondria accelerates the import of a subset of precursor proteins in vitro.

Mas37p and Mas70p Exist as a Heterodimer

The observations described so far suggest that Mas70p or Mas37p are partner subunits of an oligomeric import receptor. To test this possibility directly, we labeled yeast cells with radioactive sulfate, isolated the mitochondria, solubilized the membranes with the nondenaturing detergent TX-100, and separated the solubilized proteins on a detergent-containing sucrose gradient. Mas37p and Mas70p comigrated in the gradient (Fig. 7 A) and were both precipitated from the gradient fractions by antibodies monospecific for Mas70p (Fig. 7 B). The identity of the coimmunoprecipitated 37-kD protein was confirmed by blotting the immunoprecipitates with antibodies against a Mas37p fusion protein (Fig. 7 C). Quantitation of the immunoprecipitates by phosphorimage analysis, after correcting for the fact that
there are 16 sulfur-containing residues in Mas70p and 11 in Mas37p, indicated that Mas70p and Mas37p were precipitated in equimolar amounts.

Mas70p and Mas37p could be quantitatively solubilized with 1.0% octyl polyoxyethylene (data not shown) which is well below the critical micelle concentration of this detergent. We therefore sought to determine if the equimolar Mas37p/Mas70p complex was a heterodimer or a higher-ordered structure. Calibration of the octyl polyoxyethylene-containing gradients revealed that the Mas37p/Mas70p complex sedimented corresponding to a mass of \(~100-110\) kD (Fig. 8). This value is close to the sum of the sizes of each protein, suggesting that Mas37p and Mas70p form a heterodimer.

Discussion

Mas37p Functions as a Mitochondrial Protein Import Receptor

Until recently, only Mas70p (and its N. crassa homologue MOM72) and Mas20p (and its N. crassa homologue MOM19) were known to be receptors for protein import into mitochondria. Their identification as import receptors is based
Import Machinery

Mas37p Is a Novel Component of the Import Machinery

The pet3027Δ Δmas37 mutants display pleiotropic effects on mitochondrial function, such as reduced levels of mitochondrial made phospholipids, as indirect effects of the loss of a component of the protein import machinery. Mas37p shows no obvious amino acid sequence similarity to any known protein. A 38-kD protein (MOM38) is a component of the translocation complex in the outer membrane of N. crassa, but MOM38 is homologous to the yeast protein Isp42p (Baker et al., 1990; Kiebler et al., 1990).

Mas37p and Mas70p Function as a Heterodimer

The genetic and biochemical data reported here show clearly that Mas37p and Mas70p interact functionally and can form a heterodimer. We propose that the heterodimer is the functional receptor, although direct assays with the purified components are required to confirm this hypothesis. The hetero-

Figure 8. Mas37p and Mas70p form a heterodimer. Mitochondria (50 µg protein) were solubilized with 1.0% octyl-polyoxyethylene and the solubilized proteins were sedimented for 16 h in a sucrose gradient containing 1.0% octyl-POE instead of TX-100 (see Fig. 5). Migration of Mas37p, Mas70p and of the marker proteins alcohol dehydrogenase (homotrimer of 40-kD subunits), citrate synthetase (heterodimer of 47-kD subunits) and aconitate (monomer, 85 kD) was determined by immunoblotting of gradient fractions. As the density of octyl-POE is 1.0, its contribution to the sedimentation of detergent-bound proteins can be ignored.

on the following criteria: (a) They are outer membrane proteins whose cytosolic domains are degraded by the same low levels of protease that also inhibit protein import (Riezman et al., 1983a; Sölter et al., 1989; Kiebler et al., 1993; Lithgow et al., 1994a). (b) Antibodies monospecific for either of these proteins partly inhibit protein import into isolated mitochondria (Sölter et al., 1989, 1990; Hines et al., 1990; Ramage et al., 1993; Moczko et al., 1994). (c) Mitochondria lacking either Mas20p/MOM19 or Mas70p have defects in the import of specific precursor proteins, including the inability to form surface-bound import intermediates (Hines et al., 1990; Steger et al., 1990; Hines and Schatz, 1993; Harkness et al., 1994; Lithgow et al., 1994a; Moczko et al., 1994). Mas37p meets all of these criteria, and is thus a third receptor protein for mitochondrial protein import.

Molecular Organization of the Yeast Mitochondrial Receptor Complex

Mas20p did not copurify with either Mas37p or Mas70p under our experimental conditions. Upon more gentle solubilization, however, Mas70p and Mas20p can copurify as a complex which contains at least nine different subunits (Moczko et al., 1992; Sölter et al., 1992), and preliminary results with the yeast two-hybrid system suggest a direct interaction between the cytosolic domains of Mas20p and Mas70p (V. Haucke, unpublished observation). The combined results thus suggest that yeast mitochondria contain at least two hetero-oligomeric receptors with distinct, but overlapping precursor specificity: the Mas37p/Mas70p dimer described here, and Mas20p which probably forms a complex with the recently discovered subunit Mas22p (termed MOM22 in N. crassa; Kiebler et al., 1993; Lithgow et al., 1994b; M. Horst and T. Lithgow, manuscript in preparation). Based on these combined observations, we suggest that these two receptor complexes interact with each other in a dynamic fashion, in part through the cytosolic domains of Mas20p and Mas70p. Under most solubilization conditions, the resulting “super-complex” dissociates into its subcomplexes such as the Mas37p/Mas70p dimer described here.

Further evidence for an interaction between the receptor subcomplexes comes from the observation that the ts-petite phenotype of Δmas37 cells, and the lethal phenotype of Δmas37, Δmas70 cells, is at least partially due to an associated loss of Mas22p: in fact, if the level of Mas22p is maintained viable yeast can be recovered after deletion of any two of the three subunits Mas20p, Mas37p, and Mas70p (Lithgow et al., 1994b). As each of these doubly disrupted strains can grow on non-fermentable carbon sources, the mitochondrial receptor system remains at least partly functional without any two of these three subunits. Despite earlier predictions (Schneider et al., 1991), there is no evidence for a hierarchy of receptor function. Mas20p is not a “master receptor,” as its deletion has no obvious effect as long as nor-
chaperones in the cytosol, and do not need to interact with the or HSP60) do not need to remain associated with ATP-dependent gered at the mitochondrial surface.

Figure 9. Cooperation of receptors during protein import into mitochondria. Mitochondrial precursor proteins are made in the cytosol and the nascent polypeptides associate with chaperones (shaded). (A) Some precursors are retained on the chaperones; release from the chaperones requires extramitochondrial ATP and may be triggered at the mitochondrial surface (black) by the Mas37p/Mas70p receptor. This pathway appears to be followed by the adenine nucleotide carrier, cytochrome c1, the F1 ATPase β subunit and alcohol dehydrogenase III. (B) Other precursors (such as Su9-DHFR or HSP60) do not need to remain associated with ATP-dependent chaperones in the cytosol, and do not need to interact with the Mas37p/Mas70p receptor. These precursors bind to the "acid bristles" on Mas20p and Mas22p, probably through ionic interactions with the positively-charged mitochondrial targeting sequence.

Normal levels of its partner Mas22p can be maintained (Lithgow et al., 1994b). We suggest that there is a division of labor between the different receptor subunits and that the need for multiple subunits is dictated by the different properties of the various precursors that must be imported by mitochondria.

Precursor Specificity of the Different Subcomplexes

In vitro assays suggest that import of many precursors (such as those of HSP60 and Su9-DHFR) does not require extramitochondrial ATP and probably not cytosolic chaperones (Wachter et al., 1994). Import of these precursors is only little influenced by Mas37p/Mas70p (Hines and Schatz, 1993; this study), but requires Mas20p (Söllner et al., 1989; Ramage et al., 1993; Lithgow et al., 1994a; Moczko et al., 1994). Mas20p (Ramage et al., 1993; Moczko et al., 1994) and Mas22p (Lithgow et al., 1994b) have highly acidic regions which could readily bind the basic targeting sequences of mitochondrial precursors. The well-studied DHFR fusion proteins require Mas20p but not Mas37p/Mas70p for import, suggesting that their attached targeting sequence is both necessary and sufficient for interacting with Mas20p. Binding of these precursors to Mas20p (and perhaps also to the Mas20p/Mas22p dimer) depends on an electrostatic interaction between an acidic surface of Mas20p (or its complex with Mas22p) and the positively-charged presequence (Hauke et al., 1995).

In contrast, the precursors of ADHIII, the adenine nucleotide carrier, cytochrome c1, and of other proteins requiring extramitochondrial ATP for import appear to rely largely on cytosolic chaperones for delivery to mitochondria (Wachter et al., 1994). These precursors interact with Mas20p (Söllner et al., 1992; Ramage et al., 1993; Moczko et al., 1994) but also with Mas37p/Mas70p (Hines et al., 1990; Hines and Schatz, 1993; this study). Release of the ADHIII precursor from cytosolic chaperones requires ATP, but bypassing this coordinated release by unfolding the precursor with 8 M urea makes interaction with the Mas37p/Mas70p receptor a rate-limiting step in the import pathway.

Fig. 9 presents a model that summarizes these various ideas. The two classes of mitochondrial precursors depicted in Fig. 9 probably represent two ends of a spectrum; many precursors might normally interact with the Mas37p/Mas70p receptor, yet still be imported at near normal rates in the absence of one of its subunits. The model depicts two discrete, but heterodimeric receptors which are loosely bound to each other within a larger hetero-oligomeric complex.

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