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.

IMPORT 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., 1983*a*; 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 F_1-ATPase β subunit, the ADP/ATP translocator and cytochrome c_1, but not that of artificial precursors containing dihydrofolate reductase (DHFR)[1] 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., 1983*b*; 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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The sequence of the *MAS37* gene has been submitted to the EMBL database and is available under the accession number X62565.

1. *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; DASPMI, 4-(4-dimethylaminostyryl)-*N*-methylpyridinium iodide; TX-100, Triton X-100.

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 (Burkl 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 ^{32}P-labeled phosphate. Cells were sedimented by centrifugation, washed with ice-cold distilled water, resuspended in 50 μl $MgCl_2$ (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 2.5×10^4 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 rescreened 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*^{ts} mutant with wild-type strain w303. 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 YEpl3 (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 replica-plating 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 (YSGI, *MATα, ura3, leu2, his4, lys2, mas37::HIS4*), the linear EcoRI/HindIII fragment containing the disrupted gene was transformed (Ito et al., 1983) into strain YTJB4 (Lithgow et al., 1994*a*) and His$^+$ transformants were selected. YTJB4 is isogenic with the *Δmas20* and *Δmas70* strains previously reported (Ramage et al., 1993). The phenotype of the *HIS4* disruptant was identical to that of the original *pet3027*^{ts}. Correct integration of the *HIS4* gene at the *MAS37* locus was established by Southern and Northern blotting, and genetic analysis. Diploids generated by crossing *pet3027*^{ts} to *Δmas37* cells were unable to grow on lactate medium at 37°C.

Yeast Cultures and Transformation

The plasmids used for overexpression experiments were: p70 (YEplac181 digested with BamHI, into which was ligated the entire *MAS70* gene; Ramage et al., 1993); p37 (YEp352 was digested with SmaI and EcoRI, into which was ligated the entire *MAS37* gene); p20 (YEplac195 cut with BamHI, into which was ligated the entire *MAS20* gene; Ramage et al., 1993). Published methods were used for the preparation of total cell extracts (Daum et al., 1982; Lithgow et al., 1994*a*), isolation of total membranes, mitochondria and microsomal fractions (Zinser et al., 1991), purity of membrane fractions by immunoblotting using antisera against a microsomal 40-kD protein, porin (outer mitochondrial membrane) and the adenine nucleotide carrier (inner mitochondrial membrane) (Daum et al., 1982), storage of mitochondria (Glick et al., 1992), preparation of mitoplasts by osmotic shock (Glick et al., 1992) and isolation of mitochondrial outer membrane vesicles (Ramage et al., 1993).

Fluorescence Microscopy

Yeast mitochondria were stained with 25 μg/ml of the potential-sensitive dye 4-(4-dimethylaminostyryl)-*N*-methylpyridinium iodide (DASPMI, Bereiter-Hahn, 1976; McConnell et al., 1990). Fluorescence was recorded by confocal laser scanning microscopy, using a Leica TCS 4D confocal microscope, set up for FITC fluorescence. For microscopy, yeast cells were immobilized in 0.6% agarose and supplemented with complete medium in an aerated microscopy cell (to be described elsewhere). Microcolonies of double mutants were cut out of YPD plates after tetrad dissection, and agar blocks were incubated with a final concentration of 25 μg/ml DASPMI or 1.5 μg/ml acridine orange (Dawid, 1969) before fluorescence microscopy. In the presence of acridine orange, dead yeast cells are characterized by a bright red/orange fluorescence upon excitation with blue light.

In Vitro Import Assays

Except where otherwise noted, import of ^{35}S-labeled precursors into isolated mitochondria was as previously described (Lithgow et al., 1994*a*). Urea-denaturation of precursor proteins before import was as described by Rospert et al. (1994). Scanning densitometry of fluorograms was used to quantify the results of the import assays.

Affinity-purification of Anti-Mas37p Antibodies

The 1.05-kb HincII-EcoRI fragment encoding the 209 carboxy-terminal residues of Mas37p was subcloned into the *E. coli* expression vector pATH2. Antibodies were raised against the 61-kD TrpE-Mas37p fusion protein in rabbits. A 1.3-kb fragment of the *MAS37* gene, encoding 90% of Mas37p, was subcloned into the vector pMALc2 (New England Biolabs, Beverly, MA) and the 76.8-kD MBP-Mas37p fusion protein purified from *lon*-deficient *E. coli*. The purified fusion protein was coupled to CNBr-Sepharose beads and the antiserum was incubated with these beads overnight at 4°C. The antibodies were eluted from the washed beads as described by Scherer et al. (1992).

Separation of ^{35}S-labeled Proteins

Wild-type yeast (D273-10B) were grown overnight on sulfate-free media containing 2% lactate and 50 μCi/ml $Na_2[^{35}S]O_4$. The cells were harvested in mid-logarithmic phase for isolation of mitochondria. Sucrose step gradients (500 μl each of 20%, 15%, 10% and 5% (wt/vol) sucrose in Triton X-100 (TX-100) buffer: 1% TX-100, 20 mM Hepes-KOH, pH 7.4, 250 mM NaCl, 10% glycerol, 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 0.25 μg/ml elastinal, 5 μg/ml pepstatin, 0.5 mM PMSF) were prepared in 2-ml centrifuge tubes (Beckman Instrs., Inc., Fullerton, CA) 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 mg/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.

Coimmunoprecipitation of Mas37p and Mas70p

IgGs were coupled to protein A–Sepharose with dimethylpimelimidate according to the manufacturer's instructions (Pierce Chem. Co., Rockford, IL). Samples of mitochondria (500 μg protein) were diluted with TX-100 buffer to 1 ml, 50 μl of a 50% slurry of CL-4B Sepharose (Pharmacia LKB Nuclear, Gaithersburg, MD) was added and the mixture was incubated for 30 min at 4°C. The beads were removed by centrifugation, and the supernatants were mixed with 50 μl of a 50% slurry of the antibody protein A–Sepharose beads. After incubation for 3 h at 4°C, the beads were washed three times with TX-100 buffer, and proteins were eluted with 75 μl of SDS-containing sample buffer lacking reducing agent.

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 (Bordier, 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 $^{32}P_i$ into cellular lipids at the restrictive temperature. The collection comprised 288 complementation groups (Burkl et al., 1976). One of the mutants, *pet3027*^{ts}, was highly temperature sensitive for incorporation of $^{32}P_i$ into cardiolipin and had lowered levels of cardiolipin and phosphatidylethanolamine if grown at the restrictive temperature. As cardiolipin is specific for mitochondria, and as cardiolipin and phosphatidylethanolamine are the only phospholipids made by yeast mitochondria, the *pet3027*^{ts} 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 *pet3027*^{ts} 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 subcloned, and the complementing activity was localized to a 2.2-kb EcoRI/EcoRV fragment that potentially encoded a 328-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 *RNA14* (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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-780  GTTGCTCCTCCCATTTGTTGAATGGCTTCCACTGCTCTAAAAAATTGAGATATTCGTTCC  -721
-720  AAAAAGAAGATGATTTGGGTTCAAAAATTGCACACTTTTGCATAACTAGTTGGAATGCCT  -661
-660  TGACAATAACAGCTCTCGCCTCTTGTCCACCAGTAATTAAGTTGTTTTTTCTGCGTATGT  -601
-600  AGTCCAAATATGTTGACCAAAGAGATAGGTCATTATTTTCCAACTTGCCAGAAAGACATT  -541
-540  GAGCCAAAATCTTCTCAACAGTCTCAAATTCATCTCTTGCCAATTCACCCTTTAGTTGCA  -481
-480  AAGTCCACGCAGGTGAATAAAACGGGAATGTGTTATGAAATTGCTCGTATACTTCTCTCA  -421
-420  CCTTAGCGTATGACTCTTGAGTTTCCAAATATTGAATAAGCTGGAAGTATGATAAAATAT  -361
-360  TCGTGGGATTGTCTTTAATCCTTTCTCTAAGTCGTAGTTCATCTCCATGTATATTGTCAC  -301
-300  TAGGCTCTGCGACTTTGTCCGCAGAGGGATATAGTAAATCAGGAGTCGTAGAGCTGGACA  -241
-240  TATCTCTTGTTTGACTCTCCAGTTTGGCCTTTTATTTCGTTGTAGAATACTCTTTCATCA  -181
-180  CACAAGCTACACTTTTCTTCTTAATTATTACAAGGTAACCGACAATTTTGTCACGATTGT  -121
-120  TATCATTTTAAACGAAGTGGGAAATGTAATAAGAAATAAGCTATTTAGAATGGCTACTAT  -61
 -60  TTAATGCTGGAATTTGTATTTTTCAAATAAACGCAAAGCAAGGCTATTATCGAGTCAGTA  -1
   1  ATGGTTAAAGGTAGTGTGCATTTATGGGGAAAGGATGGCAAAGCATCGTTGATTTCAGTG  60
      M  V  K  G  S  V  H  L  W  G  K  D  G  K  A  S  L  I  S  V
  61  GACAGCATTGCGCTTGTATGGTTTATCAAGTTATGTACTTCAGAAGAGGCAAAGAGTATG  120
      D  S  I  A  L  V  W  F  I  K  L  C  T  S  E  E  A  K  S  M
 121  GTTGCGGGATTGCAGATCGTATTCTCAAACAATACAGACCTATCATCAGATGGAAAACTA  180
      V  A  G  L  Q  I  V  F  S  N  N  T  D  L  S  S  D  G  K  L
 181  CCAGTGTTAATTTTAGATAATGGAACGAAAGTATCTGGTTACGTGAACATCGTACAATTC  240
      P  V  L  I  L  D  N  G  T  K  V  S  G  Y  V  N  I  V  Q  F
 241  TTACATAAAAATATATGTACTAGCAAATATGAGAAAGGTACGGATTATGAAGAAGATTTA  300
      L  H  K  N  I  C  T  S  K  Y  E  K  G  T  D  Y  E  E  D  L
 301  GCAATTGTTAGAAAAAAGGATCGCCTTTTAGAGTATTCCCTATTGAATTATGTTGACGTT  360
      A  I  V  R  K  K  D  R  L  L  E  Y  S  L  L  N  Y  V  D  V
 361  GAAATTTCCAGACTAACGGACTACCAGCTCTTTTTGAACACCAAAAATTACAATGAATAT  420
      E  I  S  R  L  T  D  Y  Q  L  F  L  N  T  K  N  Y  N  E  Y
 421  ACCAAGAAGTTGTTTTCAAAATTGCTATATTTTCCTATGTGGTATAATACGCCATTGCAA  480
      T  K  K  L  F  S  K  L  L  Y  F  P  M  W  Y  N  T  P  L  Q
 481  CTAAGATCGCAAGCACGTGAAAATTGTGAGGAGATTATAGGCTCACTGACCCTTGAGGAT  540
      L  R  S  Q  A  R  E  N  C  E  E  I  I  G  S  L  T  L  E  D
 541  GATGAAGAATTTGTAGAAAGTAAAGCAATGGAGTCAGCCTCGCAACTAGCACAATCAAAG  600
      D  E  E  F  V  E  S  K  A  M  E  S  A  S  Q  L  A  Q  S  K
 601  ACCTTCAAAATTGCGCATAAGAACAAAATTAAGGGTAAGCAAGAGCTACAACAGGTGAAA  660
      T  F  K  I  A  H  K  N  K  I  K  G  K  Q  E  L  Q  Q  V  K
 661  TATAATCTTCAATTTGATAATAGACTACAGAGTTGCGTCAGCAATTGGTTGGCTGCCCGT  720
      Y  N  L  Q  F  D  N  R  L  Q  S  C  V  S  N  W  L  A  A  R
 721  AAAAAACTGGATGATTCTGTAATACTTTCCTCCGACCTTCTTTTCCTTGCAAATCTTTAC  780
      K  K  L  D  D  S  V  I  L  S  S  D  L  L  F  L  A  N  L  Y
 781  GTTCAGTTAGGCCTACCTGATGGTAACCGCATCCGTTCAAAACTGGAACAAACCTTTGGA  840
      V  Q  L  G  L  P  D  G  N  R  I  R  S  K  L  E  Q  T  F  G
 841  AGTGAATTATTGAATAGTATGTCGAACAAAATTGATGACTTCGTCCATAGACCAAGTAAT  900
      S  E  L  L  N  S  M  S  N  K  I  D  D  F  V  H  R  P  S  N
 901  AACTTGGAGCAAAGAGATCCTCAATTTAGAGAGCAAGGAAACGTTGTGATGTCATTATAT  960
      N  L  E  Q  R  D  P  Q  F  R  E  Q  G  N  V  V  M  S  L  Y
 961  AATTTAGCCTGTAAATACATATAATAAAAATAAACAAAATAATGCAGAAAGGTGTCTTTA  1020
      N  L  A  C  K  Y  I  *
1021  AGTTCATTTTTCAATTTCTTTTCGGTTTTCGTACTCCTTTATAAACGAAGTAATAAACAA  1080
1081  TTGTACCATCATCGTTAATTATGGCCAGCAGTATTCTTTCAGTAGATGGACTACATAAGT  1140
1141  GAGTAAATGTCTGGCTCAAGCATTCCAAAGTGAGAAAATTCTCTTTGTACTGTGACATAT  1200
1201  TGATGGGTAATATGAACTCTAGCGGGCGTGGCGATGACAAGCTATTTTCATGCTCTGTGT  1260
1261  CTACATCGTTGCTCAGCTTGTGAGGCGGACGACCTCTCAGTAATCTCAGGCTATTTCCCT  1320
1321  TCCATGATAAATCGTCTTGTAAAATTTCCACTTCCGTCCACATTTGGAAATAGAGTAAAT  1380
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Figure 1. Nucleotide sequence of the *MAS37* gene and deduced amino acid sequence of the gene product. The first nucleotide of the translation start site is designated as position 1. The sequence data are available from EMBL/GenBank/DDBJ under accession number X62565.

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., 1983*b*; 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., 1983*b*; 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 NH_2-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 $FSKLLYF_{151}$ 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 Δ*mas37* 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 effected 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_1 by 70% and 30%, respectively, but did not affect import of the HSP60 precursor (Fig. 4, *wild-type*). This inhibition pattern closely resembles that 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, Δ*mas37*).

Similarly, Δ*mas37* 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 undenatured 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: Δ*mas37* mitochondria are barely able to import the urea-denatured ADHIII precursor, but imported the urea-dena-

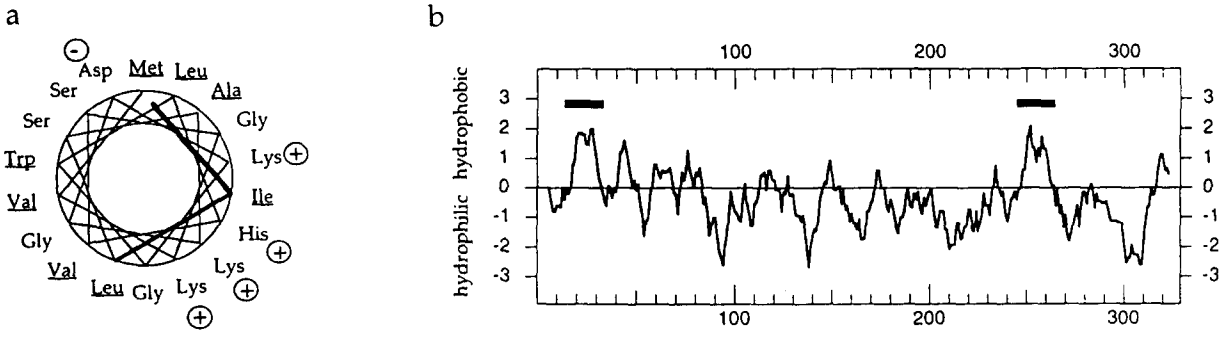

Figure 2. Structural predictions for Mas37p. (*A*) Helical wheel representation of the NH_2-terminal amino acids of Mas37p. Hydrophobic amino acids are underlined. (*B*) Hydropathy plot (Kyte and Doolittle, 1982) of Mas37p. Putative membrane-spanning regions are indicated by bars.

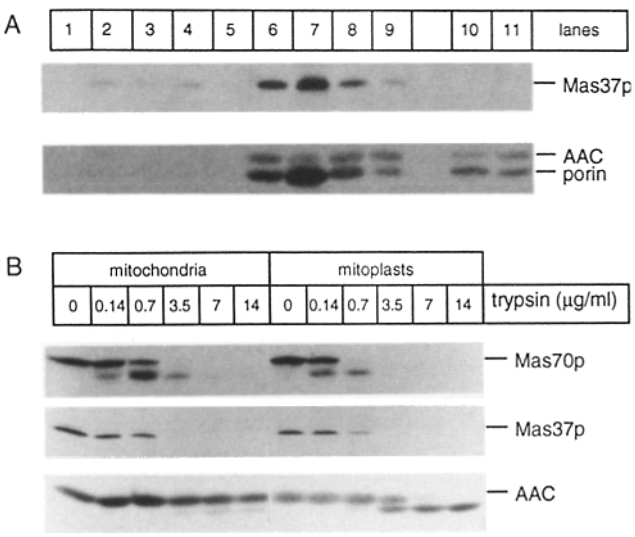

Figure 3. Mas37p is tightly bound to the mitochondrial surface. (*A*) Subcellular localization of Mas37p. 30 μg protein of each subcellular fraction was analyzed by SDS-PAGE and immunoblotting for porin (outer mitochondrial membrane), the adenine nucleotide carrier (*AAC*, inner mitochondrial membrane) 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 Δ*mas37* cells; lane *11*, mitochondria prepared from cells harboring the *pet3027*^{ts} 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).

tured Su9-DHFR precursor to the same extent as wild-type mitochondria (Fig. 5 *B*). The urea-denatured ADHIII precursor thus interacts with both Mas70p and Mas37p, and this interaction is rate limiting for import into mitochondria. To directly assess the role of Mas37p in mediating the productive binding of different precursor proteins, we have made use of a precursor binding assay. In the absence of a membrane potential the adenine nucleotide carrier precursor binds to the surface of wild-type mitochondria, but is not imported; this prebound precursor can then be chased into the mitochondria by restoring the membrane potential (Fig. 5 *C*). The effectiveness of the chase reaction provides a measure of the amount of precursor that has bound productively to receptors on the mitochondrial surface (Pfanner et al., 1987; Hines et al., 1990). No such productive binding of the adenine nucleotide carrier precursor was seen with Δ*mas37* mitochondria (Fig. 5 *C*). Δ*mas37* mitochondria thus resemble Δ*mas70* mitochondria in their inability to bind this precursor productively (Hines et al., 1990). However, Δ*mas37* mitochondria are as active as wild-type mitochondria in productive binding of Su9-DHFR (Fig. 5 *C*). The reduced efficiency for the import of the adenine nucleotide carrier into Δ*mas37* mitochondria is due to a severely reduced receptor function.

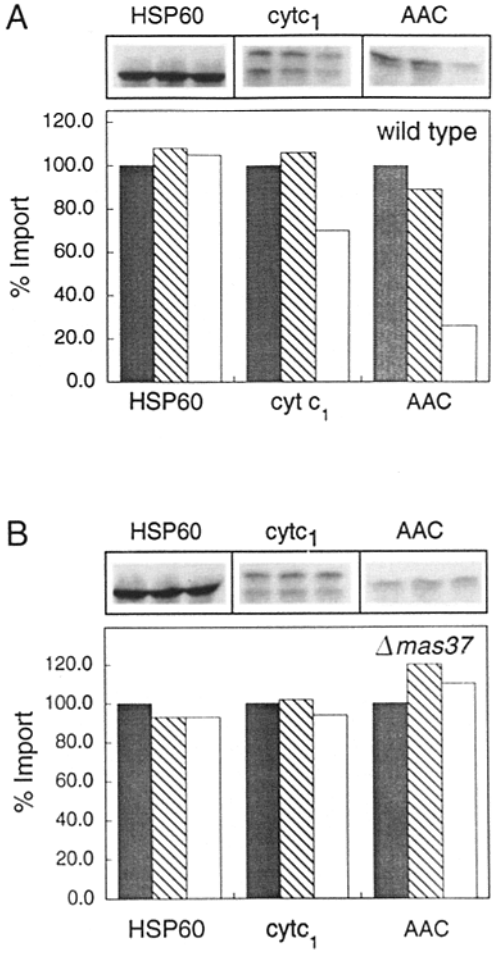

Figure 4. Affinity-purified antibodies against Mas37p inhibit mitochondrial protein import. Mitochondria (10 μg protein) from wild-type cells (*A*) or Δ*mas37* cells (*B*) were pretreated with buffer (*filled bars*) or with 100 ng antibodies against porin (*hatched bars*), or with 100 ng antibodies against 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_1 (*cyt* c_1) 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_1 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 *MAS70* 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.

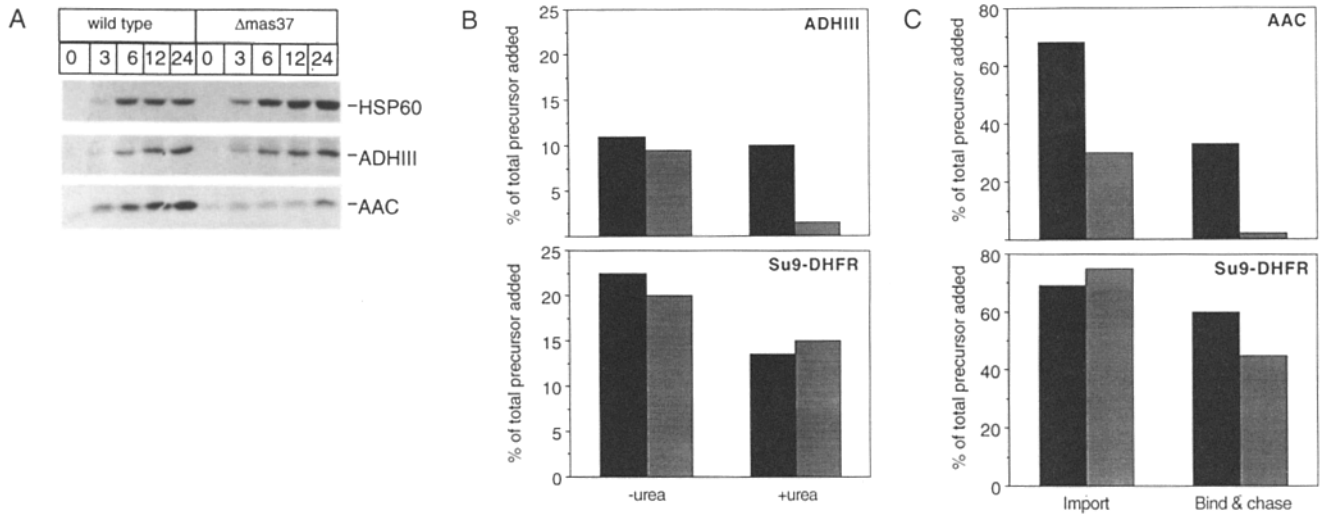

Figure 5. Mitochondria lacking Mas37p have specific defects in protein import. (*A*) Mitochondria (20 μg protein in a final volume of 100 μl) from wild-type or Δ*mas37* cells were assayed for the import of the precursor to the 60-kD heat-shock protein (*HSP60*) at 10°C, for import of the mitochondrial isoform of alcohol dehydrogenase (*ADHIII*) at 12°C or for import of the adenine nucleotide carried (*AAC*) at 14°C. At the indicated time points (min), import was stopped with carbonylcyamide 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 F_1F_0-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 Δ*mas37* (▨) 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 Δ*mas37* (▨) 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 reisolated, 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 reisolated 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 stoichiometric 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

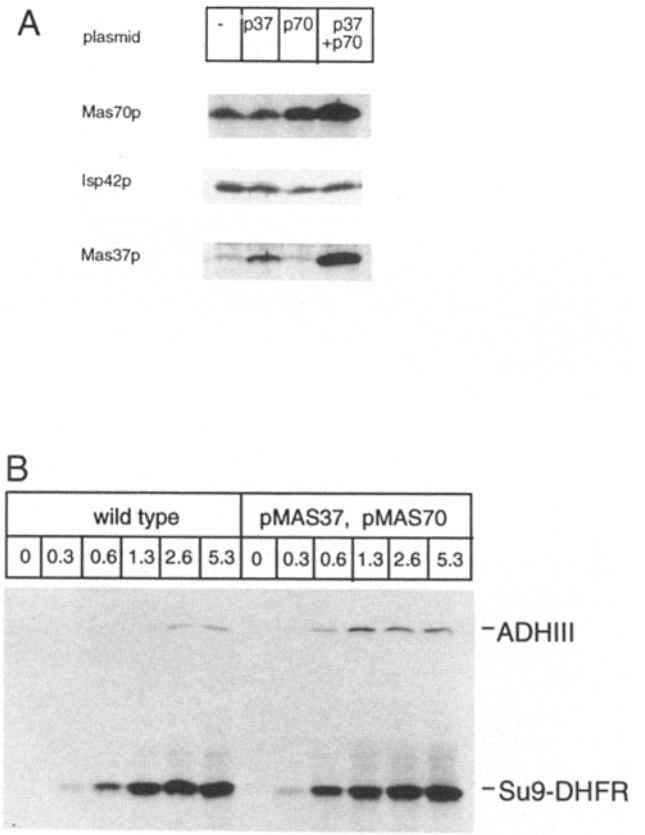

Figure 6. Overexpression of the Mas37p/Mas70p import receptor. (*A*) Yeast cells transformed with a multi-copy plasmids carrying either the *MAS37* gene (p37) or the *MAS70* gene (p70), or cotransformed with both plasmids (p37 + p70), were grown to late logarithmic phase on synthetic medium containing glucose. Total cell extracts were prepared for immunoblot analysis using antibodies against Mas37p, Mas70p, Isp42p. Overexpression of Mas37p and Mas70p was measured by densitometry of multiple exposures of the immunoblots. (*B*) Mitochondria were prepared from wild-type yeast or from cells overexpressing Mas37p and Mas70p (pMAS37, pMAS70), and assayed at 15°C for their ability to import urea-denatured ADHIII and Su9-DHFR. At the indicated time points (min), import was stopped by transferring 500-μl aliquots of the assay mixture (containing 10 μg mitochondria) to tubes containing 40 μg mitochondria pretreated with FCCP. The mixture was incubated with proteinase K (50 μg/ml) for 20 min on ice, and PMSF was then added to 200 μg/ml. Coimport of the two precursors was analyzed by SDS-PAGE fluorography.

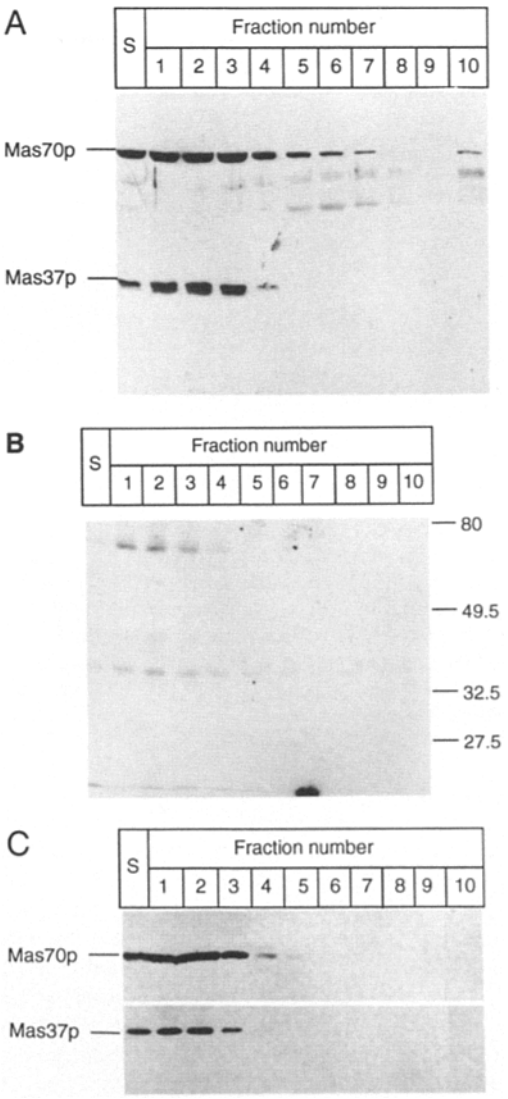

Figure 7. Mas37p and Mas70p are bound to each other. Mitochondria (500 μg protein in a final volume of 100 μl) were solubilized with 1% TX-100 and the lysate was centrifuged in Triton-containing sucrose density gradients. 10 200-μl fractions were collected for analysis. Lane *S*, one-sixth of the material applied to the gradient. (*A*) The gradient fractions were analyzed by immunoblotting with antibodies against Mas37p and with antisera against Mas70p. (*B*) As in *A*, except that the mitochondria were uniformly labeled with [^{35}S] and that the gradient fractions were subjected to immunoprecipitation with antibodies against Mas70p. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. (*C*) Proteins in the immunoprecipitated samples were separated by SDS-PAGE and immunoblotted for Mas37p and Mas70p.

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

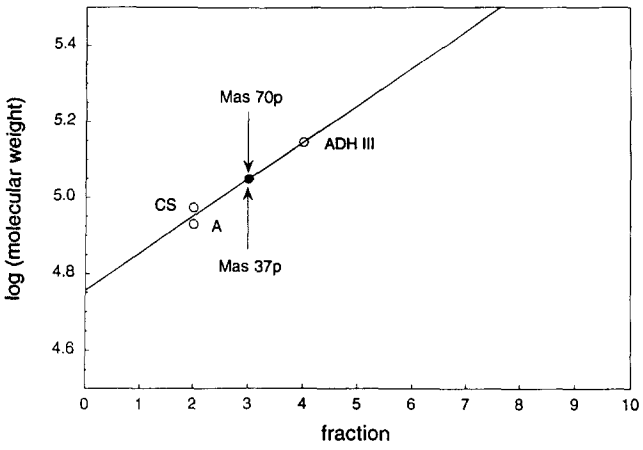

Figure 8. Mas37p and Mas70p form a heterodimer. Mitochondria (50 μg protein) were solubilized with 1.0% octyl-polyoxyethylene and the solubilized proteins were sedimented for 16 h in a sucrose gradient containing 1.0% octyl-POE instead of TX-100 (see Fig. 5). Migration of Mas37p, Mas70p and of the marker proteins alcohol dehydrogenase (homotrimer of 40-kD subunits), citrate synthetase (homodimer of 47-kD subunits) and aconitase (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., 1983*a*; Söllner et al., 1989; Kiebler et al., 1993; Lithgow et al., 1994*a*). (*b*) Antibodies monospecific for either of these proteins partly inhibit protein import into isolated mitochondria (Söllner 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., 1994*a*; Moczko et al., 1994). Mas37p meets all of these criteria, and is thus a third receptor protein for mitochondrial protein import.

Mas37p Is a Novel Component of the Import Machinery

The *pet3027*^{ts} *Δmas37* mutants display pleiotropic effects on mitochondrial function, such as reduced levels of mitochondrially 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* mitochondria, 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 heterodimer described here represents a promising basis for studying the interaction of purified precursors with their mitochondrial receptors in a defined system.

Recently, the receptor domain of Mas70p was overproduced in *E. coli* and used in in vitro assays for precursor binding (Schlossman et al., 1994). Whereas in intact mitochondria the functional form of the Mas70p receptor has been shown to mediate import of the adenine nucleotide carrier (Hines et al., 1990; Steger et al., 1990), preF$_1\beta$ (Hines et al., 1990), MOM38 (Keil et al., 1993) and cytochrome c_1 (Hines et al., 1990), competition assays between the monomeric domain of Mas70p and intact mitochondria showed that the Mas70p monomer competes for the binding of the adenine nucleotide carrier, only weakly for the binding of cytochrome c_1, and not at all for the binding of preF$_1\beta$ or MOM38. In addition, a 200–500-fold molar excess of the monomeric Mas70p domain was required to inhibit binding of the adenine nucleotide carrier by 50% (Schlossman et al., 1994). Our proposal that the functional receptor is a higher-ordered structure would explain the relatively poor performance of the monomeric receptor and suggests that studies with the Mas70p subunit alone might not correctly define the affinity and specificity of the Mas70p receptor for precursors in vivo.

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; Söllner 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., 1994*b*; 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 "supercomplex" 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., 1994*b*). 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-

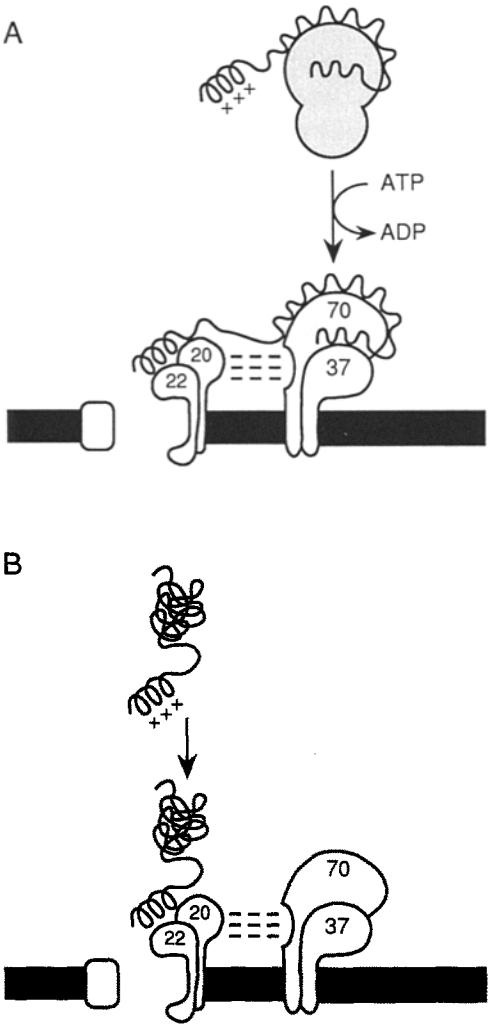

Figure 9. Cooperation of receptors during protein import into mitochondria. Mitochondrial precursor proteins are made in the cytosol and the nascent polypeptides associate with chaperones (*shaded*). (*A*) Some precursors are retained on the chaperones; release from the chaperones requires extramitochondrial 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 c_1, the F_1 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.

mal levels of its partner Mas22p can be maintained (Lithgow et al., 1994*b*). 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 also 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., 1994*a*; Moczko et al., 1994). Mas20p (Ramage et al., 1993; Moczko et al., 1994) and Mas22p (Lithgow et al., 1994*b*) 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 (Haucke et al., 1995).

In contrast, the precursors of ADHIII, the adenine nucleotide carrier, cytochrome c_1, 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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