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The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast

TREVOR LITHGOW*, TINA JUNNE*, KITARU SUDA*, SABINE GRATZER†, AND GOTTFRIED SCHATZ*‡

*Biozentrum, Universität Basel, CH-4056 Basel, Switzerland; and †Institut für Biochemie, Technische Universität Graz, A-8010 Graz, Austria

Contributed by Gottfried Schatz, August 25, 1994

ABSTRACT We have cloned the gene encoding the protein Mas22p, which spans the outer membrane of yeast mitochondria. Cells that completely lack Mas22p are inviable. The plasmid-borne *MAS22* gene suppresses several defects resulting from the deletion of one or more of the mitochondrial protein import receptors. Defects of Mas20p-deficient cells are explained by the reduced level of Mas22p in these mutants. Mas22p has one acidic domain in the cytosol and a second acidic domain in the mitochondrial intermembrane space. We suggest that these domains of Mas22p on either side of the outer membrane function as a relay system for transferring the basic targeting sequences of precursor proteins into the mitochondria.

The mitochondrial outer membrane contains several proteins that appear to function as "receptors" for precursor proteins destined to be imported into mitochondria. In the yeast *Saccharomyces cerevisiae*, these proteins are termed Mas20 protein (Mas20p), Mas37 protein (Mas37p), and Mas70 protein (Mas70p) (refs. 1 and 3; S.G., T.L., S. Kohlwein, V. Haucke, T.J., G.S. and M. Horst, unpublished data). The *Neurospora crassa* homologs of Mas20p and Mas70p are termed MOM19 and MOM72, respectively (4, 5); a *N. crassa* homolog of Mas37p has not been described. In addition, *N. crassa* mitochondria contain the outer membrane protein MOM22, which has been reported to function at some step after the binding of precursors to the protein import receptors (6).

Studies with *N. crassa* and yeast seemed to suggest that Mas20p/MOM19 functions as a "master receptor" that mediates the import of most mitochondrial proteins, including that of the other import receptors Mas70p/MOM72 and MOM22 (7–10). Our results with yeast are more compatible with a "division of labor" model in which the Mas20p, Mas70p, and Mas37p subunits cooperate to form a receptor system that ensures specific and efficient precursor binding (refs. 1 and 3; S.G. *et al.*, unpublished data). Deletion of Mas20p, Mas37p, or Mas70p is not lethal and inhibits protein import only partially, demonstrating that none of these three receptor subunits is essential for import. However, loss of any two of the three subunits is lethal (ref. 1 and S.G. and S. Kohlwein, unpublished data).

While Mas20p is not essential, disruption of the *MAS20* gene initially causes severe mitochondrial defects, including the inability of the Δ*mas20* cells to grow on nonfermentable carbon sources (1, 11). The present study was prompted by the observation that several days after disruption of the *MAS20* gene, Δ*mas20* cells regain normal growth (12). Thus, cells can adapt within days to the loss of Mas20p. A similar suppression of the respiration and protein import defects is caused by the mutation of a single nuclear gene. In both cases, the suppression also restores viability and mitochondrial protein import to cells lacking both Mas70p and Mas20p. We proposed that both of these suppression mechanisms increase the level or activity of a putative outer membrane protein, Mas*X*p, which restores mitochondrial protein import to Mas20p-deficient cells (12).

We have now cloned the *MASX* gene and show that it encodes an essential 17-kDa outer membrane protein that migrates with an apparent molecular weight of 22,000 in SDS/polyacrylamide gels. As Mas*X*p is homologous to MOM22 from *N. crassa*, we have renamed it Mas22 protein (Mas22p). We show that the defects of Δ*mas20* cells are not caused by the absence of Mas20p but are caused by a lowered level of Mas22p and that cells lacking only Mas20p have no mutant phenotype. Our results suggest that Mas22p functions as a fourth subunit of the precursor receptor system in the outer membrane and that Mas22p is the only subunit that is essential in itself.

MATERIALS AND METHODS

Yeast Strains. Strains used were as follows: Δ*mas20, res*$^-$ (YTJB5) (*MAT***a**, *leu2, ura3, his4, mas20*::*URA3*); Δ*mas20, res*$^+$ [YTJB5 that had adapted to respiratory growth after 10 days on rich medium containing ethanol and glycerol as carbon sources (12)]; Δ*mas20, SUPX*, a respiring YTJB5 revertant that appeared immediately after plating on rich glucose-containing medium. Respiration is the result of an extragenic suppressor (12). The diploid Δ*mas22* strain YTJB73 (*MAT*α/**a**, *ade2, ura3, leu2, his3, trp1, mas22*::*ADE2*/*MAS22*) was derived as follows: a polymerase chain reaction (PCR) was used to incorporate a *Sal* I site into the open reading frame of *MAS22*. A *Bgl* II fragment encompassing the entire *ADE2* gene was treated with the Klenow fragment of DNA polymerase I and inserted into the Klenow-treated *Sal* I site in *MAS22*, thereby disrupting the coding sequence after 8 bp. The *Nae* I–*Pst* I fragment of the disrupted gene was transformed into diploid cells and Ade$^+$ transformants were selected. Disruption of the *MAS22* gene was confirmed by Southern blot analysis.

Cloning of the *MAS22* Gene. Chromosomal DNA from YTJB36 [Δ*mas20*, Δ*mas70*, *SUPX* (12)] was partially digested with *Sau*3A and size-selected on a sucrose gradient. Fragments greater than 10 kb were ligated into the single-copy vector YCplac33, which carries the *URA3* gene. Cultures of Δ*mas20, res*$^-$ cells (YTJB64: *MAT*α, *leu2, lys2, ura3, his4, mas20*::*LEU2*) were grown to midlogarithmic phase and frozen in aliquots to prevent adaption of the cells to the loss of Mas20p prior to transformation. The cells were thawed, grown for 2 h on glucose-containing rich medium, and transformed with the DNA library (13). Transformants were selected on glucose-containing minimal medium without uracil. The plates were incubated for 10 h at 30°C and then shifted to 37°C. Two plasmids were isolated that enabled the

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‡To whom reprint requests should be addressed.
§The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. X80348 for the *MAS22* gene).

cells to grow at 37°C. To confirm complementation of the growth defects by *MAS22*, an *Eco*RV–*Mun* I fragment common to the isolated plasmids was subcloned into YEplac181 that had been cut with *Sma* I and *Eco*RI.

Affinity Purification of Anti-Mas22p Antibodies. A DNA fragment encoding the C-terminal extramembrane domain of Mas22p was amplified by PCR and subcloned into the vector pMALc2 (New England Biolabs). The MalE–Mas22p fusion protein was expressed in *Escherichia coli*, purified, and coupled to CNBr-activated Sepharose beads. Antiserum raised against outer membrane proteins was incubated with these beads overnight and antibodies specific for Mas22p were eluted at low pH (14).

Miscellaneous. Transformation of yeast cells directly from plates, the preparation of total yeast cell extracts, purification of mitochondria, immunoblot analysis, and SDS/PAGE have been described and fully referenced (12). Trypsin treatment of mitochondria will be described elsewhere (S.G. *et al.*, unpublished data), and mitochondrial outer membrane vesicles were prepared as described by Ramage *et al.* (1).

RESULTS

A Nuclear Suppressor Gene Restores Defects Caused by the Loss of Each of the Known Mitochondrial Protein Import Receptors. We have reported (12) that the defects of Δ*mas20* cells can be suppressed either by adaptation over a period of days (yielding Δ*mas20, res*$^+$ cells) or by an extragenic suppressor (yielding Δ*mas20, SUPX* cells). By crossing *SUPX* into cells without a functional *MAS37* gene, we found that *SUPX* also suppresses the temperature-sensitive respiratory defect of Δ*mas37* cells (Fig. 1).

In addition, *SUPX* suppressed the synthetic lethality of cells lacking any two of the receptor subunits Mas20p, Mas37p, and Mas70p. Fig. 2 shows an immunoblot of mitochondria isolated from the various doubly disrupted cells. These cells not only are viable without two of their import receptors but also respire and grow at about 50% the rate of wild-type cells on lactate medium (data not shown). According to our model (12), *SUPX* maintains the level of activity of a hypothetical outer membrane protein, Mas*X*p, in cells lacking one or more of the three known receptor subunits.

Cloning of the *MASX* Gene. The suppressor *SUPX* is a dominant mutation of a nuclear gene (12) and might be either an allele of *MASX* or a separate gene; both mechanisms could cause increased expression or enhanced stability of Mas*X*p. In an attempt to identify either *MASX* or *SUPX*, we constructed a genomic DNA library from Δ*mas20*, Δ*mas70*, *SUPX* cells in a single-copy plasmid. We isolated two clones from this library that could complement the defects of Δ*mas20* cells.

We also screened for clones that complemented the temperature-sensitive respiration defect of Δ*mas37* cells and

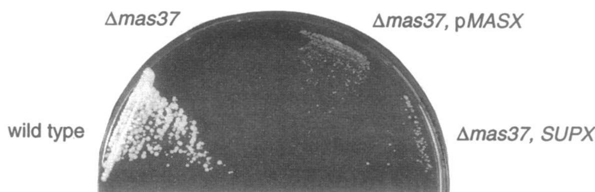

FIG. 1. Extragenic suppressor *SUPX* restores respiration-driven growth at elevated temperatures to Δ*mas37* cells. Cells were grown at 30°C on synthetic liquid medium containing 2% (wt/vol) lactate, streaked out onto rich solid medium containing 3% ethanol and 3% (vol/vol) glycerol, and allowed to grow at 37°C for 5 days. As discussed below, cells in the sector labeled Δ*mas37*, pMASX were Mas37p-deficient cells transformed with the *MAS22* gene on the multicopy vector YEplac181.

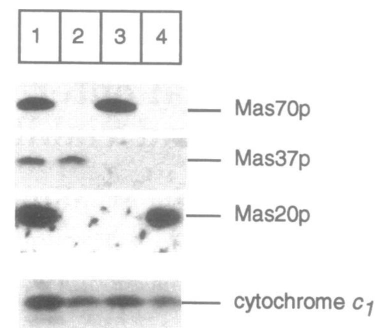

FIG. 2. Suppression of the synthetic lethal phenotype associated with the pairwise loss of Mas20p, Mas37p, and Mas70p. Mitochondria were prepared from lactate-grown cultures of the following yeast strains: wild-type cells (lane 1); Δ*mas20*, Δ*mas70*, *SUPX* cells (lane 2); Δ*mas20*, Δ*mas37*, *SUPX* cells (lane 3); Δ*mas37*, Δ*mas70*, *SUPX* cells (lane 4). Mitochondrial protein (100 μg) was separated by SDS/PAGE and analyzed on immunoblots with antisera against Mas70p, Mas37p, Mas20p, or cytochrome c_1 (an inner membrane protein used as a control).

isolated three groups of plasmids. One group carried the wild-type *MAS37* gene; the second group will be described elsewhere; and the third group shared a 3-kb *Eco*RI fragment with the plasmids that complemented the Δ*mas20* defects. Subcloning of the 3-kb *Eco*RI fragment common to the plasmids recovered from both library screens revealed that this subfragment complemented growth of Δ*mas20, res*$^-$ cells on ethanol/glycerol medium (data not shown). By hybridizing the radioactive subfragment to a library of wild-type genomic DNA, we then isolated the corresponding wild-type gene.

The 3-kb *Eco*RI fragment of genomic DNA (Fig. 3*A*) included an open reading frame that encoded a 17-kDa protein with 25% identity to the *N. crassa* protein MOM22 (Fig. 3*B*). We therefore named this gene *MAS22*. Subcloning

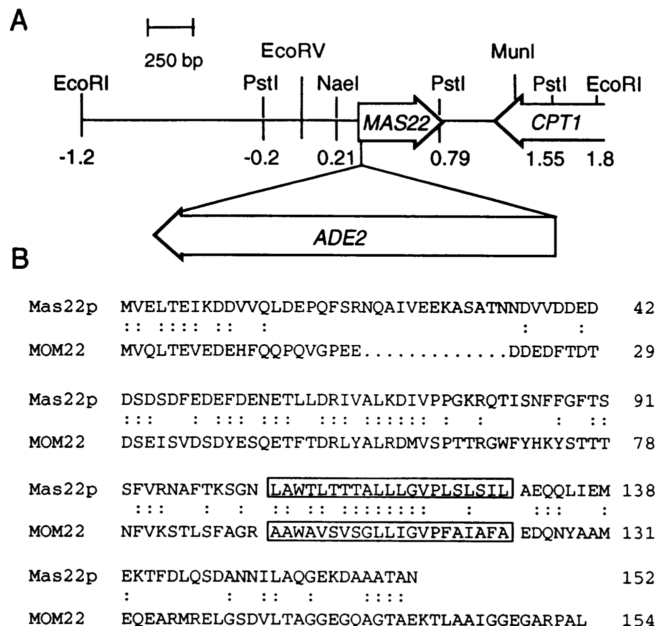

FIG. 3. *MAS22* is *MASX*. (*A*) Restriction map of the 3-kb *Eco*RI fragment common to all of the plasmids that can suppress the defects of both Δ*mas20* cells and Δ*mas37* cells. The genomic DNA fragment partially overlaps the *CPT1* gene (15). The *Eco*RV and *Mun* I sites flank the small complementing subclone, subsequently shown to carry *MAS22*. The insertion of *ADE2* to disrupt *MAS22* function is also shown. (*B*) Sequence alignment of MOM22 (6) and Mas22p. The predicted transmembrane region of each protein is boxed.

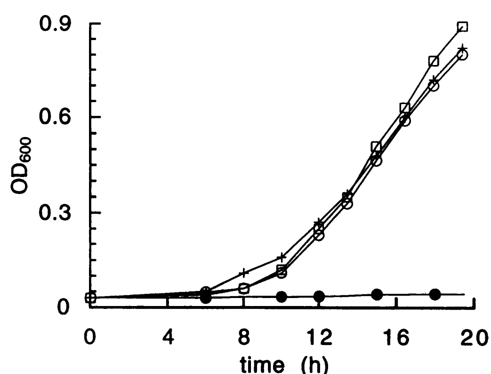

FIG. 4. Plasmid-encoded *MAS22* fully suppresses the growth defect of Δ*mas20* cells. Cultures were first grown in synthetic medium with glucose as a carbon source and then diluted into complete medium containing 2% lactate and 0.5% glucose: +, wild-type cells; ●, Δ*mas20, res*⁻ cells; ○, Δ*mas20, SUPX* cells; □, Δ*mas20* cells carrying plasmid-borne copies of *MAS22*. At the indicated times, samples were removed and their OD_{600} was determined.

of the *Eco*RV–*Mun* I fragment containing *MAS22* showed that it fully complemented the respiratory defects of Δ*mas20* cells (Fig. 4) and that it accounted for the suppression of Δ*mas37* defects (Fig. 1; Δ*mas37*, pMASX).

Mas22p Exposes Acidic Domains on Both Sides of the Outer Membrane. Sequence analysis of Mas22p revealed a predicted transmembrane domain from Leu^{98} to Leu^{118} and two acidic extramembrane regions. Antibodies specific for the C-terminal domain of Mas22p (Leu^{118} to Asn^{152}) recognized an outer membrane protein with an apparent molecular weight of 22,000 that was overexpressed in cells carrying the *MAS22* gene on a multicopy plasmid (Fig. 5*A*). Treatment of mitochondria with trypsin reduced Mas22p to a fragment that was still recognized by antibodies specific for the C-terminal domain (Fig. 5*B*). In mitoplasts, where the outer membrane barrier was breached, trypsin had access to the intermembrane space and the fragment was destroyed. Like MOM22, Mas22p thus spans the outer membrane, with an N-terminal domain in the cytosol and a C-terminal domain in the intermembrane space.

Mas22p Is Mas*X*p. One or two additional copies of *MAS22*, from the CEN-based library, restored some respiration to Δ*mas20* cells, and multiple copies of *MAS22* allowed the Δ*mas20* cells to grow as fast as wild-type cells on nonfermentable carbon sources. We conclude that Mas22p is the postulated outer membrane protein Mas*X*p (12) whose elevated level or activity suppresses the defects of cells lacking Mas20p or Mas37p.

Δ*mas20, res*⁻ cells had also lost most of their Mas22p, and suppression of these cells by *SUPX* restored Mas22p to wild-type levels (Fig. 6*A*). A similar restoration of Mas22p levels was observed in Δ*mas20, res*⁺ cells that had adapted to respiratory growth. Thus, the defects of Δ*mas20* cells are not simply attributable to the loss of Mas20p, and restored respiration correlates with restored levels of Mas22p.

These results suggested that loss of Mas20p destabilizes Mas22p and that adaptation or the *SUPX* gene prevent, or compensate for, this destabilization. One mechanism to restore steady-state levels of Mas22p is increased expression of *MAS22* that we have achieved by introducing additional

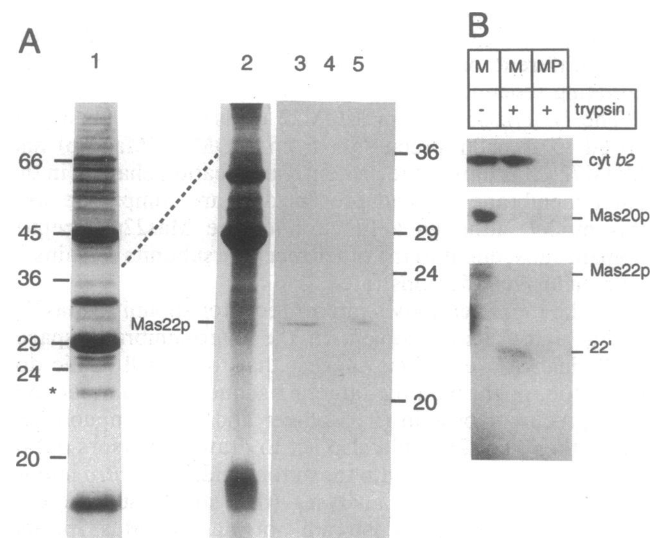

FIG. 5. Localization and topology of Mas22p in the mitochondrial outer membrane. (*A*) Mitochondrial outer membrane proteins (50 μg, lane 1) were separated in the presence of SDS on a 10–15% polyacrylamide gradient gel (1). To resolve the 22- to 23-kDa protein band (*), a sample was separated on a 30-cm 15% polyacrylamide gel and either stained with silver (lane 2) or transferred to nitrocellulose and labeled with affinity-purified antibodies (lane 3). The standards are total cell extracts (50 μg) from Δ*mas20, res*⁻ cells (lane 4) or Δ*mas20* cells transformed with plasmid-borne copies of *MAS22* (lane 5). (*B*) Mitochondria (150 μg) were analyzed directly (M, −), after trypsin treatment (M, +), or after disruption of the outer membrane and trypsin treatment (MP, +). The trypsin concentration was 150 μg/ml. The immunoblot was probed with antibodies recognizing Mas22p, Mas20p, or the intermembrane space protein cytochrome b_2 (cyt b_2). 22′, C-terminal fragment of Mas22p.

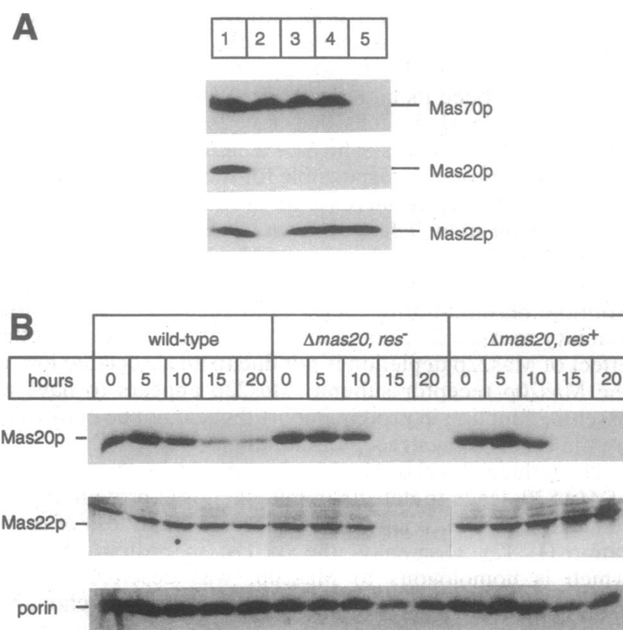

FIG. 6. Respiration defect of Δ*mas20* cells correlates with the loss of Mas22p. (*A*) Mitochondria were prepared from cultures of the following yeast strains grown on rich medium with glucose as a carbon source: wild-type cells (lane 1); Δ*mas20, res*⁻ cells (lane 2); Δ*mas20, SUPX* cells (lane 3); Δ*mas20, res*⁺ cells (adapted to growth on ethanol/glycerol medium, lane 4); and Δ*mas20*, Δ*mas70, SUPX* cells (lane 5). Total proteins extracted from the cells were analyzed by SDS/PAGE and on immunoblots with antisera against Mas70p, Mas20p, Mas22p, or porin. (*B*) Wild-type cells, Δ*mas20, res*⁻ cells, or Δ*mas20, res*⁺ cells (adapted to growth on ethanol/glycerol medium) were transformed with the plasmid pYADEMAS20. Cultures were grown to early logarithmic phase in synthetic medium plus galactose and then shifted to synthetic medium plus glucose and grown at 30°C. At the indicated time, total cell proteins were extracted and analyzed for Mas20p, Mas22p, and porin (an outer membrane protein unrelated to protein import receptors) on immunoblots.

copies of *MAS22* into the Δ*mas20* cells. However, Northern blot analysis of the various strains analyzed in Fig. 6*A* revealed that neither the *SUPX* mutation nor adaptation to respiratory growth influenced the transcription of the *MAS22* gene: all of these cells had similar levels of MAS22 mRNA (data not shown). Alternatively, adaptation or the suppressor gene might restore steady-state levels by decreasing turnover of Mas22p. Our attempts to measure the turnover of Mas22p by pulse-labeling cells with ^{35}S were unsuccessful; Mas22p has only one methionine and no cysteines, and its N-terminal methionine is removed (data not shown). As an alternative approach, we transformed Δ*mas20, res*$^-$ cells with a plasmid-borne copy of *MAS20* controlled by a glucose-repressible promoter (16). When the transformants were depleted of Mas20p by growth on glucose, Mas22p was also lost. No loss of Mas22p was observed in Δ*mas20, res*$^+$ cells that had previously adapted to respiratory growth (Fig. 6*B*).

Mas22p Is Essential for Viability. The experiments detailed above showed that decreased levels of Mas22p cause severe defects in mitochondrial function. To investigate the consequences of a complete loss of Mas22p, we disrupted one copy of the *MAS22* gene of diploid yeast cells (see Fig. 3). The cells were induced to sporulate, and the spores from 28 tetrads issued by five diploids were dissected and analyzed. The *mas22::ADE2* spores germinated, divided 10–12 times, and then stopped growing. Mas22p is thus essential for vegetative growth of yeast.

DISCUSSION

The *SUPX* Gene. *SUPX* is an extragenic suppressor of the respiratory defects associated with cells lacking either Mas20p or Mas37p. In our attempt to clone the *SUPX* gene, we instead cloned its target *MAS22*. *SUPX* restores Mas22p to wild-type levels in cells that have lost one or two of the other receptor subunits. The *SUPX* mutation might alter the activity of a protease responsible for the turnover of Mas22p or modify Mas22p to increase its intracellular half-life.

The Respiration Defect of Δ*mas20* Cells Is Caused by the Loss of Mas22p. A complete loss of Mas22p, brought about by disruption of the *MAS22* gene, is lethal to yeast cells. Multiple plasmid-borne copies of *MAS20* could not restore viability to Δ*mas22* cells (data not shown). Thus, the lethal effect of Mas22p depletion is not due to an associated loss of the Mas20p receptor subunit. Mas22p appears to have an essential function in protein import that cannot be performed by the other known receptor subunits.

The reduced level of Mas22p, brought on by the disruption of *MAS20*, leads to defects in mitochondrial protein import, reduced levels of functional cytochromes, and loss of respiration (1, 12). Originally, the *N. crassa* protein MOM19, which is homologous to Mas20p, was described as the "master receptor" and as being crucial for mitochondrial biogenesis (7, 10). Silencing the *MOM19* gene does cripple respiration of *N. crassa*, but it also reduces the level of the MOM22 protein (10). Unlike their *N. crassa* counterparts, Δ*mas20* yeast cells subsequently recover the ability to maintain Mas22p in the absence of Mas20p. We have shown that several mechanisms that restore wild-type levels of Mas22p to Δ*mas20* yeast cells render these cells normal. These mechanisms are an extragenic suppressor, adaptation of the cells leading to a decreased turnover of Mas22p, or overproduction of Mas22p from plasmid-borne copies of *MAS22*. Mitochondrial biogenesis is no more defective in the absence of Mas20p than in the absence of either Mas70p or Mas37p, provided that the level of Mas22p is maintained.

The Function of Mas22p. The results presented here further support the concept of a receptor system in which Mas20p, Mas37p, and Mas70p cooperate in the binding of precursor proteins. We propose to add Mas22p to this system of receptors: Mas22p and Mas20p contain highly acidic regions and appear to bind the basic amphipathic targeting sequences of mitochondrial precursor proteins (1, 6, 11, 17). By this definition, Mas22p acts as a mitochondrial surface receptor for precursor proteins (Fig. 7).

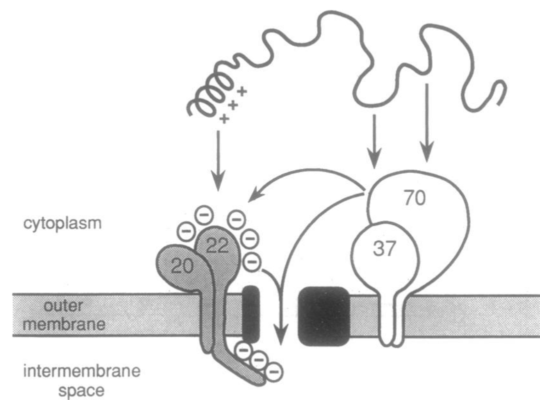

FIG. 7. Mas22p might function on both sides of the outer membrane. Mitochondrial precursor proteins are recognized at the mitochondrial surface by the import receptors Mas20p, Mas37p, and Mas70p (S.G. *et al.*, unpublished data). The receptor domain of Mas22p might bind to targeting sequences during or subsequent to the other receptors. The intermembrane space domain of Mas22p would be positioned to draw the targeting sequences into the mitochondria.

By analogy to the "methionine bristles" that might enable the signal recognition particle to recognize the hydrophobic character of the targeting sequences in secretory proteins (2), we propose that "acid bristles" in Mas22p and Mas20p might enable these proteins to recognize the positively charged amphipathic mitochondrial targeting sequences. The flexible side chains of the aspartic and glutamic acid residues should be able to deform sufficiently to accommodate the very different primary structures found in mitochondrial targeting sequences.

The *N. crassa* protein MOM22 (Mas22p) has been proposed to transfer precursors from MOM19 (Mas20p) and MOM72 (Mas70p) to the protein translocation channel in the outer membrane (6). Our present data are compatible with this model, although a function of the Mas22p receptor domain subsequent to the other receptor subunits remains to be conclusively demonstrated.

Unlike the other known import receptor subunits, Mas22p has a highly acidic domain in the intermembrane space. Preliminary observations suggest that this small acidic domain, too, might bind the targeting sequence of a mitochondrial precursor protein (L. Bolliger and T.L., unpublished data); thus Mas22p could also act to draw precursors out of the import channel and into the mitochondria. *In vitro* import assays (6) and genetic analyses (this study) suggest that Mas22p functions "downstream" of Mas70p, Mas37p, and Mas20p. In a purely spatial sense, a function of the intermembrane space domain of Mas22p would necessarily be downstream of that of the receptors on the mitochondrial surface. Mas22p may thus mediate protein import by acting on both sides of the mitochondrial outer membrane.

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