**Abstract.** To identify new components that mediate mitochondrial protein import, we analyzed mas6, an import mutant in the yeast *Saccharomyces cerevisiae*. mas6 mutants are temperature sensitive for viability, and accumulate mitochondrial precursor proteins at the restrictive temperature. We show that mas6 does not correspond to any of the presently identified import mutants, and we find that mitochondria isolated from mas6 mutants are defective at an early stage of the mitochondrial protein import pathway. MAS6 encodes a 23-kD protein that contains several potential membrane spanning domains, and yeast strains disrupted for MAS6 are inviable at all temperatures and on all carbon sources. The Mas6 protein is located in the mitochondrial inner membrane and cannot be extracted from the membrane by alkali treatment. Antibodies to the Mas6 protein inhibit import into isolated mitochondria, but only when the outer membrane has been disrupted by osmotic shock. Mas6p therefore represents an essential import component located in the mitochondrial inner membrane.

Most mitochondrial proteins are encoded in the nucleus, synthesized in the cytoplasm, and imported into mitochondria (Attardi and Schatz, 1988; Hartl and Neupert, 1990). Imported mitochondrial proteins must be recognized by mitochondria, cross one or both of the mitochondrial membranes, and sort themselves into one of four compartments: the outer membrane, the inner membrane, the intermembrane space, and the matrix. Many imported proteins destined for the inner mitochondrial compartments are synthesized in the form of precursors with cleavable amino-terminal presequences. These presequences contain all the information necessary to direct proteins into the mitochondria (Hurt et al., 1984, 1985; Horwich et al., 1985; van Loon et al., 1986).

Import of proteins into the mitochondrial matrix occurs via a multistep process that includes binding of precursors to receptors on the surface of the mitochondria (Pfeller and Neupert, 1987; Sollner et al., 1989, 1990; Hines et al., 1990), translocation of precursors across both membranes by a process requiring ATP and an inner membrane potential (Schleyer et al., 1982; Chen and Douglas, 1987; Pfanner et al., 1987; Eilers et al., 1987; Hwang and Schatz, 1989), and processing of precursors to their mature form (Mcaida and Douglas, 1982; Bohin et al., 1983). Translocation of precursor proteins has been shown to occur, at least initially, at contact sites between the inner and outer membranes (Schleyer and Neupert, 1985; Pon et al., 1989). In addition, some precursors require cytosolic factors, including the 70-kD heat shock proteins, for efficient import (Deshaias et al., 1988; Murakami et al., 1988). After import, many matrix proteins are proposed to be folded into their native conformation by matrix-localized chaperone proteins (Cheng et al., 1989; Ostermann et al., 1989; Kang et al., 1990).

Several components of the import pathway have been isolated using genetic approaches. Six mutants, *mas1-mas6*, were isolated as temperature-sensitive yeast mutants that accumulated precursors at the restrictive temperature (Yaffe and Schatz, 1984). We previously showed that *mas1* and *mas2* are defective in the activity of the matrix-localized processing protease, which removes the presequences from imported mitochondrial proteins (Yaffe et al., 1985; Jensen and Yaffe, 1988). We further showed that *MAS1* and *MAS2* encode the two subunits of this protease (Jensen and Yaffe, 1988; Witte et al., 1988; Yang et al., 1988). *mas3* mutants are defective in a transcription factor for the genes encoding many heat-shock proteins (Smith and Yaffe, 1991). *MAS5* encodes a non-essential, cytoplasmic dnaJ-like protein, that may play a chaperone role in import (Attencio and Yaffe, 1992). Subsequent genetic screens yielded new alleles of *mas1* and *mas2*, as well as a mutation in an hsp60-like protein (*mif4*) of the mitochondrial matrix (Cheng et al., 1989). *MIF4* is required for the ATP-dependent refolding and assembly of proteins imported into the matrix (Ostermann et al., 1989). A mutation in the matrix-localized hsp70 protein leads to a defect in translocation of proteins into the matrix, and in the folding of imported proteins to their native conformation (Kang et al., 1990). Recently, *MP11*, encoding a membrane-bound protein required for import has been identified (Maarse et al., 1992).

Several potential import components located in the mitochondrial outer membrane have recently been identified. Antibodies to a 42-kD protein, ISP42, inhibit import of...
proteins into isolated mitochondria (Obba and Schatz, 1987a; Baker et al., 1990). In addition, a precursor protein “jammed” in the import machinery can be cross-linked to, or coimmunoprecipitated with, the ISP42 protein (Westeger et al., 1989; Scherer et al., 1990). Antibodies to two outer membrane proteins, MOM19 and MOM72, were shown to inhibit import into isolated Neurospora crassa mitochondria (Söllner et al., 1989, 1990). MOM19 appears to be the receptor for most mitochondrial proteins synthesized with amino-terminal presequences, whereas MOM72 is required for the import of the ATP/ADP carrier protein, an imported protein that does not carry a cleavable presequence. MOM38, which is homologous to the yeast ISPs2 protein, is proposed to function as the general insertion protein (GIP), which interacts with all imported precursors at a step after the initial binding of precursors to the mitochondrial surface (Pfaller et al., 1988; Kieber et al., 1990). The yeast MA570 gene encodes the functional homologue of Neurospora MOM72 (Hines et al., 1990).

Although a number of components of the mitochondrial protein import pathway have been identified, import components located in the inner membrane are conspicuously absent. For example, although precursors are thought to be translocated across the mitochondrial membranes through an aqueous channel (Pfanner et al., 1987), no inner membrane proteins of this putative channel have yet been identified. As described below, we find that a previously uncharacterized mutant, mas6, is defective in mitochondrial protein import, and that MAS6 encodes an essential protein located in the mitochondrial inner membrane.

Materials and Methods

Strains and Relevant Genotypes

Strain AH216 (MA5a leu2-3 leu2-112) and the mas2 and mas6 mutants have been described previously (Yaffe and Schatz, 1984). mas6 strains JE4-3c (MA5a mas6-1 leu2-3 leu2-112) and JE8-1b (MA5a mas6-1 leu2-3 leu2-112) were obtained by backcrossing the mas6 mutant to AH216. JE4-5b (MAB mas6-1 trpl) was isolated by crossing JE8-1b to MA5a trpl strain YPH250 (Söllner and Hieter, 1989). MA5b/MATa ura3-52/ura3-52 strain YPH501 (Söllner and Hieter, 1989), MA5b/MATa ura3-52/ura3-52 strain SM1060 (Michaelis et al., 1986), and strain D273-10B (Sherman, 1964) have been described. Standard yeast genetic techniques (Rose et al., 1988), and yeast media (Sherman et al., 1986) were used.

Cell Labeling and Immunoprecipitation

Yeast cells to be labeled were grown to an OD600 of 0.7 to 1.0 in SD medium (Sherman et al., 1982) supplemented with the appropriate amino acids. Cells were harvested and resuspended to an OD600 of 10 in fresh SD medium. 1 ml of cells were preincubated at 38°C for 30 min, then labeled for 4 min with 150 μCi of [35S]-Translabel (1,000 Ci/mmol, ICN) at 38°C. Cells were lysed and total proteins were precipitated with TCA as described (Yaffe and Schatz, 1984). TCA pellets were resuspended in 100 μl of SDS-buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS), heated at 95°C for 5 min, and diluted with 1:1 TNE buffer (150 mM sodium chloride, 5 mM EDTA; 1% Triton X-100, 50 mM Tris-HCl, pH 8.0). Samples were centrifuged at 12,000 g for 10 min, and specific proteins were immunoprecipitated as described (Jensen et al., 1992). Labeled proteins were separated by SDS-PAGE (Laemmli, 1970; Haid and Swissa, 1983), and visualized by fluorography (Chamberlain, 1979).

Isolation of the MAS6 Gene

mas6 trpl strain JE14-5b was transformed with a library of random yeast genomic sequences in the TRPI-CEN6 vector pRS200 (Connelly, C., and P. Hieter, unpublished data) as described (Schiestl and Gietz, 1989). Trp+ transformants were selected at 37°C, and then tested for growth at 37°C. From 5,000 total transformants, one plasmid, pJE1, was found that allowed growth of the temperature-sensitive mas6 strain at 37°C. Complementation of the mas6 mutation was shown to be plasmid dependent. To localize MAS6 sequences, restriction endonuclease fragments from the pJE1 plasmid were inserted into pRS200, transformed into the mas6 strain JE14-5b, and transformants were tested for growth at 37°C. The MAS6 complementing activity was localized to a 2.2-kb SacI-BamHI fragment (see Fig. 2).

To show that pJE1 contained MAS6 sequences, a 4.2-kb Xhol-BamHI fragment was subcloned into the LEU2-containing vector pRS305 (Söllner and Hieter, 1989) to form plasmid pJE4. pJE4 was cut with HindIII to target the site of integration (Rothstein, 1991), and transformed into the MAS6 strain AH216. Stable Leu+ integrants were crossed to mas6 strain JE8-1b, and the meiotic products of the diploids were analyzed. In 28 tetrads, no recombination between LEU2 and MAS6 was found indicating that the LEU2-containing plasmid, pJE4, had integrated into 1.3 map units of MAS6.

DNA Sequence of MAS6

pJE2 and pJE3 were constructed by inserting the 2.2-kb SacI fragment of MAS6 into plasmid pRS200 (Söllner, R., and P. Hieter, unpublished data) in both possible orientations. A series of overlapping deletions of the MAS6 fragment was made using exonuclease III digestion (Henikoff, 1984). To prevent exonuclease digestion of vector sequences, pJE2 and pJE3 were digested with XhoI, and the ends were filled in with ϕ8-phosphorothioate nucleotides (Stratagene, La Jolla, CA) using DNA polymerase (Pnlley et al., 1981). MAS6 sequences carried on pJE2 and pJE3 were exposed to exonuclease digestion by Clal digestion. Single-stranded DNA was isolated by SI nuclease digestion, and plasmids were circularized by ligation. Using this collection of plasmids, both strands of the MAS6 gene were completely sequenced (Sanger et al., 1977; Jensen and Yaffe, 1988) using oligonucleotide primers specific to the pRS200 vector.

MAS6 Gene Disruptions

A precise deletion of MAS6 coding sequences was constructed as follows. First, a unique NotI site was engineered into the amino-terminus of MAS6. PCR fragment A, which contains the upstream region of MAS6, was isolated from plasmid pJE2 using oligonucleotide No. 21 (5'-ATAACACCTCACTA- AAAG-3'), oligonucleotide No. 60 (5'-GCCCACCCTGAGTGTGTTGATCT-3'), and the polymerase chain reaction (Saiki et al., 1985). Similarly, PCR fragment B containing the MAS6 open reading frame (ORF) and downstream sequences was isolated using oligonucleotide No. 20 (5'-AATACGACTCACTAG-3') and oligonucleotide No. 59 (5'-GGCGCC- GCCCTCCTGCTTGGTGGAGAT-3'). PCR fragment A was digested with NotI and SacI, and PCR fragment B was digested with NotI and BamHI. Both fragments were ligated into SacI-BamHI digested pRS315 (Söllner and Hieter, 1989) to form plasmid pJE5. pJE5 contains a unique NotI site immediately following the stop codon of MAS6.

A unique NotI site was engineered into the carboxy terminus of MAS6 as follows. PCR fragment C, which contains sequences downstream of MAS6, was isolated from plasmid pJE2 using oligonucleotide No. 20 (5'-AATACGACTCACTAG-3') and oligonucleotide No. 51 (5'-GCGCGCCCTCCTGCTTGGTGGAGAT-3'). PCR fragment D containing the MAS6 ORF and upstream sequences was isolated using oligonucleotide No. 21 (5'-ATACACCTCACTAAG-3') and oligonucleotide No. 50 (5'-GGCGCCACCCTGAGTGTGTTGATCT-3'). Fragment C was digested with NotI and BamHI, and PCR fragment D was digested with NotI and SacI. Both fragments were ligated into SacI-BamHI digested pRS315 to form plasmid pJE7. pJE7 contains a unique NotI site immediately preceding the stop codon of MAS6.

Plasmid pJE9, which has the MAS6 coding sequences deleted, was constructed by removing a NotI–SacI fragment carrying the MAS6 ORF and upstream sequences from plasmid pJE7, and replacing them with a NsiI–SacI fragment (lacking the MAS6 ORF) from PCR product A (see above). pJE10, which contains the MAS6 gene whose coding sequences were replaced by URA3, mas6: URA3, was constructed as follows. A 1.2-kb HindIII fragment containing the yeast URA3 gene was isolated from plasmid YEP24 (Botstein et al., 1979). The DNA ends were filled in with DNA polymerase, and the fragment was blunt-end ligated into the NotI site of plasmid pJE7. From 5,000 total transformants, one plasmid, pJE11, was isolated. pJE11 was constructed by PCR product B, carried on a 2.7-kb SacI–KpnI fragment, was used to replace one copy of MAS6 in the MAB/MATa diploid strains SM1060 or YPH301 (Rothstein, 1983). Stable Ura+ transformants were isolated and the meiotic products of two independently isolated diploids were analyzed at 22°C. Colonies resulting from viable spores were
tested for growth on medium lacking uracil. Of 18 tetrads, all gave rise to no more than two viable spores, even after 2 wk of incubation at 22°C. The viable spores in every tetrad were Ura-. Southern analysis of the diploids confirmed that one of the two copies of MAS6 had been replaced by mas6::URA3 (not shown).

In addition to the exact deletion of the MAS6 ORF described above, a disruption of MAS6 was made using the Tn10-LUK transposon (Huisman et al., 1987). Briefly, pJEE1, which contains MAS6 on a 4.2-kb Clal-BamHI fragment in plasmid pRS200, was transformed into bacterial strain DBI329. DBI329 was then infected with phage AKN1224, which contains the Tn10-LUK transposon. Transposon “hops” onto the MAS6-containing plasmid. The plasmid DNA was prepared from individual “hops.” One transposon was found in the MAS6 open reading frame, near the SalI site. The plasmid containing this transposon was cut with KpnI and the DNA fragment containing mas6::Tn10-LUK was transformed into the diploid strain SM1060. Ura+ transformants (TUl0-LUK carries the URA3 gene) were selected, and the meiotic products analyzed at 22°C. Of 10 tetrads, all gave rise to two viable spores, both of which were Ura-.

**Isolation of Antiserum to the MAS6 Protein**

A fusion between the E. coli maltose binding protein (MBP) and the entire MAS6 protein was created by ligating a 1.5-kb Not1-BamHI fragment from pJE5 into the EagI-BamHI site of pMAL-c (New England Biolabs Inc., Beverly, MA). Bacterial cells carrying this construct were induced to express the protein. The fusion protein homogenates were isolated as per manufacturer’s instructions. Proteins were separated by SDS-PAGE, stained with Coomassie blue, and the band containing the MBP-MAS6 fusion protein was excised with a razor blade. The gel slices were frozen in liquid nitrogen, ground in a mortar and pestle, and lyophilized. Samples were mixed with adjuvant and injected into rabbits as described (Carroll and Laughon, 1987).

**Subcellular and Submitochondrial Fractionation**

Subcellular fractionation, isolation of mitochondria, and submitochondrial fractionation were done as described (Daum et al., 1982; Jensen and Yaffe, 1988) except that the breaking buffer consisted of 0.6 M mannitol, 10 mM EDTA, 20 mM Hepes-KOH, pH 7.4, when mitochondrial membranes were being fractionated. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters (Haid and Suissa, 1983), immune decorated with antisera, and visualized with chemiluminescence (ECL, Amersham). To separate mitochondrial inner and outer membrane vesicles, 49 mg mitochondria isolated from strain D273-10B were converted to mitoplasts by osmotic shock, and membrane vesicles isolated as described (Pon et al., 1989). The membrane fraction was layered on top of a 32-ml step gradient containing 8 ml each of 0.85, 1.1, 1.35, and 1.6 M sucrose in 10 mM KCl, 5 mM Hepes-KOH, pH 7.4. Gradients were centrifuged at 100,000 g for 17 h, and 1.7 ml fractions were collected.

**Immunoelectron Microscopy**

Isolated mitochondria were incubated in 2x BB (1.2 M Sorbitol buffered with 40 mM Hepes-KOH, pH 7.4) which condensed the matrix, and separated the inner and outer membranes (Porn et al., 1989). Mitochondria were then pelleted for 3 min at 12,500 g, and the mitochondrial pellet was fixed in 2x BB containing 3% paraformaldehyde and 0.5% glutaraldehyde for 2 h at 0°C. After several washes in PBSS (1.2 M Sorbitol, 140 mM sodium chloride, 10 mM sodium phosphate, pH 7.4), the pellets were stained with 0.25% tannic acid for 60 min and washed again with PBSS. The pellets were then washed three times (10 min each) with M3 buffer (0.1 M sodium maleate, 4% sucrose), stained with 2% uranyl acetate in M5 buffer for 60 min, and washed once in M5 buffer. Subsequently, the mitochondrial pellets were dehydrated with 10 min washes of 50, 60, and 70% ethanol. The pellet was equilibrated in a 2:1 mixture of LR White resin (Ted Pella, Inc., Redding, CA) and 70% ethanol for 60 min, followed by equilibration by (rotation) in 100% LR white resin overnight at 4°C (with several changes). Finally, the resin was polymerized by incubation at 50°C overnight in gelatin capsules.

Ultrasound sections of mitochondria were obtained using a Diatome diamond knife (Diatome Inc., Fort Worth, PA) and mounted on formvar-coated 200-mesh nickel grids. All immunolabeling steps were performed as described (Berryman et al., 1992) with the following modifications. All grids were stained overnight at 4°C with rabbit serum to Mas6p, OM45p, or Fj8 antigens diluted 1:500 in 1% BSA/TBS with 0.3% Tween 20, and stained with a 1:50 dilution of 10-nm colloidal gold-labeled anti-rabbit IgG (Janssen Pharmaceutica, Beerse, Belgium). After antibody staining, grids were washed for 10 min in TBS, followed by five washes with water. Subsequently, grids were incubated in 2% glutaraldehyde for 5 min, washed with water, stained for 15 min with 2% osmium tetroxide, stained for 5 min with 0.3% lead citrate, and washed five times with water. Specimens were examined at 60 kV on a Zeiss 10A electron microscope.

**Imports into Isolated Mitochondria and Mitoplasts**

Mitochondria were isolated from wild-type cells and mas6 mutants was measured as described (Elliott et al., 1987). Briefly, mitochondria were suspended in 0.6 M mannitol, 20 mM Hepes-KOH, pH 7.4, at 10 mg/ml total protein concentration. Assays using the fluorescent dye (3,3’-dipropylthiocarbocyanine iodide (Molecular Probes Inc., Eugene, OR) were carried out at 22°C in 0.6 M mannitol, 10 mM magnesium chloride, 0.5 mM EDTA, 20 mM potassium phosphate, pH 7.4, with 1 mg/ml BSA. The dye was diluted 1,000-fold from a 2-mM stock solution in DMSO. Measurements were performed in a fluorimeter (model 650-10S; Perkin-Elmer Corp., Norwalk, CT) with excitation at 620 nm, emission at 670 nm, and slit widths of 6 nm. The final concentration of mitochondria in each reaction was 200 μg/ml.

**Construction of MAS6 under the Control of the GAL1 Promoter**

The MAS6 gene was placed under the control of the yeast GAL1 promoter as follows. A 393-bp Msel fragment, which contains the entire MAS6 open reading frame and 20-bp of upstream sequences, was isolated from plasmid pJE2. The DNA ends were filled in with DNA polymerase, and the fragment was blunt-end ligated into the HindIII site of the Bluescript II SK+ plasmid (Stratagene) to form pH1. The MAS6 gene was excised from pH1 by XhoI-BamHI digestion and inserted downstream of the GAL1 promoter in plasmid pRS315 (Sikorski and Hieter, 1989). The diploid was transformed with the pGAL-MAS6 plasmid, and the transformants that contained both the disruption and the MAS6-LEU2 plasmid. This strain was sporulated, and a haploid segregant was isolated that contained both the mas6::Tnl0-LUK disruption (see below) and the MAS6-LEU2 plasmid. This strain was transformed with the pgal-MAS6 plasmid, and the transformants transferred to medium containing galactose. Mitotic segregants that contained only the pgal-MAS6 plasmid, and not the MAS6-LEU2 plasmid were then isolated.

**Inhibition of Import Using anti-Mas6p Antibodies**

Immunoglobulin from antiserum to Mas6p and preimmune serum were diluted 1:500 in 1% BSA/TBS with 0.5% Tween 20, and stained with a 1:50 dilution of 10-nm colloidal gold-labeled anti-rabbit IgG (Janssen Pharmaceutica, Beerse, Belgium). After antibody staining, grids were washed for 10 min in TBS, followed by five washes with water. Subsequently, grids were incubated in 2% glutaraldehyde for 5 min, washed with water, stained for 15 min with 2% osmium tetroxide, stained for 5 min with 0.3% lead citrate, and washed five times with water. Specimens were examined at 60 kV on a Zeiss 10A electron microscope.

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Mitochondrial Precursor Proteins

To identify additional components of the mitochondrial protein import pathway, we have analyzed mas6, a mutant isolated in a genetic screen that yielded the previously characterized mas1 and mas2 mutants (Yaffe and Schatz, 1984). As shown in Fig. 1, mas6 mutants are defective in the import of a mitochondrial matrix protein, subunit IV of cytochrome oxidase (Cox4p). Compared to wild-type cells (WT), mas6 mutants accumulated a significant amount of the Cox4p precursor, at least as much as the previously characterized mas2 mutant (Yaffe and Schatz, 1984; Jensen and Yaffe, 1988). In pulse-chase experiments, we showed that the rate of Cox4p import was reduced 10-20-fold in mas6 strains relative to wild-type strains (not shown). In similar labeling studies, we also found that mas6 strains were defective in the import of two other mitochondrial proteins, the β subunit of the F1-ATPase (F1β) and the citrate synthase protein (not shown).

We found that the temperature-sensitive growth defect in mas6 strains, and the defect in mitochondrial protein import cosegregated in genetic crosses (not shown). Hence the import defect in mas6 mutants is due to a single genetic lesion. mas6 mutants were found to complement all previously identified import mutants: mas1, mas2, mif4 (Cheng et al., 1989), and sscl (Kang et al., 1990). Furthermore, a plasmid encoding the ISP42 protein failed to rescue the temperature-sensitive growth defect of our mas6 mutant (J. Entage and R. Jensen, unpublished data). mas6 therefore represents a new mutant defective in mitochondrial protein import.

Isolation of the MAS6 Gene

The MAS6 gene was cloned by genetic complementation of the temperature-sensitive mas6 mutant. Briefly, mutant cells were transformed with a genomic DNA library of wild-type DNA fragments in the shuttle vector pRS200 (Sikorski, R., and P. Hieter, unpublished) as described in Materials and Methods. One plasmid was isolated that suppressed the temperature-sensitive growth defect of the mas6 mutant. To verify that the complementing activity of the isolated plasmid was due to the wild-type MAS6 gene, a DNA fragment from the original plasmid was subcloned into the plasmid pRS305 (Sikorski and Hieter, 1989), integrated into the yeast chromosome by homologous recombination, and the site of integration genetically mapped. The plasmid integrated at, or close to, the MAS6 locus, indicating that the original complementing plasmid carried the bona fide MAS6 gene.

Subcloning of the original 18-kbp DNA fragment located the MAS6 complementing activity to a 2.2-kbp SacI-BamHI fragment (Fig. 2). This DNA fragment was completely sequenced (Fig. 3A), revealing an open reading frame of 666 bp, encoding a protein of 222 amino acids with a molecular mass of 23.2 kD. Two observations suggest that this open reading frame represents the MAS6 locus. First, insertion of the transposable element, Tn10-LEK (Huisman et al., 1987) into this open reading frame abolished the complementing activity (see Materials and Methods). Second, when MAS6 coding sequences were placed downstream of the yeast GAL1 promoter region, MAS6 activity was found to be galactose-dependent (see below and Fig. 9).

Hydropathy analysis (Kyte and Doolittle, 1982) of the MAS6 coding sequence suggested that Mas6p is a membrane protein (Fig. 3B). The carboxy-terminal half of Mas6p contains several potential membrane spanning domains. In con-
MAS6 Is an Essential Gene

*mas6-1* is a temperature-sensitive lethal mutation, suggesting that *MAS6* encodes an essential protein that is inactive at the nonpermissive temperature in *mas6* mutants. However, some temperature-sensitive lethal mutations affect components that are only required at high temperatures (Atencio and Yaffe, 1992). To determine whether *MAS6* encodes a gene product required at all temperatures, we constructed two disruptions of the *MAS6* gene. First, the entire *MAS6* open reading frame was replaced by the yeast URA3 gene, and the *mas6::URA3* construction was used to replace one of the two copies of *MAS6* in a *ura3/ura3* diploid cell (see Materials and Methods). When these diploid cells were sporulated and the haploid progeny allowed to grow at 22°C on glucose-containing medium, only Ura- spores were found to be viable. Hence all viable spores carried an intact *MAS6* gene, indicating that *MAS6* is essential at 22°C. When germination of the spores inferred to carry the *mas6::URA3* mutation was observed microscopically, all spores were seen to arrest in their growth after three to five divisions. Strikingly, >90% of the cells arrested as unbudded cells. Similar results were seen when the chromosomal *MAS6* gene was replaced with a *mas6* gene disrupted by a Tnl0-LUK insertion.

The MAS6 Protein Is Located in the Mitochondrial Inner Membrane

Although mitochondria isolated from *mas6* mutants are defective for import, the *MAS6* gene product does not carry a typical amino-terminal mitochondrial presequence. To determine the intracellular location of Mas6p, we raised antibodies to a fusion protein consisting of the *E. coli* maltose binding protein and the entire Mas6p protein. When this antisera was tested against total yeast proteins by immune blotting with antiserum to Mas6p, hexokinase, and the F1-ATPase β subunit (Fβ),
Figure 6. Immunoelectron microscopy indicates that Mas6p is a mitochondrial inner membrane protein. Mitochondria were swelled, fixed and stained as described in Materials and Methods. After embedding in LR White resin, ultrathin sections of mitochondria were taken and decorated with antisera to Mas6p, OM45 (an outer membrane protein), and F1\(\beta\) (an inner membrane protein). Antibody-antigen interactions were detected using a gold-coupled secondary antibody and electron microscopy. Bars, 100 nm.

blotting, a single 23-kD protein was identified. Two observations suggest that this antiserum recognized Mas6p. First, overproduction of Mas6p (due to its expression from the GALU promoter region) resulted in overproduction of the 23-kD antigen (not shown). Second, the antiserum immunoprecipitated the protein produced by in vitro transcription/translation of the cloned MAS6 gene (not shown).

Immune decorations of yeast cell fractions indicate that Mas6p is a mitochondrial protein. When a yeast cell homogenate was separated into a mitochondrial pellet and crude cytosol, Mas6p cofractionated with the mitochondrial F1\(\beta\) protein (Fig. 4). No Mas6p was found in the supernatant fraction, which contains most of the cytosol, as indicated by the hexokinase enzyme. The mitochondrial location of Mas6p was also seen in immunofluorescent labeling of permeabilized yeast cells (not shown), and in immunofluorescent labeling of mammalian COS-7 cells transiently expressing Mas6p (Jensen, R., unpublished results).

Several observations demonstrate that Mas6p is an integral membrane protein located in the mitochondrial inner membrane. First, Mas6p could not be extracted from mitochondrial membranes with 0.1 M sodium carbonate, whereas F1\(\beta\), a peripheral membrane protein, was readily extracted with carbonate (not shown). Second, when mitochondria were disrupted by sonication and the membrane vesicles separated on sucrose gradients, Mas6p cofractionated with the inner membrane-bound F1\(\beta\) protein (Fig. 5), as well as the inner membrane Cox4p and the ATP/ADP carrier protein (not shown). Little or no Mas6p was found in the sucrose gradient fractions that contained OM45p, a mitochondrial outer membrane protein (Yaffe et al., 1989). Third, immunoelectron microscopy of mitochondrial sections showed that Mas6p was located in the inner membrane, along with the F1\(\beta\) protein (Fig. 6). Essentially no Mas6p was found to colocalize with OM45p on the mitochondrial surface. Fourth, Mas6p synthesized by transcription/translation of the cloned gene was imported into the inner membrane of isolated mitochondria (not shown).

Mitochondria Isolated from mas6 Mutants Are Defective at an Early Stage in the Import Pathway

To determine the step in import that is defective in mas6 mutants, mitochondria were isolated from mas6 strains and tested for the ability to import \(^35\)S-labeled precursor proteins. The Cox4p precursor was efficiently imported and processed to the mature form in wild-type mitochondria at 23, 30, and 37°C (Fig. 7 A). In contrast, mas6 mitochondria were defective for import of Cox4p at all temperatures. Similarly, mas6 mitochondria were defective in the import of the F1\(\beta\) precursor protein, at 23°C (Fig. 7 B), and at 30 and 37°C (not shown). We also found that the import of two additional precursors, citrate synthase and cytochrome c1, was defective in mas6 mitochondria (not shown). Although mas6 strains are temperature-sensitive for viability, isolated mas6 mitochondria are defective in import even at room temperature. We suggest that the altered Mas6 protein is more labile after subcellular fractionation than in intact cells. Similarly, mitochondria isolated from temperature-sensitive masl strains are defective in import at both the permissive and restrictive temperatures (Yaffe et al., 1985).

In contrast to wild-type mitochondria, very little Cox4p or F1\(\beta\) protein pellets with the mas6 mitochondria after the import reaction (compare total recovery of precursor and mature in wild-type and mas6 mitochondria in Fig. 7). Hence the import defect in mas6 mutants appears to be early in the import pathway. The mas6 import defect, however, does not seem to be due to a defect in the initial binding of precursors to the mitochondrial surface. In particular, previous studies have shown that precursor binding to mitochondrial outer membrane import components does not require
Mitochondria isolated from mas6 mutants are defective at an early stage of the import pathway. Mitochondria were isolated from mas6 and wild-type AH216 cells and incubated with an [35S]-labeled Cox4p precursor (A) or the precursor to the F\textsubscript{1}-ATPase β subunit, F\textsubscript{1}β (B) at the indicated temperatures. After 20 min, mitochondria were reisolated by centrifugation, and proteins were solubilized in SDS-sample buffer. Proteins were separated on SDS-polyacrylamide gels, and the radiolabeled Cox4p and F\textsubscript{1}β proteins were identified by fluorography. -Δψ indicates no inner membrane potential due to the addition of valinomycin to the import reaction. Precursor (p) and mature (m) forms of the imported proteins are indicated. 20% of the precursor added to each import reaction is also shown.

mas6 Mutants Are Directly Blocked in the Import Pathway

We have shown that the import defect in mas6 mutants is not an indirect effect, for example due to defective mitochondrial energy metabolism. (a) The potential across the mitochondrial inner membrane, measured with a potential-sensitive fluorescent dye (Sims et al., 1974; Eilers et al., 1987), was not significantly different from that of wild-type mitochondria (Fig. 8). The addition of the respiratory substrates, succinate and malate, elicited a near maximal potential in both wild-type and mas6 mitochondria.

mas6 Mutants Are Directly Blocked in the Import Pathway

We have shown that the import defect in mas6 mutants is not an indirect effect, for example due to defective mitochondrial energy metabolism. (a) The potential across the mitochondrial inner membrane, measured with a potential-sensitive fluorescent dye (Sims et al., 1974; Eilers et al., 1987), was not significantly different from that of wild-type mitochondria (Fig. 8). The addition of the respiratory substrates, succinate and malate, elicited a near maximal potential in both wild-type and mas6 mitochondria.

mas6 and wild-type mitochondria. (b) Coupling ratios measured with an oxygen electrode (Yaffe et al., 1985) showed no significant differences between wild-type, mas1, and mas6 mitochondria (not shown). (c) Respiration-driven protein synthesis (Yaffe and Schatz, 1984), and aliquots containing 80 μg protein were run on SDS–polyacrylamide gels. The Mas6 protein and the F\textsubscript{1}-ATPase α subunit were identified by immune blotting. Relative amounts of the Mas6p (●), and the precursor form of the F\textsubscript{1}α protein (●) were determined by densitometry.

Depletion of MAS6 from Cells Results in the Accumulation of Mitochondrial Precursor Proteins

mas6 mutants are defective in import presumably due to the inactivation of Mas6p at the restrictive temperature, 37°C. To determine the effect of Mas6p inactivation at lower temperatures, we placed the MAS6 gene under the control of the galactose-inducible GAL1 promoter region. We introduced this construct into cells deleted for the chromosomal MAS6 gene and the pGAL-MAS6 plasmid and grew them on YEP-galactose medium. At the indicated times, cultures were harvested, and cell pellets were resuspended in glucose medium to an OD\textsubscript{600} of 0.1. The indicated times, total cell proteins were extracted (Yaffe and Schatz, 1984), and aliquots containing 80 μg protein were run on SDS–polyacrylamide gels. The Mas6 protein and the F\textsubscript{1}-ATPase α subunit were identified by immune blotting. Relative amounts of the Mas6p (●), and the precursor form of the F\textsubscript{1}α protein (●) were determined by densitometry.

Figure 8. mas6 mitochondria are not defective in establishing or maintaining the potential across the inner membrane. Mitochondria isolated from mas6 or wild-type AH216 cells were incubated with the potential sensitive dye, diS-C\textsubscript{3}-(5), and the fluorescence was recorded. A downward deflection indicated an increase in the inner membrane potential. The following were added to the mitochondria as indicated: ATP (2 mM); potassium malate (20 mM), potassium succinate (15 mM); and the potassium ionophore, valinomycin (10 μg/ml).

Figure 9. Cells depleted of the Mas6 protein accumulate mitochondrial precursor proteins. Cells carrying the mas6::Thi10-LUK disruption and the pGAL-MAS6 plasmid were grown at 30°C on YEP-galactose medium to an OD\textsubscript{600} of 1.0. Cells were centrifuged and pellets were resuspended in glucose medium (YPEP) to an OD\textsubscript{600} of 0.1. At the indicated times, total cell proteins were extracted (Yaffe and Schatz, 1984), and aliquots containing 80 μg protein were run on SDS–polyacrylamide gels. The Mas6 protein and the F\textsubscript{1}-ATPase α subunit were identified by immune blotting. Relative amounts of the Mas6p (●), and the precursor form of the F\textsubscript{1}α protein (●) were determined by densitometry.
gene (see Materials and Methods), and examined mitochondrial protein import when the expression of MAS6 was inhibited. When cells that contain the GALI-MAS6 construct were grown in galactose-containing medium, Mas6p was overproduced ~10–20-fold (not shown). When these cells were shifted to glucose medium, which inhibits the expression of MAS6, a striking correlation between the level of Mas6p and mitochondrial protein import was seen (Fig. 9). 7 h after shifting to glucose-containing medium, the amount of Mas6p was slightly below wild-type levels. By 12 h, Mas6p was no longer detectable by immune blotting. Concomitant with the loss of Mas6p, the precursor to the α subunit of the F_{1}-ATPase protein accumulated. This defect in mitochondrial protein import was not simply due to cell inviability since cells containing the GALI-MAS6 construct continued to divide for at least 24 h after their shift to glucose-containing medium (not shown). In similar experiments, we found that import of the F_{1}/β and Cox4 proteins was blocked when expression of MAS6 was inhibited by glucose (not shown). Therefore, depletion of Mas6p from cells leads to a defect in mitochondrial protein import.

Antibodies that Recognize the MAS6 Protein Inhibit Import into Mitoplasts

Our studies with different mas6 mutants suggest that Mas6p is an essential import component. To test this conclusion, we asked whether antibodies directed against the Mas6p protein inhibit mitochondrial protein import in vitro. Since MAS6 encodes an inner membrane protein, we examined import of precursors into mitoplasts, mitochondria whose outer membranes have been disrupted. In mitoplasts, precursors can be translocated directly across the inner membrane, bypassing outer membrane import components (Ohba and Schatz, 1979b; Hwang et al., 1989). For our inhibition experiments, we inactivated outer membrane import components by digestion with trypsin, and mitoplasts were then formed by breaking open the outer membrane by osmotic shock (see Materials and Methods). When 7 μg Mas6p IgG was added to 100 μg of mitoplasts, a slight decrease in the import of F_{1}/β was seen (Fig. 10). 35 μg of Mas6p IgG markedly inhibited import, and increasing the amount of Mas6p IgG to 70 μg led to a virtual block in import, with little or no mature-sized F_{1}/β protein produced. 120 μg of preimmune IgG did not inhibit the import of F_{1}/β. We obtained similar results when we examined the import of another precursor, Cox4 (not shown). As an additional control, we showed that neither Mas6p IgG nor preimmune IgG inhibited the import of precursors into mitochondria whose outer membranes remained intact (Fig. 10). Therefore, the inhibition of import with Mas6p antibodies complements our studies with the mas6 mutants described above, and strongly suggests that the Mas6p protein is an inner membrane component acting directly in the import pathway.

Discussion

We have analyzed a new import mutant, mas6, which carries a mutation that causes both temperature-sensitive growth and defective mitochondrial protein import. Several genetic observations show that MAS6 encodes an essential import component: (a) the mas6 mutant is a temperature-sensitive lethal and accumulates the precursor form of several imported mitochondrial proteins; (b) mitochondria isolated from mas6 cells are defective in the import of at least four different proteins; and (c) depletion of Mas6p from cells results in cell death and a defect in the import of mitochondrial precursor proteins.

Our results strongly suggest that MAS6 plays a direct role in import, and that the import defect in mas6 mutants is not due to a defect in some other mitochondrial function such as energy metabolism. First, we showed that mas6 strains, and mitochondria isolated from mas6 mutants, are not markedly defective in establishing or maintaining the potential across the inner membrane. Second, we find that wild-type Mas6p function can be inhibited in mitoplasts using IgG directed against Mas6p. Under these conditions we also find no detectable decrease in inner membrane potential (Emtiage, J., unpublished data). Third, we find that MAS6 is essential for viability, even when cells are grown on glucose-containing medium. All previously characterized mitochondrial components that are required for electron transport and ATP synthesis are only necessary for growth on nonfermentable carbon sources (Tzagoloff and Dieckmann, 1990). Hence MAS6 joins a family of essential mitochondrial proteins (Baker and Schatz, 1991), all of which are components of the mitochondrial protein import pathway: MAS7 (Witte et al., 1988) and MAS2 (Jensen and Yaffe, 1988), subunits of the matrix-localized processing protease; MAS4 (Cheng et al., 1989), a groEL homologue located in the matrix; MAS5 (Kang et al., 1990), a matrix-localized chaperonin protein; and MAS7 (Baker et al., 1990), an import component located in the outer membrane; and MAS11 (Maarse et al., 1992), a membrane-bound protein required for import. Since we have localized Mas6p to the inner membrane, MAS6 represents the first essential inner membrane import component.

The Mas6p protein, predicted from its DNA sequence, contains two domains. The amino-terminal half of Mas6p is hydrophilic, and the carboxy-terminal half contains several potential membrane spanning regions. Preliminary experiments using "epitope-tagged" Mas6p constructs suggest that the extreme carboxy terminus and the hydrophilic amino-terminal domain of Mas6p face the intermembrane space.
Hence Mas6p appears to be anchored in the inner membrane with its amino-terminal domain facing the outer membrane. In addition, the Mas6p protein does not itself contain a typical amino-terminal presequence, and following import into the inner membrane, Mas6p is not processed to a lower molecular weight form. Therefore, the signal to target Mas6p to the mitochondria and to localize it to the inner membrane must be located within the mature protein. Preliminary experiments have shown that at least one targeting signal in Mas6p is carried within its first putative transmembrane domain (Ryan, K., unpublished observations).

When an in vitro synthesized precursor protein such as Cox4p or the F1β protein is incubated with mitochondria isolated from mas6 strains, very little precursor or mature-sized protein pellets with mas6 mitochondria whose inner membrane potential has been dissipated with valinomycin. These results suggest that mas6 mitochondria are defective at a step in the import pathway after the binding of precursors to outer membrane components. Apparently in mas6 mitochondria, the precursor binds to outer membrane components, but falls off the mitochondria when the precursor reaches the defective Mas6p protein. Consistent with this hypothesis, time course of import studies with mas6 mitochondria indicate that the imported precursor initially binds to the mitochondrial surface, but fails to pellet with the mitochondria at later times during import (Emtage, J., unpublished observations). Since MAS6 encodes an inner membrane protein, our results raise the exciting possibility that import may be reversible even after the precursor has penetrated the outer membrane import machinery. It is important to note, however, that we have not directly shown that the precursor initially bound to the surface of mas6 mitochondria is subsequently released. Consequently, we cannot exclude the possibility that the radiolabeled precursor is simply being degraded by mas6 mitochondria.

There are several possible roles for the Mas6p protein in import. For example, Mas6p may be required for the binding of precursors to the inner membrane translocation machinery. The amino terminus of MAS6 contains a large number of acidic amino acids (17%), with some of these aspartate and glutamate residues clustered. We speculate that one of the functions of Mas6p may be to interact with the arginine- and lysine-rich presequences of imported mitochondrial proteins as they come through the outer membrane translocation machinery. Another possibility is that Mas6p may translocate precursor proteins across the inner membrane. Mas6p is an integral membrane protein containing several potential membrane spanning domains. Hence Mas6p may form part of a protein-translocating channel in the inner membrane. Furthermore, since half of the Mas6p protein is predicted to reside in the membrane, while the other half is hydrophilic, Mas6p may have more than one function.

Since Mas6p is an essential inner membrane import component, it provides a valuable tool to learn more about the mechanism by which proteins are translocated into mitochondria. Experiments are in progress to determine the step in the import pathway in which precursors come into physical contact with the Mas6p protein. For example, we have recently shown that a precursor partially translocated across the inner membrane can be chemically cross-linked to Mas6p (Ryan, K., and R. Jensen, manuscript in preparation). Furthermore, since protein import through the mitochondrial outer membrane requires many different proteins (Kiebler et al., 1990; Söllner et al., 1992), we anticipate that Mas6p does not act alone in the translocation of proteins through the inner membrane. Hence we are using both biochemical and genetic approaches to identify new inner membrane proteins with which Mas6p may interact.

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