MAS6 Encodes an Essential Inner Membrane Component of the Yeast Mitochondrial Protein Import Pathway

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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 (Pfaller and Neupert, 1987; Söllner 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 (McAda and Douglas, 1982; Böhni 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 (Deshaies 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 (Atencio 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, *MPI1*, 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 (Ohba and Schatz, 1987*a*; 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 (Vestweber 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 ISP42 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; Kiebler et al., 1990). The yeast *MAS70* 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 (*MATa leu2-3 leu2-112*) and the *mas2* and *mas6* mutants have been described previously (Yaffe and Schatz, 1984). *mas6* strains JE4-3c (*MATa mas6-1 leu2-3 leu2-112*) and JE8-1b (*MATα mas6-1 leu2-3 leu2-112*) were obtained by backcrossing the *mas6* mutant to *AH216*. *JE14-5b* (*MATa mas6-1 trp1*) was isolated by crossing JE8-1b to *MATa trp1* strain YPH250 (Sikorski and Hieter, 1989). *MATa/MATα ura3-52/ura3-52* strain YPH501 (Sikorski and Hieter, 1989), *MATa/MATα 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., 1982) were used.

Cell Labeling and Immunoprecipitation

Yeast cells to be labeled were grown to an OD_{600} 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 OD_{600} 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 ^{35}S-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 ml TNET (150 mM sodium chloride; 5 mM EDTA; 1% Triton X-100, 50 mM Tris-HCl, pH 8.0). Samples were centrifuged at 12,500 *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 Suissa, 1983), and visualized by fluorography (Chamberlain, 1979).

Isolation of the MAS6 Gene

mas6 trp1 strain JE14-5b was transformed with a library of random yeast genomic sequences in the *TRP1–CEN6* vector pRS200 (Connelly, C., and P. Hieter, unpublished data) as described (Schiestl and Gietz, 1989). Trp^+ transformants were selected at 22°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-kbp *SacI–BamHI* fragment (see Fig. 2).

To show that pJE1 contained *MAS6* sequences, a 4.2-kb XhoI-BamHI fragment was subcloned into the *LEU2*-containing vector pRS305 (Sikorski 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 leu2* strain AH216. Stable Leu^+ integrants were crossed to *mas6* strain JE8-1b, and the meiotic products of the diploid were analyzed. In 38 tetrads, no recombination between *LEU2* and *MAS6* was found indicating that the *LEU2*-containing plasmid, pJE4, had integrated within 1.3 map units of *MAS6*.

DNA Sequence of MAS6

pJE2 and pJE3 were constructed by inserting the 2.2-kbp SacI fragment of *MAS6* into plasmid pRS200 (Sikorski, 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 α-phosphorothioate nucleotides (Stratagene, La Jolla, CA) using DNA polymerase (Putney et al., 1981). *MAS6* sequences carried on pJE2 and pJE3 were exposed to exonuclease digestion by ClaI digestion. Single-stranded DNA was removed by S1 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′-ATTAACCCTCACTAAAG-3′), oligonucleotide No. 60 (5′-GGGCGGCCGCTCATGATTGTGTGTGATCT-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′-AATACGACTCACTATAG-3′), and oligonucleotide No. 59 (5′-GGGGCGGCCGCTCGTGGCTTTTTGGAGAT-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 (Sikorski and Hieter, 1989) to form plasmid pJE5. pJE5 contains a unique NotI site immediately following the start 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′-AATACGACTCACTAT-3′) and oligonucleotide No. 51 (5′-GCGGCGGCCGCTGAGCAACACAAGAACCT-3′). Similarly, PCR fragment D containing the *MAS6* ORF and upstream sequences was isolated using oligonucleotide No. 21 (5′-ATTAACCCTCACTAAAG-3′) and oligonucleotide No. 50 (5′-CGGGCGGCCGCCTTTTTCAAGTAGTCTTTT-3′). PCR 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 NotI–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 pJE9 to form plasmid pJE10. *mas6*::*URA3*, carried on a 2.7-kb SacI–KpnI fragment, was used to replace one copy of *MAS6* in the *MATa/MATα* diploid strains SM1060 or YPH501 (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, pJE11, which contains *MAS6* on a 4.2-kb ClaI–BamHI fragment in plasmid pRS200, was transformed into bacterial strain DB1329. DB1329 was then infected with phage λNK1224, which contains the Tn10-LUK transposon. Transposon "hops" onto the *MAS6*-containing plasmid were selected, and 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 (Tn10-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 NotI–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 fusion protein, and crude 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, and 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 pellet was resuspended in 0.5 ml 0.45 M sucrose, 45 mM mannitol, 20 mM Hepes-KOH, pH 7.4. 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 2× 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 (Pon et al., 1989). Mitochondria were then pelleted for 3 min at 12,500 *g*, and the mitochondrial pellet was fixed in 2× 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 MS buffer (0.1 M sodium maleate, 4% sucrose), stained with 2% uranyl acetate in MS buffer for 60 min, and washed once in MS 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 (with 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.

Ultrathin sections of mitochondria were obtained using a Diatome diamond knife (Diatome U.S., Fort Washington, 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 $F_1\beta$ proteins diluted 1:500 in 1% BSA/TBS with .05% 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* strains as described (Yaffe et al., 1985; Jensen and Yaffe, 1988), except that 1 μg/ml aprotinin and leupeptin were used in addition to 1 mM PMSF. Mitochondria were either used immediately or after storage at −70°C (Murakami et al., 1988). Mitochondrial precursor proteins were transcribed from cloned genes using SP6 polymerase (Melton et al., 1984) and translated in the presence of [^{35}S]methionine using reticulocyte lysate (Promega, Madison, WI) according to the manufacturer's directions. Import reactions contained 100μg mitochondrial protein and ~0.5 μCi precursor protein (corresponding to ~0.1 pmol) in a total volume of 100 μl. Import buffer contained 0.6 M mannitol, 15% rabbit reticulocyte lysate, 50 mM potassium chloride, 1 mM magnesium chloride, 1.2 mM GTP, 1 mM ATP, 1 mM phosphoenol pyruvate, 1 mM methionine, 15 mM potassium succinate, 20 mM potassium malate, 20 mM Hepes-KOH, pH 7.4, and 0.1 mg/ml rabbit pyruvate kinase (Boehringer Mannheim Corp., Indianapolis, IN). Mitoplasts were prepared by diluting mitochondria with 9 vol of 20 mM Hepes-KOH, pH 7.4, followed by incubation on ice for 25 min. Mitoplasts were recovered by centrifugation (12,500 *g* for 10 min), and were resuspended in import buffer. Import reactions were terminated by cooling the reaction tubes on ice. After import, mitochondria and mitoplasts were reisolated by centrifugation through 1 ml 0.625 M sucrose, 20 mM Hepes, pH 7.4. Proteins were separated by SDS-PAGE, and visualized by fluorography.

Measurement of Mitochondrial Inner Membrane Potential

The inner membrane potential of mitochondria isolated from wild-type cells and *mas6* mutants was measured as described (Eilers 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 939-bp *MseI* 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 *HincII* site of the Bluescript II SK$^+$ plasmid (Stratagene) to form pBT1. The *MAS6* gene was excised from pBT1 by XhoI–BamHI digestion and inserted downstream of the *GAL1* promoter in plasmid pRS314GU (Nigro et al., 1992) to form the plasmid pGAL-MAS6. To construct a strain dependent on pGAL-MAS6 expression, a SM1060 diploid heterozygous for the *mas6*::*Tn10-LUK* disruption (see above) was transformed with a plasmid that carries *MAS6* on the *LEU2*-containing plasmid pRS315 (Sikorski and Hieter, 1989). The diploid was sporulated, and a haploid segregant was isolated that contained both the *mas6*::*Tn10-LUK* disruption 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

purified as described (Ey et al., 1978; Harlow and Lane, 1988). Briefly, 1 ml serum was heated to 55°C for 20 min to inactivate complement, and then mixed with 10 ml 0.1 M Tris-HCl, pH 8.0. Serum was passed through a 2-ml column of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), and then through a 0.5 ml column of protein A–Sepharose CL-4B (Pharmacia). The Protein A column was washed with 10 vol 100 mM Tris-HCl, pH 8.0, followed by a wash with 10 vol 10 mM Tris-HCl, pH 8. IgGs were eluted from the protein A column by the addition of 100 mM glycine, pH 3.0. 0.5-ml fractions were collected and neutralized with 50 μl 1 M Tris-HCl, pH 8. IgG-containing fractions were pooled and dialyzed against PBS (140 mM sodium chloride, 10 mM sodium phosphate, pH 7.4). IgG was concentrated to ~10 mg/ml using Centricon 30 columns (Amicon Corp., Danvers, MA) according to manufacturer's instructions.

Antibody Inhibition Using Mitochondria. Mitochondria were isolated from wild-type strain AH216, and aliquots representing 100 μg of mitochondrial proteins in 100 μl import buffer (see above) were preincubated with either 70 μg Mas6p IgG, or 120 μg IgG isolated from preimmune serum. After a 90-min incubation on ice, an ^{35}S-labeled precursor to the $F_1\beta$ protein was added, and the reaction tubes shifted to 25°C for 20 min. Mitochondria were reisolated by centrifugation, proteins subjected to SDS-PAGE, and the $F_1\beta$ protein identified by fluorography.

Antibody Inhibition Using Mitoplasts. Isolated mitochondria were incubated with 1 mg/ml trypsin for 30 min on ice to inactivate outer membrane import components (Ohba and Schatz, 1987*a*). After the addition of soybean trypsin inhibitor (STI) to 10 mg/ml, the mitochondria were isolated by centrifugation and resuspended in breaking buffer (0.6 M mannitol, 20 mM Hepes-KOH, pH 7.4) containing 1 mg/ml STI. The mitochondrial outer membrane was disrupted by osmotic shock and the mitoplast pellet recovered by centrifugation as described above. Aliquots representing 100 μg of mitoplast proteins in 100 μl import buffer were preincubated with the indicated amounts of Mas6p IgG, or 120 μg IgG isolated from preimmune serum. After a 90-min incubation on ice, the ^{35}S-labeled $F_1\beta$ protein was incubated with the mitoplasts for 20 min at 30°C, and analyzed by SDS-PAGE and fluorography.

Miscellaneous

pSP6-COX4, a plasmid for expressing the cytochrome oxidase subunit IV protein (Cox4p) by transcription/translation was obtained from D. Allison (University of Washington, Seattle, WA). pSP6-$F_1\beta$, a plasmid expressing the β-subunit of F_1-ATPase was obtained from D. Bedwell (University of Alabama, Birmingham, AL). The injection of MBS–MAS6 fusion protein into rabbits, and the isolation of antiserum was carried out by Hazleton Research Products, Inc. (Denver, PA). Standard methods were used for restriction endonuclease digestions, DNA ligations, bacterial transformations, and small-scale plasmid isolations from bacteria. The *MAS6* DNA and protein sequences were compared with sequences contained in the GenBank (ver. 73.1), EMBL (ver. 32), PIR-Protein (ver. 34), PIR-Nucleic (ver. 36), Swiss-Prot (ver. 23), and VecBas3 (ver. 3) databases using the Fasta and tFasta algorithms (Lipman and Pearson, 1985).

Results

mas6 Mutants Are Defective in the Import of 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 ($F_1\beta$) and the citrate synthase protein (not shown).

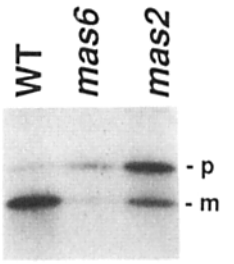

Figure 1. mas6 mutants accumulate the precursor form of an imported mitochondrial protein. Wild-type AH216, *mas6*, and *mas2* cells were grown to midlogarithmic phase, and shifted to 38°C for 30 min. Cells were labeled with [^{35}S]methionine for 4 min, and proteins were extracted and precipitated with antiserum to subunit IV of the cytochrome oxidase complex (Cox4p). Immunoprecipitates were separated on 15% SDS–polyacrylamide gels, and fluorographed. The precursor (*p*) and mature (*m*) forms of Cox4p are indicated.

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 *ssc1* (Kang et al., 1990). Furthermore, a plasmid encoding the ISP42 protein failed to rescue the temperature-sensitive growth defect of our *mas6* mutant (J. Emtage 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. 3 *A*), 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-LUK (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. 3 *B*). The carboxy-terminal half of Mas6p contains several potential membrane spanning domains. In con-

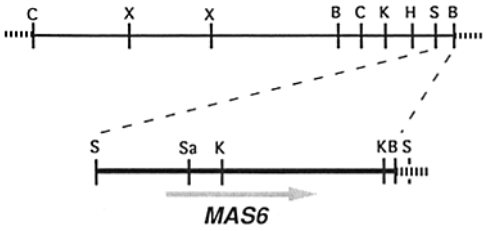

Figure 2. Restriction endonuclease map of the cloned *MAS6* gene. The top solid line indicates the relevant restriction sites in the 18-kpb insert carried in plasmid pJE1. The lower solid line shows an enlarged view of the 2.2-kbp fragment shown by subcloning to carry the *MAS6* gene. The striped lines indicate adjacent vector sequences. The shaded arrow at the bottom represents the approximate location of the *MAS6* gene. Restriction endonuclease sites: *B*, BamHI; *C*, ClaI; *K*, KpnI; *S*, SacI; *Sa*, SalI; and *X*, XhoI.

trast, the amino-terminal half of Mas6p is hydrophilic, with an unusual number of acidic residues. A comparison of *MAS6* with sequences in the available databases failed to identify any significant homologies.

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 Tn10-LUK insertion.

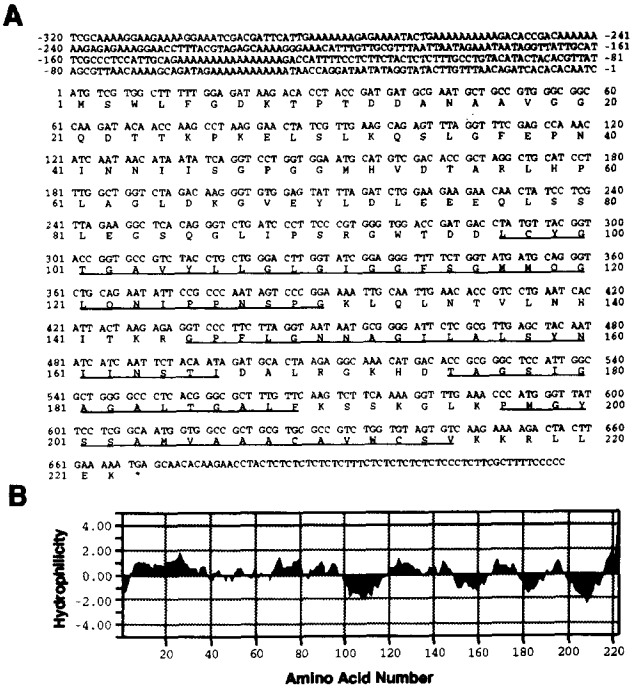

Figure 3. MAS6 encodes a 23.2-kD protein with several potential membrane-spanning domains. (*A*) DNA sequence of *MAS6* and its predicted protein product. Underlined amino acids represent potential *trans*-membrane domains predicted from hydropathy analysis. (*B*) Hydropathy plot of the predicted *MAS6* product. The Mas6 protein was analyzed using the algorithm of Kyte and Doolittle (1982) with a window size of 12. Hydrophobic stretches in Mas6p are indicated as shaded areas with negative values. These sequence data are available from EMBL/GenBank/DDBJ under accession number X71633.

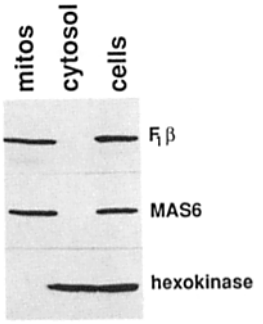

Figure 4. Mas6p is a mitochondrial protein. AH216 cells were grown to the midlogarithmic stage in semisynthetic medium containing 2% lactate, converted to spheroplasts, and homogenized. The homogenate (*cells*) was centrifuged at 2,500 *g* for 5 min, and the supernatant fractionated into a mitochondrial pellet (*mitos*), and a postmitochondrial supernatant (*cytosol*) by centrifugation at 9,600 *g* for 10 min. Aliquots of homogenate, mitochondria, and cytosol representing equivalent numbers of cells were subjected to SDS-PAGE and analyzed by immune blotting with antiserum to Mas6p, hexokinase, and the F_1-ATPase β subunit ($F_1\beta$).

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 Mas6 protein. When this antiserum was tested against total yeast proteins by immune

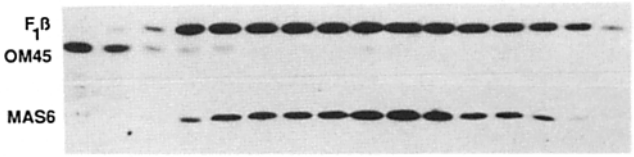

Figure 5. The Mas6 protein fractionates with the inner membrane. Mitochondria were converted to mitoplasts and sonicated (see Materials and Methods). Mitochondrial membrane vesicles were loaded onto sucrose step gradients, and centrifuged at 100,000 *g* for 17 h. Fractions were collected, and an aliquot from each fraction analyzed by immune blotting with antiserum to Mas6p, the outer membrane OM45 protein, and the inner membrane $F_1\beta$ protein. The top of the gradient is to the left.

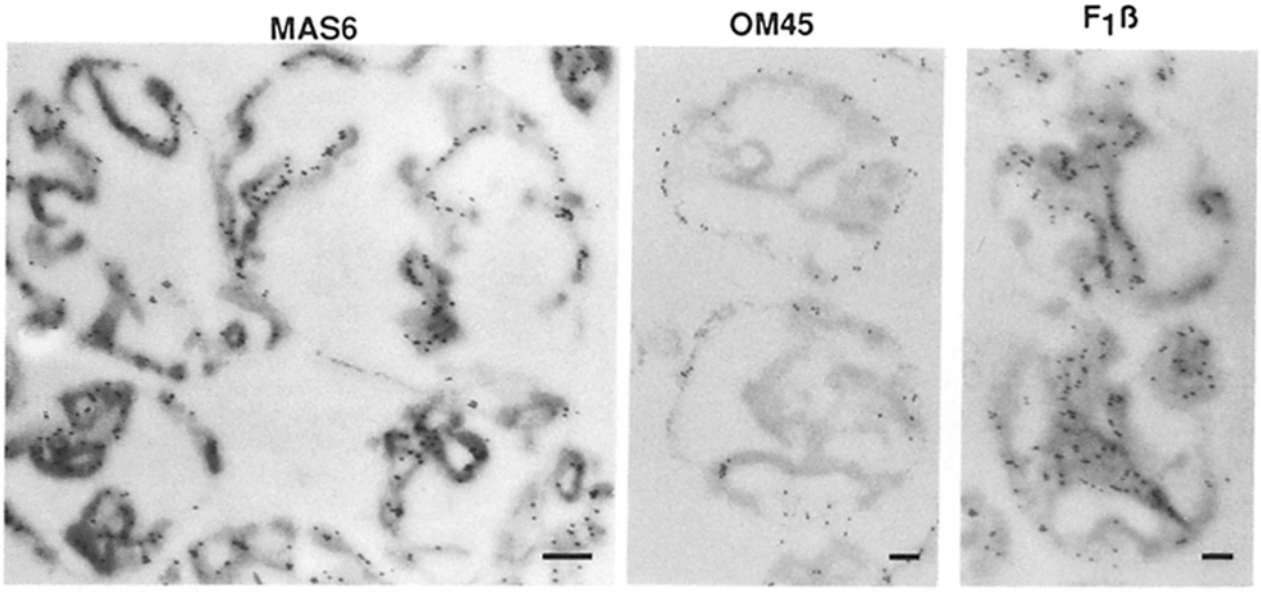

Figure 6. Immunoelectron microscopy indicates that Mas6p is a mitochondrial inner membrane protein. Mitochondria were swelled, fixed and stained as described in Materials and Methods. After imbedding in LR White resin, ultrathin sections of mitochondria were taken and decorated with antiserum to Mas6p, OM45 (an outer membrane protein), and $F_1\beta$ (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 *GAL1* 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 $F_1\beta$ 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 $F_1\beta$, 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 $F_1\beta$ 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 $F_1\beta$ 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 $F_1\beta$ 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 c_1, 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 *mas1* 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 $F_1\beta$ 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

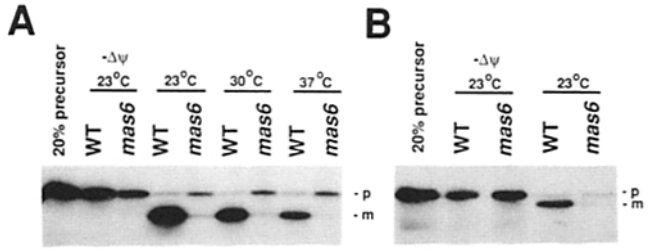

Figure 7. Mitochondria isolated from *mas6* mutants are defective at an early stage of the import pathway. Mitochondria were isolated from *mas6* and wild-type AH216 cells and incubated with an ^{35}S-labeled Cox4p precursor (*A*) or the precursor to the F_1-ATPase β subunit, $F_1\beta$ (*B*) at the 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_1B proteins were identified by fluorography. $-\Delta\psi$ 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.

an inner membrane potential (Pfaller and Neupert, 1987; Söllner et al., 1989, 1990; Hines et al., 1990). We found that when mitochondria were deenergized with valinomycin (Fig. 7, $-\Delta\psi$), similar amounts of Cox4p or the $F_1\beta$ precursor protein pelleted with 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

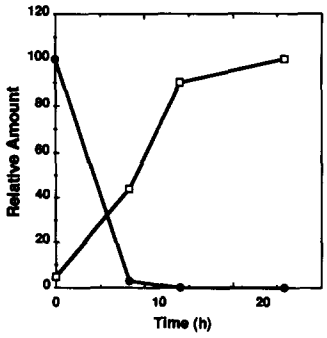

Figure 9. Cells depleted of the Mas6 protein accumulate mitochondrial precursor proteins. Cells carrying the *mas6::Tn10-LUK* disruption and the pGAL-MAS6 plasmid were grown at 30°C on YEP-galactose medium to an OD_{600} of 1.0. Cells were centrifuged and pellets were resuspended in glucose medium (YEPD) to an OD_{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_1-ATPase α subunit were identified by immune blotting. Relative amounts of the Mas6p (●), and the precursor form of the $F_1\alpha$ protein (□) were determined by densitometry.

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) was not temperature-sensitive in *mas6* strains (not shown). Finally, we found that *mas6* mitochondria are not deficient in any of their major membrane phospholipids (not shown). All of our observations, therefore, indicate that *MAS6* is directly involved in the mitochondrial protein import pathway.

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*

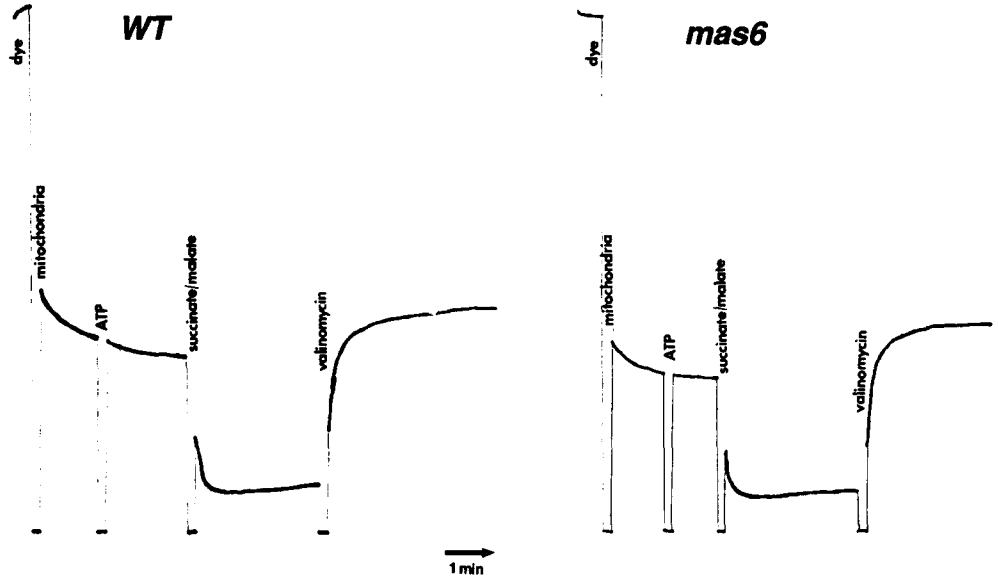

Figure 8. mas6 mitochondria are not defective in establishing or maintaining the potential across the inner membrane. Mitochondria isolated from *mas6* or wild-type AH216 cells were incubated with the potential sensitive dye, diS-C3-(5), and the fluorescence was recorded. A downward deflection indicated 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).

gene (see Materials and Methods), and examined mitochondrial protein import when the expression of *MAS6* was inhibited. When cells that contain the *GAL1-MAS6* construction 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 *GAL1-MAS6* construction 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 $F_1\beta$ 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.

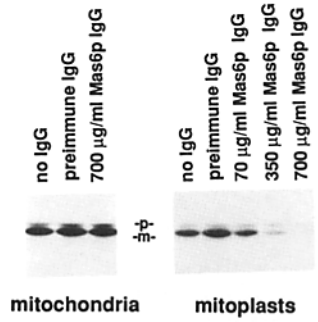

Figure 10. Antibodies directed against the Mas6 protein inhibit import into mitoplasts, but do not inhibit import into intact mitochondria. Mitoplasts were formed by first inactivating surface import components on mitochondria with trypsin, and then disrupting the mitochondrial outer membranes by osmotic shock (see Materials and Methods). Mitoplasts or intact mitochondria representing 100 μg of total mitochondrial protein were preincubated at 0°C with the indicated amounts of Mas6p IgG, or with 120 μg IgG isolated from preimmune serum. Subsequently, an ^{35}S-labeled precursor to the $F_1\beta$ protein was added, and the reaction tubes shifted to 25°C for 20 min. After the import reaction, mitochondria and mitoplasts were reisolated by centrifugation, proteins subjected to SDS-PAGE, and the $F_1\beta$ protein identified by fluorography. The precursor (*p*) and mature (*m*) forms of $F_1\beta$ are indicated.

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 Mas6 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 membranes 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 $F_1\beta$ was seen as compared to mitoplasts not treated with IgG (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 $F_1\beta$ protein produced. 120 μg of preimmune IgG did not inhibit the import of $F_1\beta$. We obtained similar results when we examined the import of another precursor, Cox4p (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 Mas6 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 (Emtage, 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: *MAS1* (Witte et al., 1988) and *MAS2* (Jensen and Yaffe, 1988), subunits of the matrix-localized processing protease; *MIF4* (Cheng et al., 1989), a groEL homologue located in the matrix; *SSC1* (Kang et al., 1990), a matrix-localized chaperonin protein; *ISP42* (Baker et at., 1990), an import component located in the outer membrane; and *MPI1* (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 Mas6 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

(Emtage, J., and J. Kalish, unpublished data). Hence Mas6p appears to be anchored in the inner membrane with its amino-terminal domain facing the outer membrane. In addition, the Mas6 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 $F_1\beta$ protein is incubated with mitochondria isolated from *mas6* strains, very little precursor or mature-sized protein pellets with the mitochondria after the import reaction (see Fig. 8). In contrast, a significant amount of precursor 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 Mas6 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 (Ematge, 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 Mas6 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 Mas6 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 Mas6 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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