The EMBO Journal vol.14 no.24 pp.6318–6326, 1995

Acidic receptor domains on both sides of the outer membrane mediate translocation of precursor proteins into yeast mitochondria

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Mitochondrial precursor proteins made in the cytosol bind to a hetero-oligomeric protein import receptor on the mitochondrial surface and then pass through the translocation channel across the outer membrane. This translocation step is accelerated by an acidic domain of the receptor subunit Mas22p, which protrudes into the intermembrane space. This *'trans'* domain of Mas22p specifically binds functional mitochondrial targeting peptides with a K_d of <1 μM and is required to anchor the N-terminal targeting sequence of a translocation-arrested precursor in the intermembrane space. If this Mas22p domain is deleted, respiration-driven growth of the cells is compromised and import of different precursors into isolated mitochondria is inhibited 3- to 8-fold. Binding of precursors to the mitochondrial surface appears to be mediated by cytosolically exposed acidic domains of the receptor subunits Mas20p and Mas22p. Translocation of a precursor across the outer membrane thus appears to involve sequential binding of the precursor's basic and amphiphilic targeting signal to acidic receptor domains on both sides of the membrane.

Keywords: biogenesis/mitochondrial protein import/organelle/protein targeting/*Saccharomyces cerevisiae*

Introduction

Transport of proteins into mitochondria is mediated by two independent protein transport systems, one in the outer and the other in the inner membrane. These systems can act independently of each other, but can reversibly link up during the transport of a protein across both membranes (Glick *et al.*, 1991; Pfanner *et al.*, 1992; Horst *et al.*, 1995).

The energetics of protein transport across the inner membrane are reasonably well understood (Neupert *et al.*, 1990; Martin *et al.*, 1991; Beasley *et al.*, 1992). Transport is initiated by an electrochemical transmembrane potential which inserts the basic and amphiphilic targeting sequence across the membrane; translocation of the remainder of the polypeptide chain is then powered by an ATP-driven 'import motor', which is composed of matrix-located hsp70, the co-chaperone GrpEp (Yge1p) and the membrane protein Isp45 (MIM44). In contrast, protein transport across the outer membrane requires neither a transmembrane electrochemical potential nor ATP hydrolysis; it is thus not clear how this transport is energized.

The evidence reported here suggests that protein transport across the outer membrane is facilitated by sequential binding of a precursor's basic targeting sequence to acidic protein domains on both sides of the outer membrane. We show that the acidic intermembrane space domain of the import receptor Mas22p (Kiebler *et al.*, 1993a; Lithgow *et al.*, 1994; Nakai and Endo, 1995) specifically binds mitochondrial targeting sequences with high affinity and that deletion of this domain strongly impairs cell growth on non-fermentable carbon sources and protein import into isolated mitochondria. We also provide preliminary evidence that binding of precursors to the mitochondrial surface is mediated by cytosolically exposed acidic domains ('acid bristle' domains) of Mas22p and the associated receptor subunit Mas20p.

Results

The C-terminal intermembrane space domain of Mas22p is important for mitochondrial function in vivo

The protein import receptor of yeast mitochondria contains at least four transmembrane subunits: Mas70p, Mas37p, Mas22p and Mas20p. Mas70p and Mas20p are both anchored to the outer membrane through a short N-terminal hydrophobic region and expose a C-terminal domain to the cytosol (Kiebler *et al.*, 1993a,b; Lithgow *et al.*, 1995). In contrast, Mas22p spans the outer membrane in the opposite orientation and has both an N-terminal cytosolic domain and a small acidic domain of 34 residues protruding into the intermembrane space. To test whether this intermembrane space domain of Mas22p functions in protein import we constructed the mutant allele *mas22-9*, which encodes Mas22p lacking the C-terminal domain. When the truncated and the wild-type Mas22p were co-expressed in the same cell the mitochondria contained both forms of Mas22p in comparable amounts, as shown by immunoblotting with an antiserum specific for the cytosolic Mas22p domain. Also, C-terminally truncated Mas22p appeared to be correctly oriented in the outer membrane, as it was cleaved by trypsin in the same way as wild-type Mas22p or the receptor subunit Mas70p (Figure 1A). Cleavage of truncated Mas22p occurred under conditions in which the intermembrane space protein cytochrome c heme lyase was inaccessible to trypsin.

Co-expression of truncated Mas22p with wild-type Mas22p was toxic, causing almost complete loss of respiratory growth. Although the transformed cells could grow on glucose, they grew poorly on galactose and failed to grow on the non-fermentable carbon sources ethanol and glycerol (Figure 1B). Mas22p lacking its intermembrane space domain has thus a dominant negative effect

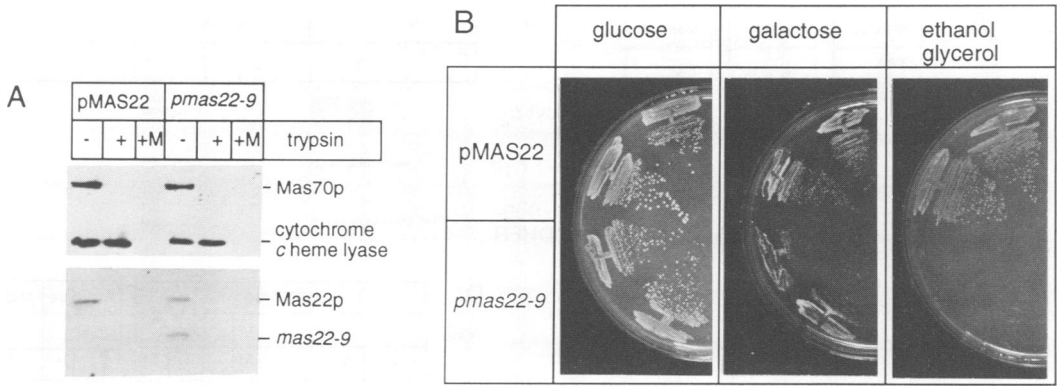

Fig. 1. Expression of Mas22p lacking its intermembrane space domain impairs mitochondrial function even in the presence of wild-type Mas22p. (**A**) The diploid *S.cerevisiae* strain (YTJB73, MATa/α *leu2/leu2 ura3/ura3 trp1/trp1 his3/his3 ade2/ade2 MAS22/mas22::ADE2*) was transformed with plasmid pYPGE1 (Brunelli and Pall, 1993) carrying either the intact *MAS22* gene (yielding strain pMAS22) or a mutant *MAS22* gene encoding the C-terminally truncated Mas22p (yielding strain pmas22-9). Both plasmid-borne *MAS22* alleles were under the control of the yeast phosphoglycerate kinase promoter. Note that both types of transformants carried one chromosomal copy of the wild-type *MAS22* gene. Mitochondria (100 μg) from the two strains were isolated and left untreated (–trypsin) or treated with 150 μg/ml trypsin (+) or treated with 150 μg/ml trypsin in hypotonic buffer to disrupt the outer membrane barrier (+M). They were then analyzed by SDS–PAGE and immunoblotting with antisera against the cytosolic domain of Mas22p, against Mas70p or against the intermembrane space marker cytochrome c heme lyase. The blots were developed with [^{125}I]protein A and autoradiography. (**B**) The diploid transformants described in (A), overexpressing either wild-type Mas22p (pMAS22; upper two sectors) or the C-terminally truncated Mas22p (pmas22-9; lower two sectors) were grown on synthetic media lacking tryptophan with either 2% glucose, 2% galactose or 2% ethanol + 2% glycerol as carbon sources.

in vivo, perturbing mitochondrial function even if the cells also express wild-type Mas22p.

Probably because of their mitochondrial defects, diploid yeast cells co-expressing wild-type and truncated Mas22p sporulated poorly. Nevertheless, we could recover some viable spores which expressed only the truncated Mas22p. These haploid *mas22-9* mutants exhibited poor viability. About 70% of the cells yielded inviable microcolonies on plates of glucose-containing rich medium and the viable cells grew at only half the rate of wild-type cells. No growth at all was seen on synthetic media containing non-fermentable carbon sources (data not shown).

Deletion of the intermembrane space domain of Mas22p strongly inhibits protein import into isolated mitochondria

Mitochondria from a haploid *mas22-9* mutant expressing only truncated Mas22p contained normal levels of the receptor subunits Mas70p, Mas37p and Mas20p (Kiebler *et al.*, 1993b; Lithgow *et al.*, 1995), as well as of the import site proteins Isp42p and Isp45p/MIM44 (Hannavy *et al.*, 1993; Figure 2A). Yet import of several precursor proteins into these mutant mitochondria was strongly impaired. The reduction in import rate varied between 3- and 8-fold (Figure 2B).

To exclude the possibility that the low rate of protein import into *mas22-9* mitochondria was caused by an abnormally low electrochemical potential across the inner membrane or a defective supply of ATP in the matrix we repeated the import experiment with mitochondria whose outer membrane had been selectively ruptured. In these mitoplasts the outer membrane system is by-passed and precursors have direct access to the inner membrane translocation system (Ohba and Schatz, 1987). As shown in Figure 2C, mitoplasts from the *mas22-9* mutant imported precursors at wild-type rates. As the membrane potential and matrix ATP are required for translocation across the inner membrane (Ohba and Schatz, 1987; Hwang and Schatz, 1989; Neupert *et al.*, 1990), the import defect of *mas22-9* mitochondria reflects a defect of the protein transport system in the outer membrane.

The C-terminal Mas22p domain is required to fix the N-terminal targeting sequence of a translocation-arrested precursor in the intermembrane space

To check directly whether the C-terminal Mas22p domain participates in protein translocation across the outer membrane we arrested translocation of the [^{35}S]methionine-labeled chaperonin 10 (cpn10) precursor across the inner membrane of isolated mitochondria by dissipating the potential across that membrane (Rospert *et al.*, 1993; Haucke *et al.*, 1995). When mitochondria that had accumulated cpn10 translocation intermediate were treated with proteinase K most of the precursor molecules were completely degraded, suggesting that they had been arrested at the mitochondrial surface. However, ~5% of the molecules were converted to a 2–3 kDa fragment (Figure 2D, lanes 2 and 11). This fragment represented the N-terminus of cpn10, since the only two methionine residues of cpn10 are at positions 1 and 15 (Rospert *et al.*, 1993). This N-terminal fragment, which represents the mitochondrial targeting sequence (Jarvis *et al.*, 1995), was degraded when the outer membrane was ruptured to allow the protease access to the intermembrane space (Figure 2D, lane 3). The low yield of this fragment suggests that not all of the bound precursor was bound productively or that some of the productively bound molecules did not span the outer membrane. The first explanation is supported by previous observations (Haucke *et al.*, 1995), as well as the results with the *mas22-9* mutant (see below); the second explanation agrees with the expectation that productive binding of the precursor to Mas20p would not generate the protected fragment.

Arrest of the precursor across the outer membrane was salt sensitive; addition of 50 mM NaCl to the incubation

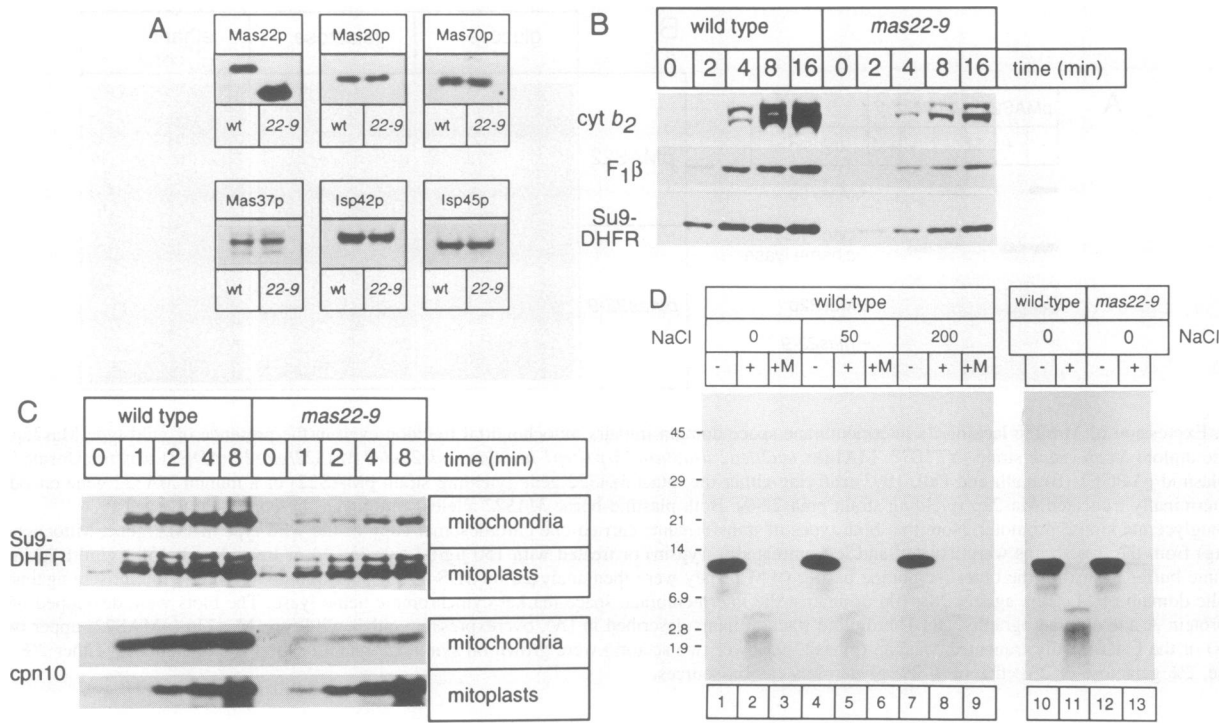

Fig. 2. Deletion of the intermembrane space domain of Mas22p impairs protein transport across the mitochondrial outer membrane. (**A**) Mitochondria from haploid wild-type cells (expressing only full-length Mas22p, wt) or from the haploid *mas22-9* mutant (expressing only Mas22p lacking its intermembrane space domain, 22-9) were analyzed by SDS–PAGE and immunoblotting for the import receptors Mas22p, Mas20p, Mas70p and Mas37p, as well as for the import site proteins Isp42p (outer membrane) and Isp45p (inner membrane). Blots were developed with [^{125}I]protein A and autoradiography. (**B**) Mitochondria from haploid wild-type cells or the *mas22-9* mutant were assayed for the indicated times for import of cytochrome b_2 (cytb_2; at 15°C), the F_1-ATPase β-subunit ($F_1\beta$; at 25°C) or Su9-DHFR (at 25°C). Import was analyzed by SDS–PAGE and fluorography. (**C**) Mitochondria or mitoplasts (10 μg protein in a volume of 100 μl) from wild-type cells or the *mas22-9* mutant were allowed to import radiolabeled cpn10 and Su9-DHFR for the indicated times at 25°C and then analyzed by SDS–PAGE and fluorography. The gel was fluorographed for different times to verify that the rate of precursor import into mitochondria and mitoplasts was essentially identical. (**D**) (Left) Mitochondria from wild-type cells were allowed to bind radiolabeled cpn10 in the presence of the uncouplers valinomycin and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone in import buffer supplemented with the indicated amount of NaCl. They were then left untreated (–) or treated with proteinase K in isotonic import buffer (+) or in hypotonic buffer which ruptures the outer membrane (+M) and analyzed for the trypsin-inaccessible N-terminal cpn10 fragment by SDS–PAGE and fluorography. Migration of the molecular weight markers (ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 21 kDa; lysozyme, 14 kDa; cyanogen bromide fragments derived from apocytochrome c, 6.9, 2.8 and 1.9 kDa) is indicated on the right-hand margin. (Right) The preceding experiment was repeated with mitochondria (50 μg) from wild-type cells or from the *mas22-9* mutant in the absence of added NaCl.

buffer reduced binding of cpn10 to the mitochondrial surface only slightly (Figure 2D, lane 4), but inhibited formation of the protected fragment by >80% (lane 5). Anchoring of the targeting sequence in the intermembrane space thus appears to involve electrostatic interactions. However, we cannot exclude the possibility that this salt effect merely reflected a lowered productive binding of cpn10 to the mitochondrial surface.

Mitochondria from the *mas22-9* mutant bound approximately the same amount of cpn10 as wild-type mitochondria (compare Figure 2D lanes 10 and 12). With the mutant mitochondria, however, no protease-protected cpn10 fragment was detected in the intermembrane space (compare lanes 13 and 11). Thus the intermembrane space domain of Mas22p appears to be required for binding of a precursor N-terminal targeting signal to the *trans* side of the outer membrane.

The intermembrane space domain of Mas22p specifically binds functional mitochondrial targeting peptides

The results shown in Figure 2 suggest that the acidic, intermembrane space domain of Mas22p binds the targeting sequence of a precursor protein and thereby assists transit of this sequence across the outer membrane. As a further test of this model we grafted the intermembrane space domain of Mas22p onto maltose binding protein from *Escherichia coli* and measured the interaction of the resulting fusion protein with synthetic peptides representing either functional or non-functional mitochondrial targeting sequences. As a negative control we also measured binding of the peptides to a fusion protein in which the α-domain of β-galactosidase had been fused to maltose binding protein. The peptides tested represented the functional mitochondrial targeting sequences of the precursors to yeast cytochrome oxidase subunit IV (COXIV; Hurt *et al.*, 1984) and to mammalian cpn60 (cpn60; Peralta *et al.*, 1993), as well as non-functional homologs of the targeting signals of COXIV (SynB2; Allison and Schatz, 1986) and of a cytochrome P450 from mammalian mitochondria (SCC1-19M; Kumamoto *et al.*, 1987; Komiya *et al.*, 1994).

The functional mitochondrial targeting peptides COXIV and cpn60 bound stably to the fusion protein containing the intermembrane space domain of Mas22p (Figure 3A, MalE–22tail, lanes 4 and 5), whereas the non-functional

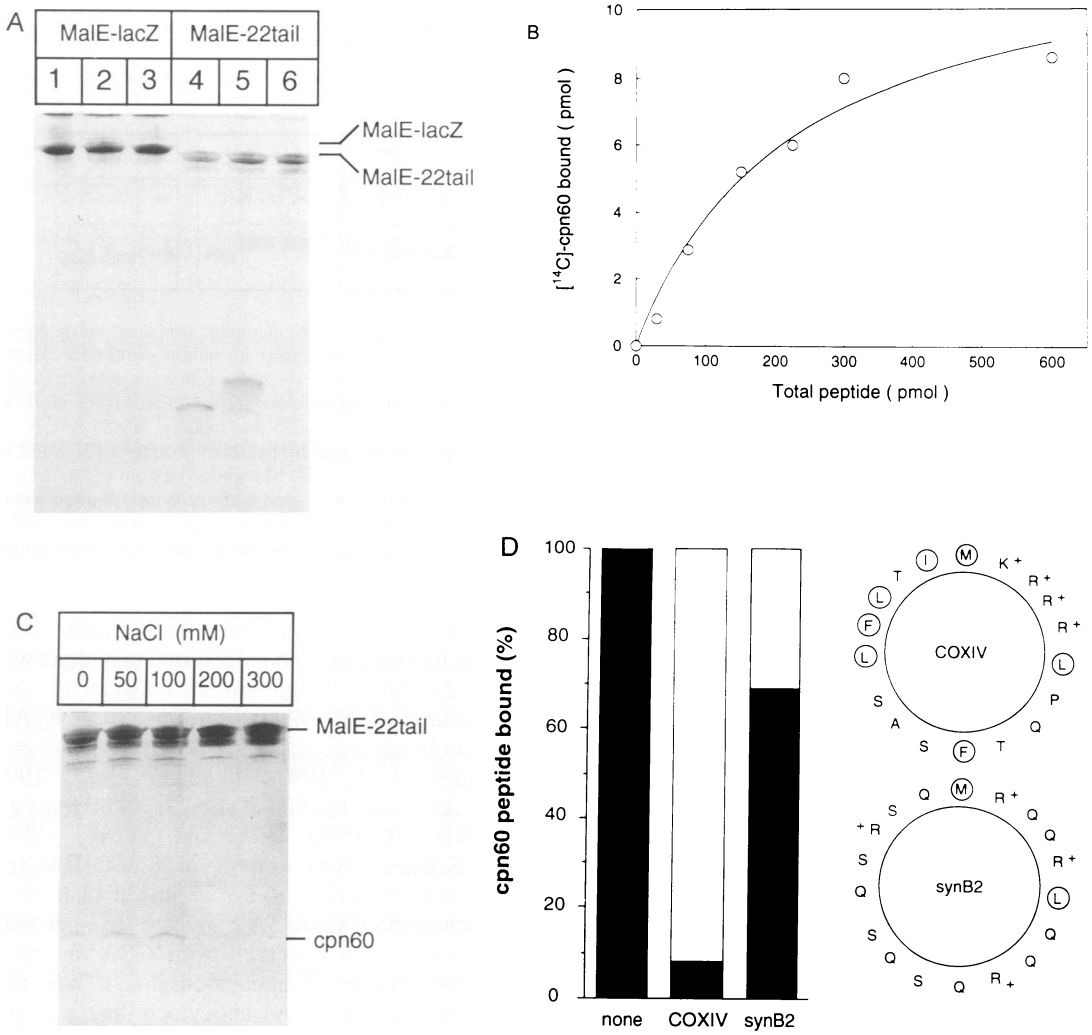

Fig. 3. The intermembrane-space domain of Mas22p specifically binds functional mitochondrial targeting peptides *in vitro*. (**A**) Equal amounts (375 pmol) of synthetic peptides representing the functional mitochondrial targeting sequences of mammalian cpn60 (lanes 1 and 4) or of yeast COXIV (lanes 2 and 5) or the non-functional, mutant mitochondrial targeting sequence of a mammalian cytochrome P450 SCC1-19M (lanes 3 and 6) were incubated with two types of fusion proteins: one containing maltose binding protein fused to the α-fragment of β-galactosidase (MalE–lacZ, lanes 1–3), the other containing maltose binding protein fused to the intermembrane space domain of Mas22p (MalE–22tail, lanes 4–6). Fusion proteins together with any bound peptides were recovered on amylose beads, eluted from the beads with SDS-containing gel sample buffer and analyzed by SDS–16% PAGE and silver staining. The two low molecular weight bands seen in lanes 4 and 5 represent the functional targeting peptides that had bound to the MalE–22tail fusion protein. (**B**) Increasing amounts of ^{14}C-labeled cpn60 prepeptide were incubated with the MalE–22tail fusion protein, which contains the intermembrane space domain of Mas22p fused to maltose binding protein. Bound and unbound peptide were separated by centrifugation and analyzed by SDS–16% PAGE and quantification in a phosphorimager. (**C**) Binding of the mitochondrial targeting peptide of cpn60 (375 pmol) to the MalE–22tail protein was analyzed as in the preceding section, but in the presence of the indicated NaCl concentrations; the SDS–PAGE gels were stained with silver. (**D**) The ^{14}C-labeled mitochondrial targeting peptide of cpn60 (300 pmol) was incubated with MalE–22tail protein in the absence of further additions ('none') or in the presence of 600 pmol of either unlabeled functional COXIV prepeptide or the peptide synB2, which has the same number of positive charges as the COXIV prepeptide, but is not amphiphilic and therefore is inactive as a targeting signal. The COXIV and SynB2 peptides are depicted on the right in helical wheel projections, with hydrophobic residues circled. Binding of the ^{14}C-labeled mitochondrial targeting peptide to the Mas22p tail domain was measured as in the experiment depicted in (B).

targeting peptide SCC1-19M did not bind (lane 6). Binding of the functional targeting peptides to the control fusion protein containing the α-domain of β-galactosidase was at least 10-fold lower than to the MalE–22tail fusion protein (Figure 3A, MalE–lacZ, lanes 1–3).

We also assayed binding of prepeptides to the intermembrane space domain of Mas22p with radiolabeled cpn60 prepeptide. Binding of the prepeptide appeared to be saturatable, half-maximal binding being observed at 800 nM prepeptide (Figure 3B). A more precise measurement was possible using fluorescence quenching to monitor interaction of the fusion protein with the fluorescently labeled prepeptide of aspartate aminotransferase from mammalian mitochondria (Schmid *et al.*, 1994); with this system we measured a K_d of 220 nM (M.Horst, unpublished).

The function of mitochondrial targeting sequences depends on both positive charge and amphiphilicity (Roise *et al.*, 1986; von Heijne, 1986; Lemire *et al.*, 1989). Both charge and amphiphilicity of the targeting peptides were also important for binding to the Mas22p domain. First, the amphiphilic, but uncharged, peptide SCC1-19M did not bind (see above). Second, binding of the functional cpn60 prepeptide was inhibited by 200 mM NaCl (Figure

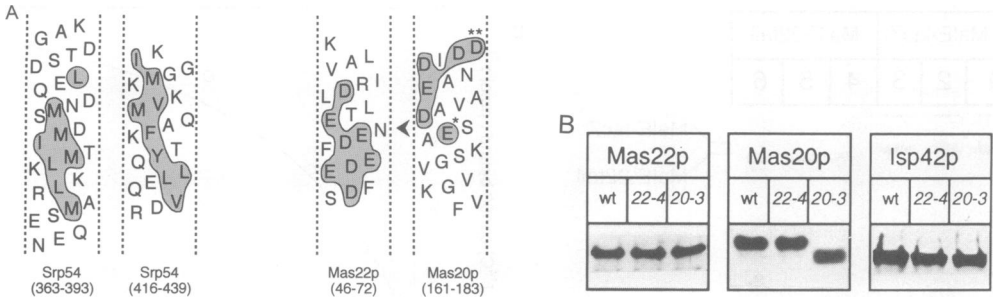

Fig. 4. The cytosolic 'acid bristle' domains of the mitochondrial protein import receptor. (**A**) (Left) The 'methionine bristles' lining presumptive helical regions of the Srp54 subunit of the signal recognition particle (Bernstein *et al.*, 1989). These regions are rich in methionine, leucine and isoleucine and have been suggested to bind signal sequences for the endoplasmic reticulum. On a helical net projection these bristles appear as patches of hydrophobicity (shaded). (Right) The regions of Mas22p and Mas20p chosen for mutagenesis are shown on analogous plots, with the acidic residues shaded. The arrowhead denotes the dipeptide N55 E56 within the Mas22p sequence, which was mutated to R55 K56 in the *mas22-4* mutant. In the *mas20-3* mutant the C-terminal 13 amino acids (E171–D183) of Mas20p were replaced by the three residues E171 Y172 R173. E171 and D183 of the wild-type Mas20p sequence are marked by single and double asterisks respectively. (**B**) Mitochondria from mutants *mas22-4* and *mas20-3* have normal levels of Mas20p and Mas22p protein. Mitochondria (100 μg protein) from wild-type yeast cells (wt) and the mutants *mas22-4* and *mas20-3* were analyzed by SDS–PAGE and immunoblotting with antisera against Mas20p, against Mas22p or, as a control for equal protein loading, for the outer membrane protein Isp42p (Baker *et al.*, 1990). The blots were decorated with ^{125}I-labeled protein A and autoradiographed.

3C). Third, binding of the functional COXIV prepeptide was competed for by the cpn60 prepeptide, but not by the hydrophilic peptide SynB2, even though SynB2 has the same number of positive charges as the COXIV prepeptide (Figure 3D). We conclude that the intermembrane space domain of Mas22p specifically binds mitochondrial targeting sequences.

The cytosolic 'acid bristle' domains of Mas20p and Mas22p may mediate precursor binding to the mitochondrial surface

Mas20p and Mas22p have been proposed to bind the great variety of basic and amphiphilic targeting sequences for mitochondria (Kiebler *et al.*, 1993b; Moczko *et al.*, 1994; Lithgow *et al.*, 1995). As at least part of this interaction appears to be electrostatic, we have considered the possibility that it involves acidic patches ('acid bristles'; Figure 4A) on the cytosolic domains of Mas20p and Mas22p. This hypothesis was inspired by the detection of methionine-rich hydrophobic patches in the 54 kDa subunit of the signal recognition particle and the suggestion that these 'methionine bristles' bind the great variety of hydrophobic targeting sequences for the endoplasmic reticulum (Bernstein *et al.*, 1989).

To test this model we constructed a Mas22p mutant (*mas22-4*) in which residues N55 and E56 of wild-type Mas22p are replaced by R55 and K56. We also constructed a Mas20p mutant (*mas20-3*) which lacks the acidic C-terminus of Mas20p (Figure 4A). Secondary structure predictions (not shown) suggested that neither of these mutations significantly alters the folding of the corresponding cytosolic domains.

Haploid cells expressing only the *mas22-4* form of Mas22p grew at ~50% of the rate of wild-type cells on non-fermentable carbon sources on which mitochondrial function is crucial (data not shown). The level of the mutant Mas22p was comparable with that of normal Mas22p in wild-type cells (Figure 4B). Haploid cells expressing only the *mas20-3* mutant form of Mas20p grew as fast as wild-type cells on rich medium containing non-fermentable carbon sources (data not shown).

The *mas22-4* and *mas20-3* mutations strongly inhibited import of those precursors into isolated mitochondria which had previously been inferred to interact with Mas20p and Mas22p; a fusion protein between the presequence of subunit 9 of *Neurospora crassa* F_1F_0-ATPase and dihydrofolate reductase (Su9-DHFR; Kiebler *et al.*, 1993b; Ramage *et al.*, 1993, Hönlinger *et al.*, 1995), cpn10 (Haucke *et al.*, 1995) and subunit Va of yeast cytochrome c oxidase (COXVa; Miller and Cumsky, 1991; Lithgow and Schatz, 1995). Import of Su9-DHFR into mutant mitochondria was 2- to 3-fold slower than into wild-type mitochondria (Figure 5A); an even stronger reduction (up to 10-fold) was observed with COXVa and cpn10. In contrast, import of apocytochrome c was not affected (Figure 5A, cyt c). As apocytochrome c is translocated across the outer membrane independently of the general protein import receptor (Stuart and Neupert, 1990), mutations in the 'acid bristle' regions of Mas20p and Mas22p appear to selectively effect the import of those precursors that are imported via the general import receptor.

Preliminary experiments suggested that this inhibition reflected a lowered productive binding of the precursors to the mitochondrial surface, as well as a lower import rate of the productively bound precursor (not shown).

Discussion

Mas22p has a presequence binding domain in the intermembrane space

Unlike the other known subunits of the mitochondrial import receptor, Mas22p has an acidic extramembrane domain facing the intermembrane space (Kiebler *et al.*, 1993a; Lithgow *et al.*, 1994; Nakai and Endo, 1995). The evidence reported here strongly suggests that this *trans* domain binds mitochondrial targeting sequences during protein import into mitochondria. Deletion of the domain strongly impairs respiration-driven growth of yeast cells and import of precursors into isolated mitochondria. Also, the domain specifically binds functional mitochondrial targeting peptides *in vitro* and the resulting complex can be purified. Such a complex offers a promising system for

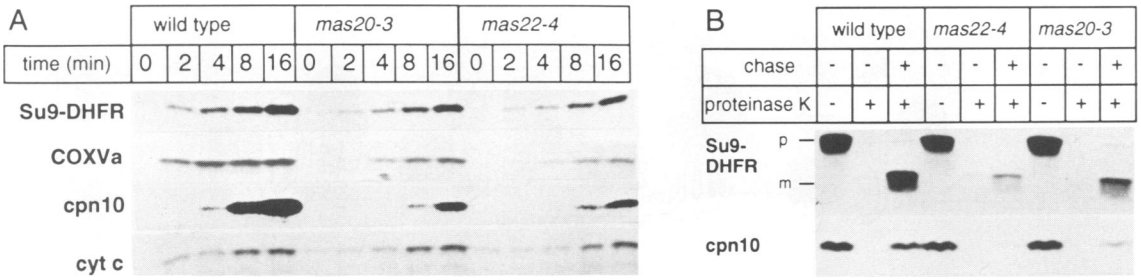

Fig. 5. Mutations in the acid bristle regions of Mas20p and Mas22p impair protein import into isolated mitochondria. (**A**) Mitochondria from wild-type yeast and from mutants *mas22-4* and *mas20-3* were assayed for import of the COXVa precursor for the indicated times at 15°C and for import of Su9-DHFR, cpn10 and apocytochrome c (cyt c) at 25°C. The mitochondria were then analyzed by SDS–PAGE and fluorography. (**B**) *In vitro*-synthesized Su9-DHFR and cpn10 were bound to de-energized mitochondria (Haucke *et al.*, 1995). The mitochondria were then divided into three aliquots. Two aliquots (– chase) were incubated for 30 min at 0°C with (+) or without (–) proteinase K respectively. The third aliquot (+ chase) was first re-energized to chase the precursor into the mitochondria and then treated with proteinase K. Finally, mitochondria from all three aliquots were washed, re-isolated and analyzed by SDS–PAGE and fluorography.

defining the three-dimensional structure of a mitochondrial receptor domain carrying its peptide ligand.

Mayer *et al.* (1995) have recently shown that mitochondrial outer membrane vesicles can partly translocate matrix-targeted precursors and bind the presequence of these precursors to an unidentified binding site on the inner face of the outer membrane. Our present results, gathered *in vivo* with isolated intact organelles and with purified proteins, indicate that the intermembrane space domain of Mas22p contributes to this function by anchoring the precursor within the intermembrane space, thereby drawing it across the import channel. Since deletion of this domain is not lethal and does not completely block mitochondrial protein import, binding of presequences to the inner side of the outer membrane is either not essential for import or is also mediated by other protein domains in the intermembrane space.

The cytosolic 'acid bristle' domains on the mitochondrial protein import receptor

Three lines of evidence suggest that Mas20p and Mas22p are presequence binding subunits of the yeast mitochondrial protein import receptor (Lithgow *et al.*, 1995). First, import of fusion proteins consisting of a mitochondrial targeting sequence fused to a non-mitochondrial passenger protein is specifically inhibited by antibodies against either Mas20p or Mas22p (Becker *et al.*, 1992; Ramage *et al.*, 1993; Mayer *et al.*, 1993; Hönlinger *et al.*, 1995). Second, once these fusion proteins are bound to the mitochondrial surface they can be cross-linked to Mas20p (Haucke *et al.*, 1995) and Mas22p (Hönlinger *et al.*, 1995). Third, productive binding of the fusion proteins to the mitochondrial surface is very sensitive to salt and to chemically synthesized presequence peptides, suggesting that this binding is largely electrostatic and involves the presequence (Haucke *et al.*, 1995).

Here we show that the cytosolic domains of Mas20p and Mas22p each contain clusters of negative charges on the surface of predicted helices. We also report that decreasing the acidity of these 'acid bristle' domains by site-directed mutation impairs import of precursor proteins into isolated mitochondria and that part of this inhibition reflects a lowered productive binding of precursors to the mitochondrial surface. Since Mas20p and Mas22p (and their *N.crassa* homologs MOM19 and MOM22) probably form a complex with each other *in vivo* (Harkness *et al.*, 1994; Lithgow *et al.*, 1994; Nargang *et al.*, 1995), the acid bristle domains of Mas20p and Mas22p may function as a unit in binding a precursor targeting signal to the mitochondrial surface. While the role of the acid bristle domains must be investigated by more extensive mutational analysis, our data suggest that transfer of a precursor protein across the mitochondrial outer membrane involves sequential binding of the targeting sequence to receptor domains on both sides of the membrane. This model is outlined in Figure 6. It predicts that Mas22p associates at least reversibly with the protein transport channel in the outer membrane. The proposed dual function of Mas22p on both sides of the outer membrane may explain why Mas22p is the only essential subunit of the mitochondrial protein import receptor (Lithgow *et al.*, 1994; Hönlinger *et al.*, 1995; Nakai and Endo, 1995; Nargang *et al.*, 1995).

Most precursor proteins are destined to travel on through the translocation channel in the inner membrane. Since binding of a targeting sequence to the intermembrane space domain of Mas22p is stable *in vitro*, it must be reversed by the attractive force of other components of the import machinery *in vivo* (X in Figure 6; Söllner *et al.*, 1992; Mayer *et al.*, 1995). We suggest that such components might be subunits of the inner membrane protein transport channel. One likely candidate for such a function is Mas6p (MIM23), which has an acidic domain facing the intermembrane space (Dekker *et al.*, 1993; Emtage and Jensen, 1993). Local pH gradients close to the surface of the mitochondrial inner membrane may also play a role in loosening the interaction between Mas22p and the targeting sequence in the intermembrane space. The experimental systems described here may help to identify additional presequence binding subunits of the mitochondrial protein import machinery.

Materials and methods

Deletion of the intermembrane space domain of Mas22p

The *MAS22* gene was truncated at the codon for L130, immediately after the predicted transmembrane region (Lithgow *et al.*, 1994), and a methionine (M131) and a lysine (K132) were added to the new C-terminus. This truncation completely eliminated the intermembrane space domain of Mas22p. We used PCR with the primers 5′-GCGCGAATTCCGGATCCATATGGTCGAATTAACTG-3′ and 5′-CGACGGATCCGGAATTCTACTTCATGGCAAGTATAGATAAGGA-3′. The mutant

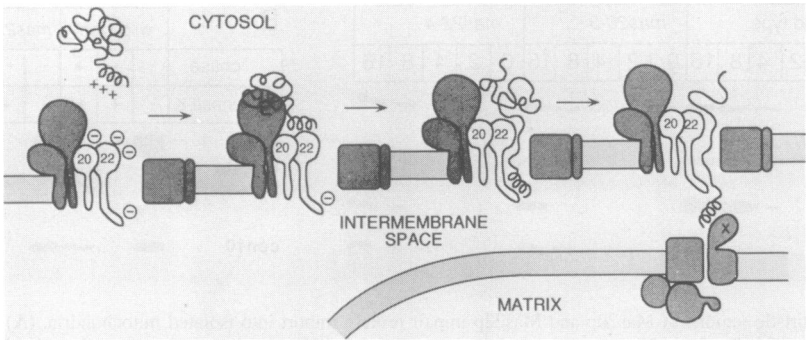

Fig. 6. Efficient transfer of a precursor's matrix targeting sequence across the mitochondrial outer membrane is mediated by acidic receptor domains on both sides of the outer membrane. Mitochondrial precursors carrying a basic and amphiphilic N-terminal targeting sequence are recognized and bound by the acid bristle domains of Mas20p (20) and Mas22p (22). Mas22p might then associate with the translocation channel across the outer membrane and direct the precursor targeting sequence to the Mas22p domain in the intermembrane space. Components of the import machinery in the inner membrane (such as the intermembrane space domain of Mas6p or as yet unidentified domains, X) could then mediate the subsequent import steps.

Table I. Strains used

Strain	Genotype
pMAS22	*MATa/α ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 MAS22/mas22::ADE2//pYPGE1::MAS22*
pmas22-9	*MATa/α ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 MAS22/mas22::ADE2//pYPGE1::mas22-9*
JK9-3d 'WT'	*MATα ura3 leu2 his3 trp1//pYPGE1*
mas22-9	*ura3 leu2 his3 trp1 ade2 mas22::ADE2//pYPGE1::mas22-9*
mas20-3	*ura3 leu2 his3 trp1 mas20::LEU2//pYCplac::mas20-3*
mas22-4	*ura3 leu2 his3 trp1 ade2 mas22::ADE2//pYPGE::mas22-4*

The mating type of the haploid strains mas22-9, mas22-4 and mas20-3 was not determined. The sign // designates a plasmid-borne gene. Strain JK9-3d is described by Kunz *et al.* (1993). WT, wild-type.

sequence was generated by PCR and the DNA fragment cloned into the vector spYPGE1 (Brunnelli and Pall, 1993). The construct was checked by DNA sequencing, transformed into the diploid *Saccharomyces cerevisiae* strain YTJB73 (MATa/α *ura3/ura3 leu2/leu2 ade2/ade2 his3/his3 trp1/trp1 mas22::ADE2*) and the transformants grown on semi-synthetic media with glucose as a carbon source. To generate the haploid yeast mutant *mas22-9* the diploids were incubated for 14 days in sporulation medium without tryptophan. Less than 1% of the cells formed spores; 16 spores were recovered, dissected onto plates of rich medium containing 2% glucose and allowed to germinate at 30°C. After 2 days Ade$^+$ *mas22-9* cells (*ura3 leu2 ade2 his3 trp1 mas22::ADE2//pYPGE1::mas22-9*) were plated and maintained on rich medium with ethanol and glycerol to select for respiratory competence. (The cells failed to grow on synthetic media containing ethanol and glycerol.) Mitochondria were isolated from *mas22-9* cells grown on rich medium containing L-lactate as the major carbon source.

Mutagenesis of the cytosolic domains of Mas20p and Mas22p

The *mas20-3* yeast mutant was created as follows. A 26 bp *Hin*fI–*Eco*RV fragment encoding the extreme C-terminus of Mas20p was removed by *Hin*fI digestion and Klenow treatment of a *MAS20* gene fragment. The resulting 26 bp *Hin*f–Klenow fragment was digested with *Eco*RI and inserted into a genomic fragment of *MAS20* which had been cut with *Eco*RI and *Eco*RV. Finally, the resulting truncated *MAS20* construct was cloned into the yeast expression vector YCplac33, which carries the *URA3* gene as a selectable marker, and the resulting recombinant vector was transformed into *S.cerevisiae* strain YTJB72 (MATα *ura3 leu2 trp1 his4 mas20::LEU2*).

To introduce mutations into the cytosolic domain of Mas22p we designed the mutagenic primer 5′-GAATTTGATGAAAA/GT/GC/AAAACATTGTTGGA-3′ and the PCR primer 5′-GGGATCCAAGCTTGAATTCAACTTTACTCTAAAGC-3′. The mutated and amplified DNA fragments were then used as megaprimers together with the primer 5′-GCGCGGATCCGTGTCGAGCGATATAAGT-3′ to amplify the complete *MAS22* open reading frame carrying a mutation. A collection of these mutant *mas22* sequences was subcloned into the yeast expression vector pYPGE1 (Brunelli and Pall, 1993), which carries the *TRP1* gene as a selectable marker, and the plasmids 'shuffled' into the haploid yeast strain YTJB77 (MATa *ura3 leu2 ade2 his3 trp1 mas22::ADE2//YCplac33::MAS22*) by growing the yeast transformants on plates containing 5-fluoroorotic acid. This procedure yielded mutant *mas22-4*, as well as other mutants not described here. In mutant *mas22-4* residues N55 and E56 of wild-type Mas22p are replaced by R55 and K56.

The genotypes of the yeast strain constructs are given in Table I.

Preparation and affinity purification of antibodies

A DNA fragment encoding the entire open reading frame of *MAS22* was amplified by PCR, checked by sequencing and subcloned into the plasmid pUEX2. The resulting recombinant plasmid allowed overproduction of an insoluble lacZ–Mas22p fusion protein in *E.coli*. A rabbit antiserum against Mas22p was raised against this fusion protein. Where indicated antibodies recognizing the cytosolic domain of Mas22p were affinity-purified on a fusion protein (T.Junne and T.Lithgow, unpublished) which contained the cytosolic domain of Mas22p fused to *E.coli* maltose binding protein.

A fusion protein corresponding to the cytosolic domain of Mas20p (D30–D183 of Mas20p) was constructed with a DNA fragment amplified by PCR, checked by sequencing and subcloned into plasmid pMalc2 (New England Biolabs). The fusion protein was expressed in *E.coli* and used to raise a rabbit antiserum recognizing the cytosolic domain of Mas20p.

Protein import into isolated yeast mitochondria

Mitochondria were isolated from the various yeast strains and stored frozen as previously described (Glick *et al.*, 1992). Protein import into trypsin-pretreated mitochondria was assayed exactly as previously described (Lithgow and Schatz, 1995). To measure the effects of mutations in Mas20p and Mas22p on protein import into isolated mitochondria we used a modified buffer for the import assay (0.6 M sorbitol, 20 mM HEPES–KOH, pH 7.4, 5 mM $MgCl_2$, 12 mM KCl, 0.5 mM EDTA; Haucke *et al.*, 1995). In order to ensure that the concentration of mitochondria was rate limiting for import the mitochondrial concentration in the import assay was 0.1 mg/ml for all precursors except for CoxVa, for which it was 0.03 mg/ml (Lithgow and Schatz, 1995). To measure the import of apocytochrome c the import buffer was supplemented with 7 mM NADPH and 1 mM dithiothreitol instead of with NADH (Dumont *et al.*, 1988). Preparation of, and protein import

into, mitoplasts were as previously described (Ohba and Schatz, 1987; Glick *et al.*, 1992). In order to generate a cpn10 translocation intermediate spanning the mitochondrial outer membrane mitochondria (0.1 mg/ml protein) were incubated in import buffer (20 mM HEPES–KOH, pH 7.4, 5 mM $MgCl_2$, 12 mM KCl, 1 mM DTT, 0.5 mM EDTA, 0.6 M sorbitol) containing carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (150 μM) and valinomycin (5 μM) and incubated with [^{35}S]methionine-labeled cpn10 for 10 min at 20°C. The samples were resolved on SDS–16% PAGE (Schägger and von Jagow, 1987) containing an additional 2% linear polyacrylamide. Productive binding of precursors to the mitochondrial surface was measured as previously described (Haucke *et al.*, 1995).

Preparation of a MalE fusion protein containing the C-terminal domain of Mas22p (MalE–22tail)

The pMalc2 plasmid (New England Biolabs) encodes the fusion protein MalE–lacZ. A DNA fragment encoding the intermembrane space domain of Mas22p was amplified by PCR with the primers 5′-GCGCGAATT-CGGATCCGCCGAACAACAGCTAA-3′ and 5′-GGGATCCAAGCTT-GAATTCAACTTTACTCTAAAGC-3′. The plasmid pARTY, encoding the MalE–22tail protein (corresponding to A121–N154 from Mas22p) was engineered by inserting the *Eco*RI-cut PCR product into plasmid pMalc2. The fusion protein was purified from *E.coli* transformants after 2–3 h growth in medium containing 1 mM isopropyl-β-D-thiogalacto-pyranoside. The cell pellet from 300 ml culture was resuspended in 15 ml 20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride and frozen in liquid nitrogen. It was rapidly thawed in a sonication water bath and further sonicated with a 0.8 cm probe (Cell Disruptor W-375, Heat Systems-Ultrasonics Inc.) on ice for six cycles of 15 s each at 80% duty. The solubilized fusion proteins were then purified on amylose resin (New England Biolabs) according to the supplier's instructions.

In vitro binding of mitochondrial targeting peptides to the MalE–22tail fusion protein

The standard binding assay (total volume 150 μl, 20 mM sodium phosphate, 85 mM NaCl, pH 6.5) contained fusion protein (1 μg) attached to amylose resin (5 μl packed volume) and, except where otherwise noted, 375 pmol peptide. After incubation at 4°C for 30 min the bound material was recovered by centrifugation and pellet and supernatants were analyzed by SDS–PAGE and silver staining (Schägger and von Jagow, 1987).

For quantitative analysis a sample of the cpn60 peptide was labeled with [^{14}C]iodoacetamide. Peptide (450 μg) was incubated under nitrogen for 4 h at 25°C in a total volume of 750 μl containing 400 mM Tris, pH 8.6, 5 mM EDTA, 20 mM 2-mercaptoethanol) to reduce any disulfide bonds. [^{14}C]Iodoacetamide (1 μmol, 50 mCi/mmol) in 100 μl 50 mM Tris, pH 8.6, was added and the mixture was incubated for an additional 4 h. The reaction was terminated by the addition of 1 μl 1 M dithiothreitol, proteins were precipitated with 5% trichloroacetic acid onto phosphocellulose paper and incorporation of ^{14}C-label into the peptide was measured by scintillation counting. The ^{14}C-labeled peptide was diluted to a stock solution of 87.5 μM peptide with a specific activity of 1 mCi/mmol.

Acknowledgements

We thank Peter Hoj and Nick Hoogenraad for the cpn60 prepeptide, Naomi Hachiya and Tsuneo Omura for the SCC1-19M peptide, Mike Hall for the yeast strain JK9-3d, Rick Pearson for phosphocellulose paper, Emmanuel Margoliash for the plasmid RC9 encoding apocytochrome c, Martin Horst and Prof. Philipp Christen for performing the fluorescence measurements, Sabine Rospert for help with analysis of the *mas20-3* and *mas22-4* mutants, Kitaru Suda for preparation of the low molecular weight protein standards, Hildegard Brütsch for technical assistance, Verena Grieder, Margrit Jäggi and Liselotte Müller for the artwork and members of the Schatz laboratory for helpful criticism. This study was supported by grants from the Swiss National Science Foundation (31-40510.94) and from the Human Capital and Mobility program of the European Union (to G.S.) and by a Long Term Fellowship from the Human Frontiers Science Program Organization (to T.L.).

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Received on July 17, 1995; revised on September 1, 1995