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The processing peptidase of yeast mitochondria: the two co-operating components MPP and PEP are structurally related

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Two proteins co-operate in the proteolytic cleavage of mitochondrial precursor proteins: the mitochondrial processing peptidase (MPP) and the processing enhancing protein (PEP). In order to understand the structure and function of this novel peptidase, we have isolated mutants of *Saccharomyces cerevisiae* which were temperature sensitive in the processing of mitochondrial precursor proteins. Here we report on the mif2 mutation which is deficient in MPP. Mitochondria from the mif2 mutant were able to import precursor proteins, but not to cleave the presequences. The MPP gene was isolated. MPP is a hydrophilic protein consisting of 482 amino acids. Notably, MPP exhibits remarkable sequence similarity to PEP. We speculate that PEP and MPP have a common origin and have evolved into two components with different but mutually complementing functions in processing of precursor proteins.

Key words: mitochondria/yeast/MPP/PEP

Introduction

During recent years a considerable amount of information has become available on how nuclear-coded proteins are imported into mitochondria (for reviews see Hay *et al.*, 1984; Wickner and Lodish, 1985; Pfanner and Neupert, 1987). It has been found that most, but not all, proteins are initially made as precursors which carry amino-terminal presequences. These presequences have a targeting function to direct the precursors to the mitochondria (Hurt *et al.*, 1984; Horwich *et al.*, 1985, 1986; Emr *et al.*, 1986; Keng *et al.*, 1986; Vasarotti *et al.*, 1987). They are cleaved during or after entry of the precursors into the mitochondrial matrix.

Mitochondria possess an import machinery which includes surface receptors, which can recognize precursors (Zwizinski *et al.*, 1984), and membrane components, which can facilitate their insertion into the outer membrane and movement into the interior of mitochondria (R.Pfaller *et al.*, submitted). Translocation contact sites in which outer and inner membranes are in close proximity are involved in channelling proteins to the inner membrane and matrix (Schleyer and Neupert, 1985; Hartl *et al.*, 1986, 1987a,b; Schwaiger *et al.*, 1987; Pfanner *et al.*, 1987a,b). Subsequent steps involve the proteolytic cleavage (Böhni *et al.*, 1980; Miura *et al.*, 1982; Conboy *et al.*, 1982; McAda and Douglas, 1982; Schmidt *et al.*, 1984; Hawlitschek *et al.*, 1988; Witte *et al.*, 1988) and concomitant sorting into the different mitochondrial subcompartments (Hartl *et al.*, 1986, 1987a). The transport pathways of a number of precursors have been described in considerable detail. Procedures have been developed to halt precursors at various distinct steps (Pfanner *et al.*, 1988). In order to understand fully the molecular mechanisms of protein import it is essential to identify and characterize the components involved. This is, however, hampered by a number of experimental difficulties. One is the presence of these proteins in only minute amounts, another is the problem of being able to assay for precise function of the various protein components of the import machinery.

A possible approach to characterize such protein components is to analyze mutations which affect the import reaction. A combined effort of molecular genetics and biochemical analysis may therefore be expected to be of great advantage in identifying the unknown components. Indeed, in previous studies mutants affecting mitochondrial protein import have been isolated and partly characterized (Yaffe and Schatz, 1984; Yaffe *et al.*, 1985).

In this report we describe a new screen for mutants of *Saccharomyces cerevisiae* temperature-sensitive (ts) defective in *m*itochondrial *i*mport *f*unctions (mif-mutants). Mutants of three complementation groups were obtained by this procedure and were characterized by accumulation of uncleaved precursor proteins at the non-permissive temperature. One of the ts-lethal mutations, mif2, was investigated in detail. By genetic crossing, mif2 was found to correspond to the mas2 mutation described by Yaffe and Schatz (1984). On the basis of our analysis of its phenotype *in vivo* and *in vitro* we conclude that the mutation affects the mitochondrial processing peptidase (MPP). This is the catalytic component of the mitochondrial processing activity, which together with the previously identified processing enhancing protein (PEP) is required for cleaving the targeting signals of precursor proteins in the mitochondrial matrix (Hawlitschek *et al.*, 1988). MPP is a protein with a mol. wt of 53 000. Its primary structure was determined by sequencing of the isolated gene. The derived amino acid sequence of MPP bears notable similarities to that of PEP, suggesting that the respective genes are of common evolutionary origin.

Results

Isolation of conditional mutants defective in mitochondrial protein import

In order to design a strategy for isolation of conditional yeast mutants affecting the mitochondrial import machinery, we first considered the phenotype of such mutants. Since a multiplicity of essential biochemical steps reside in mitochondria, critical not only to respiration and energy metabolism but also to amino acid and lipid metabolism, the absence of these enzymes in their mature, active forms, and

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the resulting blockade of these pathways, should be lethal. Implicit in this assumption is that the pathway of entry of some or all of the subunit precursors of these various enzymes is shared, such that mutations affecting components of the import machinery would affect a host of entering precursors.

To develop an efficient screening procedure, permitting rapid analysis of large numbers of ts-lethals, we exploited previous findings. Cheng *et al.* (1987) transformed a yeast strain (arg3) deficient in ornithine transcarbamylase (OTC) with a plasmid containing the coding sequence for human OTC joined with a gal1 operon promoter. Upon induction of human OTC the precursor was imported by the mitochondria, cleaved to the mature-sized subunit and assembled into active enzyme. We reasoned that in a ts-lethal mutant affecting mitochondrial import no OTC enzyme activity would be produced at the non-permissive temperature because only mature-sized subunit, assembled into the trimeric form, exhibits enzyme activity. Neither the non-cleaved precursor nor non-assembled mature-sized subunit are enzymatically active (Kalousek *et al.*, 1984). A bank of ts-lethals was produced using ethylmethanesulfonate mutagenesis. Cultures of individual mutants were subjected to an assay in which cells were shifted to non-permissive temperature (37°C) and expression of OTC precursor was simultaneously induced from the gal1 promoter. After 2 h cells were harvested and OTC enzyme activity was determined to analyze whether the import pathway was functional. The host strain as well as the majority of the ts-lethal mutants contained significant levels of activity. However, ~10% of the mutants were lacking activity.

Approximately 1500 mutants lacking OTC activity were analyzed by immunoblot analysis for accumulation of OTC precursor. Twenty-four mutants were found to accumulate precursor at non-permissive temperature. These mutants produced both mature-sized OTC subunits and OTC enzyme activity at the permissive temperature. In pairwise matings, they were found to fall into three distinct genetic complementation groups. They were designated mif1−3, for 'mitochondrial import functions'. mif2, containing 12 alleles, proved to be unable to complement an allele of the mutant mas2 previously isolated by Yaffe and Schatz (1984), indicating that these alleles involve the same gene. This was confirmed by the observation that the mif2 alleles we had isolated accumulated at non-permissive temperature the precursor of the endogenous yeast mitochondrial F_1ATPase subunit β ($F_1\beta$) (not shown).

Mitochondria from mif2 cells lack MPP activity at the non-permissive temperature

We first analyzed whether mitochondria isolated from cells bearing the mif2 allele B35.5 were able to import precursor proteins destined to different submitochondrial compartments. Mutant mitochondria which had been exposed to 37°C for 10 min were found to translocate the precursors of the Rieske Fe/S protein (complex III) and of $F_1\beta$ into a position protected against externally added proteinase K. In contrast to mitochondria isolated from wild-type cells, mutant mitochondria were unable to process proteolytically the imported precursors. The precursor of ADP/ATP carrier

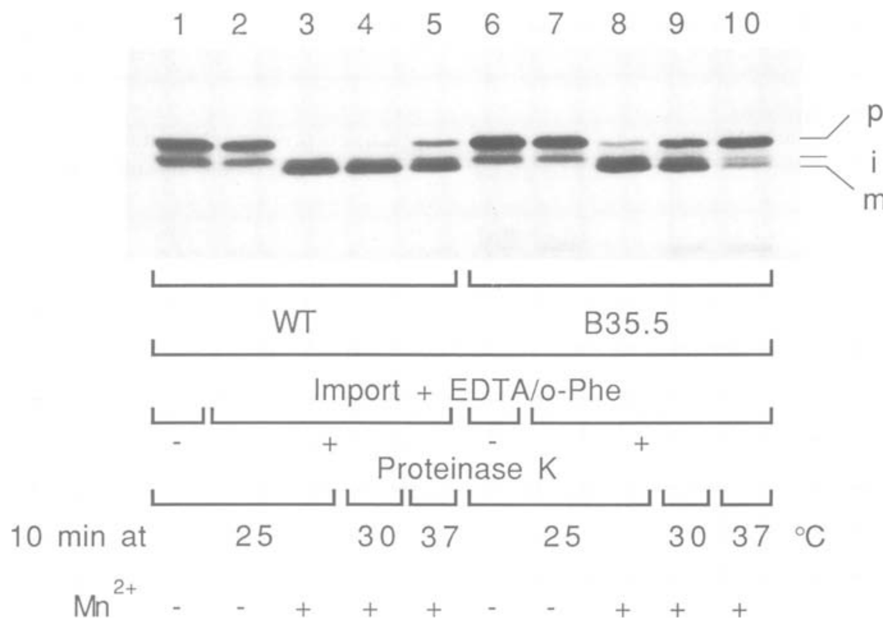

Fig. 1. Proteolytic processing of p-Fe/S accumulated in the matrix of wild-type and mutant mitochondria. Mitochondria were isolated from wild-type and B35.5 cells grown at the permissive temperature. They were incubated for 20 min at 25°C in the presence of reticulocyte lysate containing radiolabeled precursor of Fe/S protein and ATP and NADH (1 mM each). EDTA and *o*-phenanthroline (7.5 mM and 1 mM final concentrations respectively) were included in the import reaction to block the processing peptidase in the matrix. Import was stopped by addition of valinomycin, the incubations were 3-fold diluted with BSA buffer containing 1 mM EDTA and 1 mM *o*-phenanthroline and were divided into five aliquots each (**reactions 1−5** and **6−10**). **Reactions 2−5** and **7−10** were treated with proteinase K (15 μg/ml final concentration). Afterwards mitochondria were reisolated and resuspended at 250 μg protein/ml in BSA buffer containing PMSF and *N.crassa* protease inhibitor. EDTA and *o*-phenanthroline were added (0.5 mM each) to maintain the block of the processing peptidase. Reactions 1−3 and 6−8 were then incubated for 10 min at 25°C, reactions 4 and 9 at 30°C, and reactions 5 and 10 at 37°C. Then $MnCl_2$ (2 mM final concentration) was added to reactions 3−5 and 8−10 to relieve the block of the processing peptidase. Incubation was continued for 15 min at the respective temperature except for reactions 5 and 10 which were adjusted to 30°C. Mitochondria were reisolated from all reactions, and analyzed by SDS−PAGE and fluorography. Abbreviations: p, precursor; i, intermediate; m, mature.

which does not contain a cleavable presequence was also translocated with the same efficiency into mutant and wild-type mitochondria and was protected from added protease in both cases (not shown).

The proteolytic processing of precursors accumulated in the matrix space of mitochondria from mutant and wild-type was assayed independently of the membrane translocation event. Precursor of the Fe/S protein was imported into isolated mitochondria under conditions where the metal-dependent matrix-processing peptidase was inhibited (i.e. in the presence of EDTA and orthophenanthroline) (Figure 1). This led to the accumulation of p-Fe/S soluble in the matrix (Hartl *et al.*, 1986). After import at the permissive temperature, valinomycin was added to dissipate the mitochondrial membrane potential. Mitochondria were treated with proteinase K to digest precursors exposed at the surface. The mitochondria were further incubated for 10 min at 25, 30 or 37°C. Then the inhibition of the matrix-processing peptidase was relieved by addition of Mn^{2+} ions and incubation was continued for 15 min at the respective temperatures (Figure 1, lanes 3–5 and 8–10). Controls showed that both mutant and wild-type mitochondria had efficiently accumulated p-Fe/S in the matrix (Figure 1, lanes 1,2 versus lanes 6,7). In wild-type mitochondria p-Fe/S was completely processed to m-Fe/S after reactivation of the processing peptidase. If mitochondria had been exposed to 37°C, processing to m-Fe/S still occurred although at a somewhat lower rate (Figure 1, lane 5). The ability of mutant mitochondria to process accumulated precursor was slightly reduced at the permissive temperature and was completely missing at the non-permissive temperature (Figure 1, lanes 8–10 versus lanes 3–5). Notably, the second proteolytic processing step of intermediate-sized Fe/S (i-Fe/S) to m-Fe/S was not affected in the mutant (Figure 1, lane 10 versus lane 7). Proteolytic cleavage of i-Fe/S to m-Fe/S which also takes place inside the inner membrane is most probably performed by an enzyme different from processing peptidase (Hartl *et al.*, 1986; F.-U.Hartl and W.Neupert, unpublished).

These results indicated that the mutant mitochondria are defective in the activity of the metal-dependent processing peptidase in the matrix at the non-permissive temperature. To prove this further, the activity of the processing enzyme was measured in detergent extracts of mitochondria. In such extracts proteolytic cleavage of precursor proteins has been shown to be dependent on two mitochondrial components: MPP itself and PEP. The latter component strongly stimulates the activity of MPP (Hawlitschek *et al.*, 1988).

Mitochondria from wild-type and mutant were pre-incubated at 25 or 37°C and detergent extracts were then prepared. Radiolabeled precursor proteins were added and processing activities were determined (Figure 2A). Extract

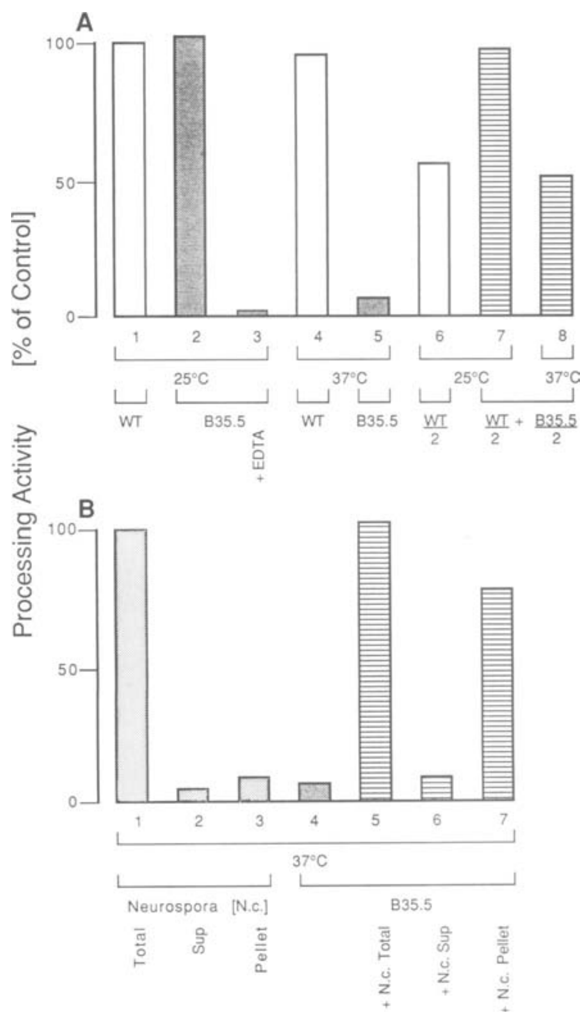

Fig. 2. Processing of cytochrome b_2 precursor by matrix extracts prepared from wild-type and mutant mitochondria. Aliquots of wild-type and mutant mitochondria (B35.5) were suspended at 5 mg protein/ml in SEM (0.25 M sucrose, 1 mM EDTA, 10 mM Mops, pH 7.2) containing 100 μg/ml of *N.crassa* protease inhibitor and 1 mM PMSF. They were incubated for 10 min at 25 or 37°C. Mitochondria were then reisolated by centrifugation and solubilized at 0°C at a concentration of 1.5 mg protein/ml in extraction buffer (1% Triton X-100, 100 mM NaCl, 30 mM Tris, 1 mM PMSF, 100 μg/ml protease inhibitor of *N.crassa*, pH 7.4). Insoluble material was removed by centrifugation for 10 min at 45 000 *g*. **(A)** Mitochondrial extracts corresponding to 15 μg of mitochondrial protein from wild-type (**columns 1** and **4**) and B35.5 cells (**columns 2, 3** and **5**) pretreated at 25°C (**columns 1–3**) or 37°C (**columns 4** and **5**) were incubated for 30 min at 25°C in the presence of 5 μl reticulocyte lysate containing radiolabeled precursor of cytochrome b_2. $MnCl_2$ and $MgCl_2$ were added (final concentration 1 mM each). The volume of the processing assay was adjusted to 100 μl with extraction buffer lacking NaCl. In reaction 3 processing activity was inhibited by addition of 5 mM EDTA. In reaction 6 extract from wild-type mitochondria corresponding to 7.5 μg protein was assayed. In reactions 7 and 8 wild-type extracts were mixed with extracts of B35.5 mitochondria (each corresponding to 7.5 μg protein) pretreated at 25°C (column 7) and 37°C (column 8). Processing reactions were stopped by addition of trichloroacetic acid (10% final concentration). After incubation for 20 min on ice, protein precipitates were recovered by centrifugation and were analyzed by electrophoresis and fluorography. Intermediate-sized cytochrome b_2 produced from the precursor was quantified by laser densitometry. The processing activity of mitochondrial extracts from wild-type mitochondria preincubated at 25°C was set to 100%. **(B)** A Triton extract of *N.crassa* mitochondria (prepared as in A) was diluted 10-fold with extraction buffer. To aliquots corresponding to 5 μg of mitochondrial protein (**column 1**) antiserum directed against MPP and protein A–Sepharose was added to deplete the extracts of processing activity. After centrifugation, processing activity was assayed in the resulting supernatant (**column 2**) and pellet (**column 3**). Extracts of 37°C pretreated B35.5 mitochondria (corresponding to 10 μg of protein) (**columns 4–7**) were assayed either separately (**column 4**) or were mixed with a total extract of *Neurospora* (**column 5**), or a *Neurospora* supernatant depleted of MPP (**column 6**), or a pellet containing the immunoadsorbed MPP (**column 7**). Processing activities were measured as in (A) and are expressed as percentages of the activity of the total *Neurospora* extract. Abbreviations: Sup, supernatant of *N.crassa* mitochondrial extract depleted of MPP; pellet, antibody–protein A–Sepharose complex with adsorbed MPP.

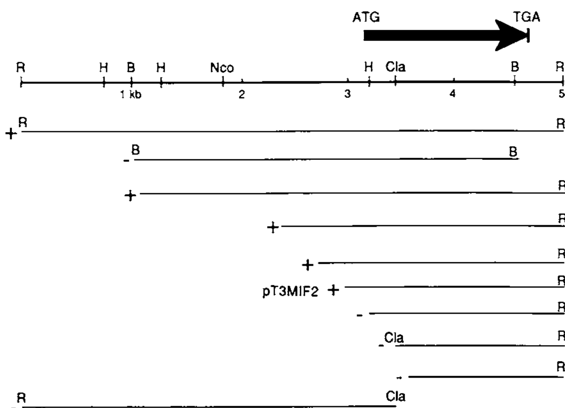

Fig. 3. Definition of MPP gene by DNA-fragment-mediated transformation of B35.5 cells. The fragments indicated were obtained as described in Materials and methods and represent deletion derivatives of an original 5.0-kb DNA fragment that could confer growth to B35.5 cells at 37°C. Rescuing fragments are designated by '+', fragments that failed to rescue by '−'. The arrow indicates the open reading frame determined by DNA sequencing.

from mutant mitochondria preincubated at 25°C was able to process precursor to cytochrome b_2 with the same efficiency as extract from wild-type mitochondria (Figure 2A, column 2 versus column 1). In contrast, extract from mutant mitochondria preincubated at 37°C was completely devoid of processing activity (Figure 2A, column 5 versus column 4). This lack of activity was not the result of non-specific proteolysis since the highly protease-sensitive precursor protein contained in these reactions was not degraded. Non-specific inhibition of processing in extracts of mutant mitochondria was excluded by the observation that processing in extracts from wild-type mitochondria was not inhibited by addition of mutant extract (Figure 2A, columns 7 and 8). In a further experiment extract from *Neurospora* mitochondria which had been depleted of MPP by immunoabsorption did not restore activity in mutant extract (Figure 2B, column 6 versus column 4). On the other hand, extracts from mutant mitochondria preincubated at non-permissive temperature stimulated the activity of *Neurospora* MPP immunoadsorbed to protein A−Sepharose (Figure 2B, column 7 versus column 3).

These observations show that in the mif2 mutant the MPP component of the processing enzyme is defective in a ts manner, whereas the other component of the processing enzyme, PEP, is present in active form. Since the B35.5 allele behaves as a single ts-nuclear mutation in genetic crosses, the affected gene is coding for MPP, the catalytic component of the processing enzyme.

Isolation and structural analysis of the MPP gene

The strain bearing the B35.5 allele was transformed with a yeast library containing genomic DNA fragments inserted into a centromere-bearing plasmid, and the transformed cells were plated at 37°C. DNA was prepared from a number of colonies that grew at 37°C and used to transform *Escherichia coli*. Plasmids from ampicillin-resistant bacterial colonies were isolated. They contained inserts ranging in size from 10 to 20 kb. Restriction analysis with *Eco*RI revealed the presence of a 5.0-kb fragment in all of these plasmids, suggesting that this segment of genomic DNA lies within or encompasses the MPP gene. To define the gene further,

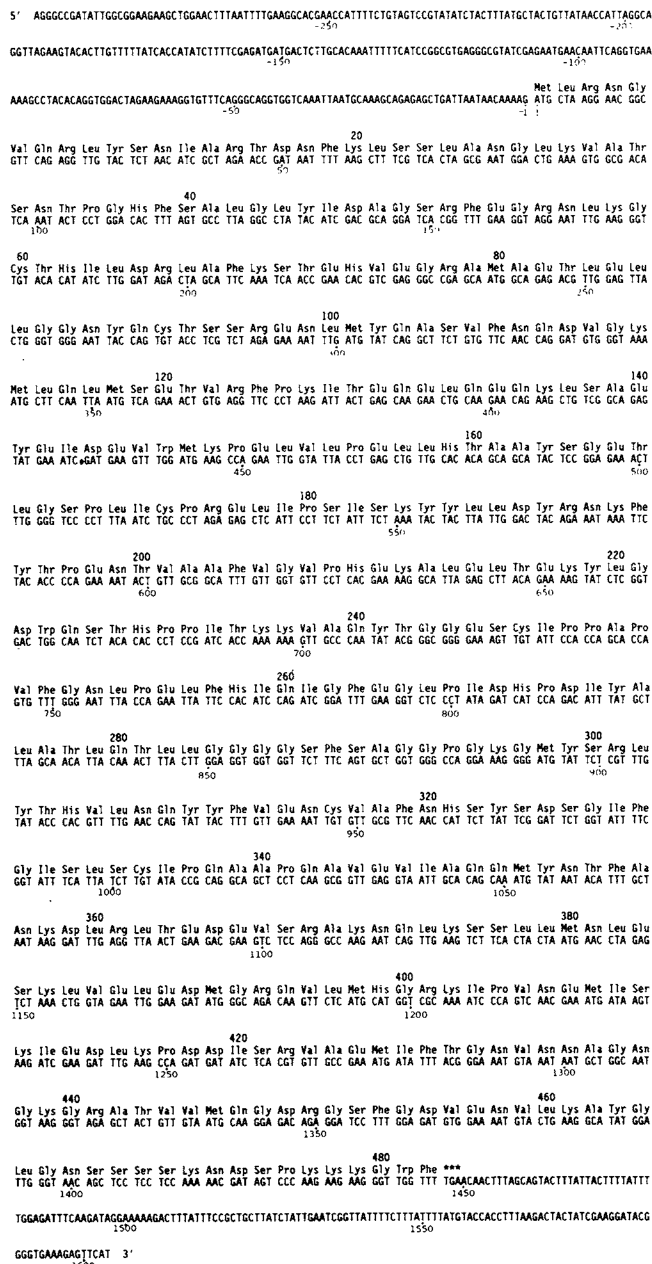

Fig. 4. Nucleotide sequence of MPP gene and deduced amino acid sequence. Numbering of the two sequences is referenced from the ATG codon that commences the open translational reading frame. A protein of 482 residues is predicted.

rescue experiments were carried out using fragments that were deletion derivatives (Figure 3). The fragments were used to transform B35.5 cells which were plated at 37°C. A number of rescuing segments were identified, the smallest of which was 2 kb in size.

The DNA sequence of this 2-kb rescuing fragment was determined (Figure 4). A continuous open reading frame comprising 1446 nucleotides was identified. This corresponded with the size of an RNA from wild-type yeast that hybridized in Northern analysis with a probe derived from the 2-kb fragment (not shown). A protein of 482 amino acid residues is predicted from the open reading frame. The sequence shows an abundance of hydrophilic amino acids and lacks stretches of hydrophobic amino acids that are characteristic of membrane-spanning proteins. The overall

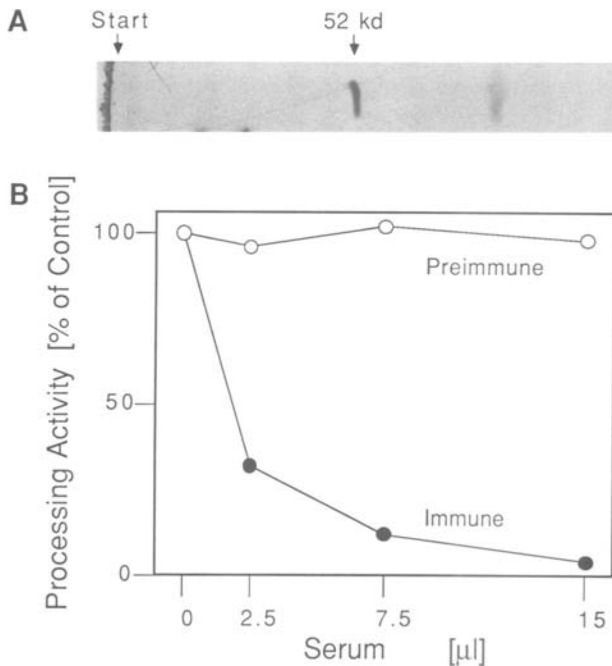

Fig. 5. Removal of processing activity from a mitochondrial extract by antibodies against the MPP gene product. (**A**) A mitochondrial pellet (50 µg) was dissociated in SDS-containing buffer, separated by SDS–PAGE and electrotransferred to nitrocellulose paper. Immunolabeling was performed with an antiserum raised in rabbits against a fusion protein containing most of the MPP gene product and part of the bacterial trpE sequence. Bound antibodies were visualized by decoration with [^{14}C]protein A and autoradiography. A band with an apparent mol. wt of 52 000 is indicated. (**B**) Immunoglobulins directed against the MPP gene product (see above) or preimmunoglobulins contained in 0, 2.5, 7.5 or 15 µl of immune or preimmune serum respectively were prebound to protein A–Sepharose. Mitochondrial Triton extracts corresponding to 15 µg of protein contained in 100 µl of extraction buffer were added to the Sepharose beads and the MPP protein was immunoadsorbed by end over end mixing for 2 h at 4°C. The beads were collected by centrifugation and the supernatants were tested for their activity in processing the precursor to cytochrome b_2 (see legend to Figure 2). Values are expressed as percentages of the activity of a control extract to which no antiserum had been added.

Table I. Subcellular localization of the MIF2 gene product

	Protein	MIF2	CS	AAC
Homogenate	100	100	100	100
PMS	88.4	7.5	10.6	2.5
Mitochondria	9.2	92.3	86.5	93.7

Spheroplasts prepared from wild-type cells by zymolyase treatment were broken in a Dounce homogenizer. Cell debris was removed by centrifugation for 5 min at 2000 *g*. The resulting homogenate was fractionated into a mitochondrial fraction and a postmitochondrial supernatant (PMS) by centrifugation for 15 min at 45 000 *g*. Fractions were analyzed for protein content and citrate synthase (CS) activity (Srere, 1969). Amounts of MIF2 gene product and ADP/ATP carrier (AAC) were determined by SDS–PAGE and immunoblotting of aliquots of the different cell fractions. Values are expressed as percentages of total content in the homogenate.

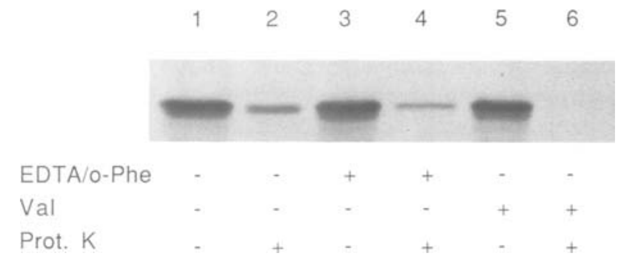

Fig. 6. Import of MPP into isolated mitochondria. In three reactions reticulocyte lysate containing radiolabeled MPP synthesized by coupled transcription/translation from the cloned gene was incubated with mitochondria from wild-type yeast (see legend to Figure 1). Reaction (**a**) served as control (**lanes 1** and **2**). Reaction (**b**) contained 7.5 mM EDTA and 1 mM *o*-phenanthroline to inhibit the mitochondrial processing peptidase (**lanes 3** and **4**). In reaction (**c**) mitochondria were de-energized by addition of valinomycin (Val) (1 µM final concentration) (**lanes 5** and **6**). After 20 min incubation at 25°C, one half of each reaction was treated with 15 µg/ml proteinase K (**lanes 2, 4** and **6**); the other halves remained untreated (**lanes 1, 3** and **5**). Mitochondria were reisolated and analyzed by electrophoresis and fluorography.

character is that of a soluble protein. At the N terminus three basic residues are present at positions 3, 8 and 15. The first acidic residue is at position 17. This may represent a mitochondrial targeting sequence, though it would be considerably shorter than the average one. N-terminal abundance of positively charged residues and absence of negatively charged residues is a feature observed for virtually all mitochondrial presequences analyzed to date. So far a consensus sequence at the cleavage site of presequences has not been identified. However, recent surveys show that the configuration of the cleavage site is often -Arg-X^1-Y- (Nicholson and Neupert, 1988). Thus, processing of the MPP precursor could potentially occur after residues 9 or 16, although this speculation can only be substantiated by radiosequencing.

The MPP gene supplies an essential function in yeast

To address whether the MPP gene is essential for yeast, a gene disruption experiment was carried out. A large part of the coding sequence (nucleotides 286–1351; see Figure 3) was removed from the plasmid carrying the entire functional MPP gene and replaced with the URA3 gene. The plasmid was then linearized within the remaining MPP coding region and used to transform a wild-type diploid strain. URA$^+$ transformants were selected and were then sporulated. Twenty-five tetrads were analyzed and in each case only two out of four spores were viable. In no case was a viable URA$^+$ spore detected. This indicates that the MPP gene serves an essential function in yeast.

The MPP gene product is located in the mitochondrial matrix of yeast and can be imported into isolated mitochondria

A fusion protein containing the bulk of the MPP gene product and part of the bacterial trpE sequence was produced in *E.coli* and injected into a rabbit to obtain antibodies against MPP. The antibody recognized in immunoblots of whole cell extracts and of mitochondrial lysates a protein with an apparent mol. wt of 52 000 (Figure 5A). This corresponds well to the mol. wt predicted by the amino acid sequence. In addition, MPP antibodies were able to remove efficiently the processing activity from mitochondrial detergent extracts (Figure 5B).

The antibody was used to determine the subcellular localization of the MPP gene product. Yeast cells were fractionated into a mitochondrial and a postmitochondrial supernatant fraction. As shown in Table I, the 52-kd protein

was recovered almost completely in the mitochondrial fraction. Upon subfractionation of mitochondria with digitonin the 52-kd protein was selectively released with soluble matrix marker enzymes (not shown). The localization of the MPP protein in the matrix agrees with our earlier finding that the catalytic component of the processing peptidase is a soluble protein of the matrix (Hawlitschek *et al.*, 1988).

The MPP gene cloned into a Bluescript derivative was transcribed with T3 RNA polymerase and the product translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. A major product of 53 kd was detected in the lysate mixture which could be immunoprecipitated with the antibody against MPP described above. Upon incubation with isolated mitochondria this translation product was imported into a location where it was inaccessible to externally added proteinase K (Figure 6, lanes 1 and 2). Import was dependent on the mitochondrial membrane potential (Figure 6, lanes 5 and 6). Newly synthesized MPP protein, both contained in the reticulocyte lysate and associated with the outer membrane of de-energized mitochondria, exhibited a slightly higher apparent mol. wt compared to the fully imported species. The reduction of mol. wt of the imported species was not observed if metal chelators (EDTA and *o*-phenanthroline) were included during the import reaction to inhibit the mitochondrial processing enzyme (Figure 6, lanes 3 and 4). This suggests proteolytic processing of MPP; however, further analysis is required. It is an intriguing question as to whether MPP is made as a precursor which is processed by the mitochondrial processing enzyme. If this is the case, the continuous presence of the active enzyme in mitochondria is a prerequisite for the formation of mitochondria, unless the precursor of MPP has the capacity for autocatalytic cleavage.

Discussion

Biochemical analysis of the mif2 mutation showed that the MIF2 (or MAS2) gene encodes MPP, the catalytic component of the mitochondrial processing peptidase. Two components, MPP itself and PEP, have been found to be required for proteolytic processing of mitochondrial precursor proteins (Hawlitschek *et al.*, 1988). The latter component is encoded by the MAS1 gene (equivalent to

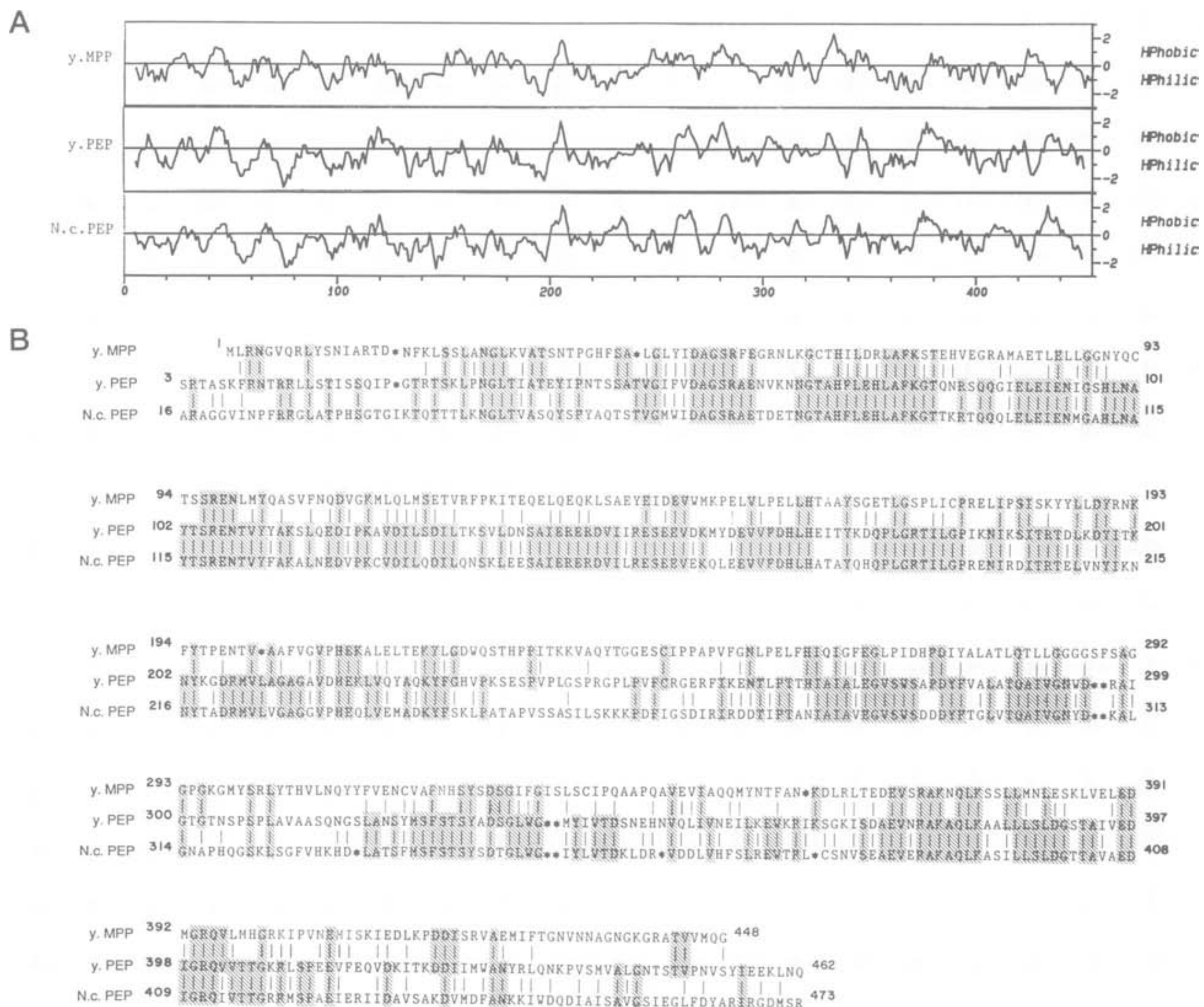

Fig. 7. Comparison of MPP with PEP from yeast (mas1 gene product) and *Neurospora*. **(A)** Hydropathy plots. The parameters as defined by the UWGCG program Pepplot (Kyte and Doolittle, 1982) were used. **(B)** Sequence alignment. Those amino acid residues in yeast MPP or *Neurospora* PEP which are identical to the corresponding residue in yeast PEP are indicated by hatched shading. Isofunctional residues are indicated by broken lines.

MIF1) (Witte *et al.*, 1988). The evidence that MIF2 specifies MPP is as follows: (i) mutant cells accumulate mitochondrial precursor proteins at the non-permissive temperature; (ii) isolated mutant mitochondria pretreated at the non-permissive temperature are able to translocate precursor proteins across the mitochondrial membranes but are unable to process them proteolytically; (iii) extracts of *Neurospora* mitochondria depleted of MPP cannot restore processing activity in extracts of mutant mitochondria; (iv) antibodies directed against the MIF2 gene product can efficiently remove the processing activity from extracts of wild-type mitochondria; (v) the MIF2 gene product is a soluble protein located in the mitochondrial matrix.

In summary, the phenotype of the mif2 mutation reflects the consequences predicted for a deficiency in MPP activity. The phenotype of the MPP mutant can be mimicked by inhibiting the processing peptidase with metal chelators. Also under these conditions import of proteins into mitochondria occurs independently of proteolytic processing (Zwizinski *et al.*, 1983; Hartl *et al.*, 1986).

Yeast MPP is a soluble protein of the mitochondrial matrix which is imported from the cytosol. The amino acid sequence deduced from the nucleotide sequence is indeed typical for a hydrophilic protein. MPP consists of 482 amino acid residues and has an apparent mol. wt of 53 000. It contains a positively charged amino terminus, a feature shared by all proteins of the mitochondrial matrix analyzed so far; however, it is not entirely clear that a short peptide is cleaved from the protein transcribed and translated from the MPP gene. We performed a structural comparison of MPP with yeast and *Neurospora* PEP whose sequences have been recently reported (Hawlitschek *et al.*, 1988; Witte *et al.*, 1988). A striking similarity is seen in the hydropathy plots, in particular in the N-terminal halves of the three proteins (Figure 7A). Alignment of the MPP sequence to yeast PEP showed 26% identity and 48% similarity if isofunctional amino acid residues are considered (Figure 7B). If *Neurospora* PEP is included in the comparison, yeast MPP has 30% identity to PEP. Identity between *Neurospora* and yeast PEP is 52% (68% similarity including isofunctional residues).

These results strongly suggest an evolutionary relationship between both components of the processing enzyme. The functional properties of MPP and PEP, however, are different. MPP alone has a very low catalytic activity which is stimulated by PEP as found with the *Neurospora* enzyme. PEP alone has no apparent cleavage activity at all. The two components, on the other hand, do not form a detectable complex. Furthermore, PEP is present in mitochondria in large excess with respect to quantity and processing-enhancing activity. A large part of PEP behaves as peripheral membrane protein associated with the inner surface of the inner membrane; in contrast, MPP is completely soluble in the matrix (Hawlitschek *et al.*, 1988). Assuming a common origin for the genes of MPP and PEP, the latter component must have lost (or never have acquired) the ability to cleave presequences but has probably acquired or preserved the ability of binding to the presequences of imported precursor proteins thus enhancing the interaction of MPP with the cleavage site. Overexpression of the MPP gene inserted into a 2 μ plasmid in the ts-mutant mif1 (identical with the previously described mas1 mutation; Yaffe and Schatz, 1984) could restore slow growth at the non-permissive temperature while overexpression of PEP in the MPP mutant did not (M.Y.Cheng and A.Horwich, unpublished). This indicates that under conditions where the intrinsic activity of MPP is sufficiently high, the processing enhancing function of PEP becomes dispensable. This could be due either to MPP being sufficient by itself or to MPP taking over some function of PEP. Recent results with the mif1 mutant suggest that in addition to stimulating cleavage, PEP has a function in facilitating the translocation of precursor proteins across the mitochondrial membranes (F.-U.Hartl, A.Horwich and W.Neupert, unpublished). This might explain why overexpression of MPP in the mif1 mutant restores growth only to rates much slower than those observed for the wild-type.

Materials and methods

Yeast strains and growth media

Strain MC3 (Matα, arg 3, ura 3-52, his 4-519, leu 2-112) was generated by crossing the previously described strain RP11 (Cheng *et al.*, 1987) with the strain JRY-438, a gift of Giorjana Barnes and Jasper Rine (Barnes and Rine, 1985). MC3:YIPGALOTC was generated by lithium acetate transformation (Ito *et al.*, 1983) of strain MC3 with *Sma*I-linearized pYIPGALOTC. YPD: 1% yeast extract, 2% peptone, 2% dextrose; YPEG: 1% yeast extract, 2% peptone, 2% ethanol and 3% glycerol. Synthetic minimal media: 0.64% yeast nitrogen base supplemented with 20 μg/ml each of arginine, leucine and histidine.

DNA manipulations

pYIPGALOTC was generated by joining a *Pvu*I–*Sal*I fragment of the plasmid pGALOTC, containing the Gal1 operon promoter linked to OTC cDNA, with a *Pvu*I–*Sal*I fragment of the plasmid pYIP5, bearing the URA3 gene. The MPP gene was isolated by plasmid rescue (Broach *et al.*, 1979) using a yeast DNA CEN library (Rose and Fink, 1987). Both the definition of functional regions of the rescuing plasmid (i.e. the rescuing plasmid with the smallest insert) and dideoxy DNA sequence analysis employed deletion constructs generated using exonuclease III and mung bean nuclease (Stratagene). Deletion plasmids were tested for their ability to restore growth at 37°C by linearizing them with *Eco*RI and transforming the mif2 allele B35.5 using the spheroplast method (Hinnen *et al.*, 1978). Cells were directly plated on YPD at 37°C. Five micrograms of plasmid DNA typically yielded 600–2000 colonies per 3 × 10^7 cells. Deletion plasmids were sequenced using the modified dideoxy method of Tabor and Richardson (1987), employing double-stranded template DNAs isolated by the alkaline lysis method (Maniatis *et al.*, 1982). Additional clones for sequence analysis were generated by complete digestion of the 5-kb rescuing DNA fragment with either *Sau*IIIA or *Hae*III followed by insertion of the collection of respective fragments into pBluescript (Stratagene).

Isolation of mutants

A culture of MC3:YIPGALOTC cells was mutagenized with 3% ethylmethanesulfonate to produce 40–60% survival (Sherman *et al.*, 1986). Portions of the culture were grown on YPEG plates at 23°C and the clones were colony purified on YPEG at 23°C and retested for absence of growth on YPD at 37°C. Temperature-sensitive colonies were next subjected to an assay involving a simultaneous shift from permissive to non-permissive temperature and from ethanol/glycerol to galactose-containing medium to induce expression of OTC. In particular, ts colonies were first grown for 3 days on YPEG plates at 23°C. Individual colonies were then resuspended in 2 ml of YPEG medium to an OD_{600} of 2 and incubated at 37°C for 30 min with vigorous agitation. Galactose was added to 2% and the culture was then grown for 2 h. One milliliter of the culture was lysed by the addition of 100 μl 1.85 M NaOH containing 7.4% β-mercaptoethanol and the extract was processed for SDS–PAGE as previously described (Yaffe and Schatz, 1984). To the second milliliter of culture, 1 ml of 'spheroplast solution' was added, containing 2.8 M sorbitol, 20 mM KP_i pH 7.4, 0.5% β-mercaptoethanol, 20 mM NaN_3 and 100 μg/ml zymolyase 100T (Miles). The mixture was incubated at 37°C for 30 min without shaking, then chilled to 4°C and centrifuged for 5 min at 1000 g to pellet the spheroplasts. Pelleted spheroplasts were resuspended in 100 μl of a solution containing 0.1% Triton X-100, 20 mM NaN_3 and 20 mM KP_i, pH 7.4. and the spheroplasts were lysed by three cycles of freezing and thawing. The extract was then assayed for OTC activity as previously described (Kalousek *et al.*, 1978) except that incubation time was extended to 1 h. Colonies exhibiting significantly

reduced levels of OTC activity in the shift assay were examined by immunoblot analysis.

Isolation of mitochondria and import in vitro

Wild-type and mutant yeast were grown for ~16 h at 23°C in YPEG until a concentration of 1 g wet weight of cells/l of culture was reached. Isolation of mitochondria was performed essentially as described previously (Daum *et al.*, 1982; Hartl *et al.*, 1987a) except that during treatment of cells with zymolyase, protease inhibitor of *Neurospora* (Schmidt *et al.*, 1984) was added to a concentration of 100 μg/ml. Growth of *N.crassa* and isolation of *Neurospora* mitochondria was carried out as published (Hartl *et al.*, 1986). Radiolabeled precursors of Fe/S protein of complex III, ATPase $F_1\beta$ and ADP/ATP carrier were synthesized by coupled transcription/translation (Pelham and Jackson, 1976; Krieg and Melton, 1984; Stueber *et al.*, 1984) of the respective cDNAs cloned into expression vector pGEM3. In the case of cytochrome b_2 a genomic clone was used (Guiard, 1985). For import *in vitro*, isolated mitochondria were incubated with reticulocyte lysates containing [^{35}S]methionine-labeled precursor proteins 5-fold diluted with BSA buffer (3% bovine serum albumin, 80 mM KCl, 2.5 mM $MgCl_2$, 10 mM Mops, pH 7.2) (Hartl *et al.*, 1987a; Pfanner and Neupert, 1987b). Treatment of mitochondria with proteinase K after import, inhibition of the mitochondrial processing peptidase and reisolation of mitochondria from the import reactions were carried out as previously described (Hartl *et al.*, 1987a).

Miscellaneous

The following procedures were performed according to published methods: *in vivo* labeling of cells with [^{35}S]methionine (Yaffe and Schatz, 1984); digitonin fractionation of mitochondria (Hartl *et al.*, 1987a); immunoprecipitation of OTC and $F_1\beta$ from cell extracts (Cheng *et al.*, 1987); immunoprecipitation of MPP from mitochondrial Triton extracts and assay of processing activities (Hawlitschek *et al.*, 1988); precipitation of protein with trichloroacetic acid (Hartl *et al.*, 1987a); protein determination (Bradford, 1976); SDS–PAGE on 8.5% (OTC) and 15% ($F_1\beta$, cytochrome b_2, Fe/S-protein, ADP/ATP carrier) polyacrylamide gels (Laemmli, 1970); immunoblotting (Burnette, 1981); quantitation of fluorographs by densitometry (Hartl *et al.*, 1986). Sequence analyses were compiled using University of Wisconsin Genetics Computer Group (UWGCG) software (Devereux *et al.*, 1984).

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