Most mitochondrial precursor proteins are processed to the mature form in one step by mitochondrial processing peptidase (MPP), while a subset of precursors destined for the matrix or inner membrane are cleaved sequentially by MPP and mitochondrial intermediate peptidase (MIP). We showed previously that yeast MIP (YMIP) is required for mitochondrial function in Saccharomyces cerevisiae. To further define the role played by two-step processing in mitochondrial biogenesis, we have now characterized the natural substrates of YMIP. A total of 133 known yeast mitochondrial precursors were collected from the literature and analyzed for the presence of the motif RX(4)(F/L/I)XXX(T/S/G)XXX(3), typical of precursors cleaved by MPP and YMIP. We found characteristic MIP cleavage sites in two distinct sets of proteins: respiratory components, including subunits of the electron transport chain and tricarboxylic acid cycle enzymes, and components of the mitochondrial genetic machinery, including ribosomal proteins, translation factors, and proteins required for mitochondrial DNA metabolism. Representative precursors from both sets were cleaved to predominantly mature form by mitochondrial matrix or intact mitochondria from wild-type yeast. In contrast, intermediate-size forms were accumulated upon incubation of the precursors with matrix from mip1Δ yeast or intact mitochondria from mip1Δ yeast, indicating that YMIP is necessary for maturation of these proteins. Consistent with the fact that some of these substrates are essential for the maintenance of mitochondrial protein synthesis and mitochondrial DNA replication, mip1Δ yeast undergoes loss of functional mitochondrial genomes.

Mitochondrial intermediate peptidase (MIP)1 is a component of the mitochondrial protein import machinery required for the maturation of a subset of nuclear-encoded precursor proteins targeted to the matrix or inner membrane (1–3) (for a review, see Ref. 4). These precursors are characterized by a three amino acid motif, RX(4)(F/L/I)XX(T/S/G)XXX(3), at the carboxyl terminus of their leader peptide (5, 6). In the matrix, the mitochondrial processing peptidase (MPP) initially cleaves the motif two peptide bonds from the arginine residue, leaving a characteristic octapeptide sequence at the protein amino terminus; the octapeptide is then cleaved by YMIP to yield mature protein (1, 2). Active YMIP is a soluble monomer of 75 kDa and presents the unusual characteristic of being a thiol-dependent metallopeptidase (7). Positioning of the octapeptide at the substrate amino terminus and a large hydrophobic residue at P-8 are essential features for cleavage by YMIP (2), a substrate specificity which is not shared by other known peptidases.

Since the molecular characterization of rat MIP (8), a family of structurally related but primarily cytosolic enzymes, thimet oligopeptidase, has been defined (9, 10). The prototype of this family is the rat testes thimet oligopeptidase EC 3.4.24.15, which has homologues in bacteria, Saccharomyces cerevisiae, and mammals (9). Although there is evidence that EC 3.4.24.15 may play a role in the processing or catabolism of pharmacologically active peptides (9), the natural substrates and biological roles of thimet oligopeptidases are not known in most cases. Likewise, the biological role of the proteolytic cleavage carried out by MIP is not yet understood, in part because only a handful of natural substrates of this peptidase are known. To date, cleavage by YMIP has been demonstrated, in vitro or in vivo, for only seven precursors from S. cerevisiae (11, 12), Neurospora crassa (13, 14), rat (15, 16), and man (1). On the other hand, several observations indicate that YMIP is important for mitochondrial function. Chromosomal disruption of the MIP1 gene causes loss of respiratory competence in S. cerevisiae (3). Moreover, this locus is conserved in eukaryotes (8, 10), and YMIP homologues from Schizosaccharomyces pombe and rat liver can rescue the phenotype of mip1Δ yeast (10), indicating that crucial substrates in this pathway must have been conserved as well.

Unlike MIP1, the S. cerevisiae MAS1/MIP1 and MAS2/MIF2 genes, which encode the two structurally related subunits of yeast MPP, are essential for yeast viability (17, 18); as such, MPP is believed to be required for global mitochondrial protein processing (19). In contrast, yeast MIP (YMIP) must be required for the biogenesis of a specific subset of mitochondrial proteins, as MIP1 inactivation leads to loss of respiratory function without affecting the viability of the facultative anaerobe S. cerevisiae (3). We have previously shown that MIP1 disruption results in failure to cleave at least two nuclear-encoded respiratory chain components, the cytochrome c oxidase subunit IV ( Cox1V) and the iron-sulfur protein of the bc1 complex (FeS) (3). In this study, we analyze the leader peptide cleavage sites of known yeast mitochondrial precursors and show that a significant fraction of these proteins contain typical MIP cleavage sites. We demonstrate that the natural substrates of YMIP include not only proteins required for respiration, but also components of the mitochondrial genetic apparatus essential for mitochondrial protein synthesis and mtDNA replication. As the latter are required for mtDNA maintenance, loss of

Yeast Mitochondrial Intermediate Peptidase*

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27366

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1The abbreviations used are: MIP, mitochondrial intermediate peptidase; YMIP, yeast MIP; MPP, mitochondrial processing peptidase; Cox1V, cytochrome c oxidase subunit IV; Cox1V, intermediate Cox1V; pCox1V, precursor Cox1V; F3β, F3β, ATPase subunit b; pF3β, pF3β, precursor F3β; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).
YMIP activity indirectly leads to mitochondrial genome instability.

**MATERIALS AND METHODS**

**Yeast Strains, Growth Media, and Plasmids**—The yeast strains used in this study are listed in Table I. The YPD, YPEG, SD, and selective media were as described previously (2). Plasmid pGEM-3Z (Promega) was used for in vitro protein expression. Two centromeric plasmids, YCp50 (21) and YCp lac22 (22), which carry URA3 and TRP1, respectively, were used for construction of the mip1(G578L)α mutant.

**Genetic Crosses**—Haploid cells of opposite mating types and different growth requirements were mixed in patches on YPD plates and left at 30°C for 8–10 h. Diploid cells were selected based on their ability to grow on SD plates lacking the growth requirements of the two parental haploid strains; they were subsequently replica-plated onto YPEG and SD plates, and growth was scored after 5 days at 30°C, as described in Myers et al. (20).

**Construction of G578Lα Mutant Yeast Strain**—A MIP1(G578L)-c-myc allele, coding for a YMIP-c-myc fusion protein with a glycine to leucine substitution at position 578 of the predicted YMIP sequence, was synthesized by site-directed mutagenesis, using two primers, sense primer 1, 5′-GCAAAATATGCGATGTAGTACAGT-3′ (bp 1,337–1,360 of MIP1), upstream from a unique KpnI site in the MIP1 gene, and antisense primer 2, 5′-GTAAGTTTTTGTTAATTTACC-3′ (bp 2,323–2,300 of MIP1), downstream from a unique EcoNI site. These two sites flank the region encoding the putative YMIP catalytic domain (10). The G578L mutation was introduced using two overlapping primers, antisense primer 2, 5′-TAGGACATTGCGATGTAGTACAGT-3′ (bp 1,870–1,841) and sense primer 3, 5′-TGCAAAGACTATACTTATGGCAGT-3′ (bp 1,841–1,870), containing the desired codon changes underlined. Two DNA fragments of 534 and 483 bp were initially amplified from wild-type MIP1 using primers 1 and 2, and primers 3 and 4, respectively. A 987-bp fragment was then synthesized in a reaction containing the two overlapping polymerase chain reaction products plus sense primer 1 and antisense primer 4. This mutated fragment was digested with KpnI and EcoNI and substituted for the corresponding wild-type fragment in a MIP1-c-myc fusion gene (3). The resulting MIP1(G578L)-c-myc gene was cloned in YCp lac22 and introduced by transformation into a trp1α mutant complemented by a wild-type allele of MIP1 on YCP50. The MIP1(G578L)-c-myc allele was exchanged with wild-type MIP1 using a URA3/5-fluoroorotic acid counterselection procedure (23). The growth phenotype of mip1(G578L)α yeast was characterized on solid YPEG medium at 25 and 37°C. The YMIP(G578L)-c-myc protein was detected by immune blotting using a monoclonal anti-c-myc (9E10) antibody, as described previously (3).

**Mitochondrial Import and Processing of Yeast Mitochondrial Precur- sor Proteins**—Mitochondria were isolated according to the procedure of Daum et al. (24). To obtain coupled mip1(G578L)α mitochondria, it was necessary to use nonpermissive conditions that cause incomplete inactivation of YMIP(G578L), such that the isolated mitochondria are defective in MIP activity but maintain some degree of respiratory function and import competence. The mip1(G578L)α yeast were grown in semi-synthetic medium at room temperature to 2–4 OD600. Spheroplasts were subsequently prepared by treatment with Zymolyase 20T for 30 min at the nonpermissive temperature (37°C). Mitochondria were then isolated at 4°C by homogenization of the spheroplasts and differential centrifugation. Either freshly isolated or previously frozen mitochondria were used in import assays, as described in Yaffe (25). Preparation of matrix fractions and determination of protein concentration were carried out as described previously (3).

The coding sequences of the previously cloned yeast genes COXIV (26), DHSA1 (27), DLDH1 (28), CYP3 (29), MRPS28 (30), tuFM (31), and RIM1 (32) were polymerase chain reaction-amplified from total genomic DNA of wild-type yeast, using primers complementary to the published sequence of these genes. All genes were cloned in pGEM-3Z downstream from the T7 polymerase promoter, and their 5′-coding regions were analyzed by DNA sequencing.

Each gene was transcribed in vitro, and the mRNA was translated in the presence of [35S]methionine using a coupled transcription/translation system (Promega Biotech Inc.) (3). The precursor for the S. cerevisiae F1-ATPase subunit β (βF1) (33) was similarly synthesized and used in control reactions. In vitro processing with crude matrix fractions, mitochondrial import assays, trypsin treatment of import reactions with or without addition of 1% Triton X-100, and reisolation of mitochondrial pellets by centrifugation were carried out as described previously (3).

Processing reactions were analyzed directly by SDS-PAGE and fluorography. The following separating gels (total length, 12.5 cm) were used: T = 12.5% for CoxIV, CYP, and RIM1; T = 10.4% for MRPS28, T = 8.3% for F1β; T = 7.3% for DLDH and tuFM, using a stock solution of acrylamide/bisacrylamide = 40:1.7, and T = 7.7% for DHSA, using a stock solution of acrylamide/bisacylamide = 30.8:1 (T denotes the total concentration of acrylamide and bisacrylamide). Separating gels were overlaid with T = 4% stacking gels. Electrophoresis at room temperature began at 180 V, was shifted to 240 V after the samples had completely entered the separating gel, and was continued for an additional 20 min (CoxIV; CYP; RIM1), 40 min (F1β), 75 min (tuFM), 90 min (DHSA; MRPS28) or 120 min (DLDH) after the samples had reached the bottom of the separating gel.

**RESULTS AND DISCUSSION**

**Cleavage Site Motifs in Yeast Mitochondrial Protein Precursors**—In two previous studies (5, 6), amino acid sequence analysis in the region surrounding the mature N terminus of about 50 mitochondrial precursor proteins from lower eukaryotes and mammals revealed that precursors cleaved in two steps by MPP and MIP share a highly conserved three-amino acid motif at the C terminus of their leader peptide. The motif, RX(2)-RX(3) (F/I)(L/V)(X)(T/S/G)(X)(X)(X)(X) (27), includes an arginine at −10, a large hydrophobic amino acid (phenylalanine, leucine, or isoleucine) at −8, and a small hydroxylated residue (serine or threonine) or glycine at −5 from the amino terminus of the mature protein (i.e. the MIP cleavage site). Because the −10 arginine is at −2 from the first cleavage site (i.e. the MPP cleavage site), eight amino acids within the motif (i.e. the octapeptide) separate the two cleavages. Not counting homologous sequences, about 13 precursors potentially cleaved in two steps by MPP and MIP were identified on the basis of this motif (5). Since then, two-step processing via formation of an octapeptide-containing intermediate has been demonstrated for seven proteins: the S. cerevisiae CoxlV (11) and FeS (12) precursors, the N. crassa FeS (13) and cytochrome A-binding protein (14) precursors, the rat malate dehydrogenase precursor (16), and the rat (15) and
human (1) ornithine transcarbamoylase precursors. Taken together, the octapeptide-containing precursors identified to date represent a rather heterogeneous group of proteins, as they include proteins targeted to the matrix or the inner membrane and involved in a variety of metabolic functions. The lack of any obvious functional correlation among these proteins represents a hindrance in understanding the role of MIP.

The identification of a larger number of octapeptide-containing precursors from a single organism may help elucidate the nature of the substrates cleaved by MIP. To characterize the octapeptide-containing precursors of S. cerevisiae, yeast mitochondrial precursor sequences were collected from Swiss Protein (May 1995). We found 133 mitochondrial matrix and inner membrane protein precursors, including 56 precursors for which the leader peptide cleavage site has been defined by amino acid sequencing of the mature N terminus (Table I, A-D), and 77 precursors for which the mature N terminus is not known (Table IIE).2

Precursors of the first group were aligned according to the mature N terminus, and the leader peptide cleavage sites were analyzed for the presence of three motifs, XRX((X/Y)S/X(R)X), XRX(T/S/G)XXXXX, and XXXI((X/Y)S/X) (R-none), which are found in precursors that are cleaved in one step by MPP (5, 6), and the motif RX(F/L)(XXT/S/G)XXXXX (R-10), which is typical of precursors that are cleaved in two sequential steps by MPP and MIP (5, 6). Eleven of the 56 precursors conformed to the R-2 motif (Table IIA); one of these precursors (CYPC) also contained an R-10 motif. Twelve precursors conformed to the R-3 motif (Table IIB); two of these precursors also contained a R-2 (CYPC) or R-10 motif (Table IIE); two of these precursors also contained a R-2 (CYPC) or R-3 (DLDH) motif. The CYPC precursor was subsequently identified as an R-2 and R-10 precursor, respectively. Another precursor (RM20) contained arginine at -10 and phenylalanine at -8 but alanine instead of serine, threonine, or glycine at -5; this precursor was also included in the R-10 group. These 11 R-10 precursors represent about 20% of the sequences analyzed. Twenty-two precursors (R-none) did not contain arginine at any of the three critical positions, -2, -3, or -10 (Table IIC). One precursor (DHA1) contained arginine at -10 but not phenylalanine, leucine, or isoleucine at -8, nor serine, threonine, or glycine at -5. Because a closely related precursor sequence (DHA2) contains asparagine at the -10 position, the DHA1 precursor was included in the R-none precursors. Together, R-none precursors represent about 41% of the sequences analyzed.

Analysis of Yeast MIP Cleavage Sites—On the basis of previous observations, the 11 R-2, 11 R-3, and 23 R-none (Table I, A-D) precursors are predicted to be cleaved in one step by yeast MPP (5, 6). When the leader peptides of these precursors were compared to the R-10 precursors, no obvious differences were observed except for the presence of the RX(F/L/I)XXX(T/S/G)XXX motif in the latter. A phenylalanine was present at the -8 position in 8 of the 11 R-10 precursors, while isoleucine and leucine were found at this position in one and two precursors, respectively. Although glycine is frequently found at the -5 position in mammalian octapeptides (5, 6), serine or threonine was found at the -5 position in 10 of the 11 R-10 yeast precursors, while alanine was found at -5 in one case (RM20). While serine appeared to be preferred at the -7 position, no particular amino acid(s) were preferred at any of the remaining 5 positions within the octapeptide. On the other hand, the 11 octapeptide sequences did seem to have a similar amino acid composition, with each containing serine or threonine at two or more positions, often clustered together. Furthermore, when the overall amino acid composition of the octapeptides was analyzed, one of the six smallest amino acids, serine, threonine, alanine, valine, proline, or glycine, was found at 47 positions (53% of the total number of amino acids). While a positively charged residue, arginine or lysine, was present at 7 positions, no acidic residues were found in any of the 11 octapeptides. No particular amino acid residue(s) appeared to be preferred at the +1 or +2 positions, nor at any other positions within the first 10 amino acids of the mature protein, consistent with the fact that no consensus sequence has been identified on the C-terminal side of known MIP cleavage sites (5, 6). Because six of the yeast R-10 precursors have now been shown to be processed by YMIP (3, 11, 12) (this study), we conclude that the octapeptides of these 11 proteins reflect the cleavage specificity of the yeast peptidase. In addition to the motif RX(F/L/I)XXX(T/S/G)XXX, the general features of these octapeptides can be used to predict new MIP substrates in yeast and other species.

Prediction of YMIP Substrates—The 56 precursors with a known mature N terminus were also analyzed for the presence of the motif RX(F/L/I)XXX(T/S/G)XXX over the entire length of their leader peptide. In addition to the 11 R-10 precursors described above, the motif was found in the leader peptide of seven precursors, but the motif was not associated with the mature N terminus of these proteins. Furthermore, while the CYPC precursor contained the motif precisely upstream from the mature N terminus, this precursor did not show two-step processing (see below). Thus, the motif was found in 19 precursors and was associated with the leader peptide cleavage site in 11 of them (58%). As processing of seven of these precursors has now been analyzed (3, 11–12) (this study) and only CYPC is not cleaved in two steps, we suggest that, in yeast sequences which contain the motif RX(F/L/I)XXX(T/S/G)XXX upstream from the mature N terminus, YMIP cleavage can be predicted with a high level of confidence. However, until a larger protein sample is analyzed, the processing pattern of putative YMIP-cleaved precursors may require confirmation by biochemical studies.

Yeast mitochondrial precursors for which the mature N terminus is not known (77 precursors; Table IIE)2 were then analyzed for the presence of the motif RX(F/L/I)XXX(T/S/G)XXX within amino acids 1–50. This region should be sufficient to include the leader peptide cleavage sites of most precursors, as suggested by the fact that, of the 56 yeast precursors with known mature N terminus analyzed in this study, only three (CCPR, RM32, and RM41) contain leader peptides longer than 50 amino acids. We found a typical RX(F/L/I)XXX(T/S/G)XXX motif in 18 of the 77 precursors (Table IIE). With the exception of the EFGM octapeptide which contained a glutamate residue, the amino acid composition of the 18 putative octapeptides was characterized in most cases by a

2 Swiss Protein accession codes of S. cerevisiae precursor proteins for which the mature N terminus is not known and which do not contain the R-10 motif within amino acids 1–50: Q01802, P32463, P22136, P22135, P32389, P21560, P14066, P32453, P18900, P27680, P00425, P21592, P21800, P32891, P15801, P32785, P08417, P37292, P38523, P09950, P19882, P22774, P33416, P25038, P07342, P06208, P36775, P32787, P35191, P32266, P20967, P19262, P32473, P33893, P02771, P10834, Q02772, P32857, P09368, P07275, P22353, Q02204, P38064, P32388, P32387, P13433, P10662, P10663, P32902, P38120, P12686, P28778, P32580, P15179, P32048, P22438, P04803, P00927, and P12887.

3 G. Isaya, unpublished data.
| TABLE II |
|-------------|

Leader peptide cleavage sites of *S. cerevisiae* mitochondrial protein precursors

Sequences were obtained from Swiss Protein (May 1995) using the program STRINGSEARCH, from the University of Wisconsin Genetics Computer Group. Precursors are aligned according to the N-terminus of the mature protein. The entire leader peptide sequence and the N-terminal 10 amino acids of the mature protein are shown for most precursors, while only the C-terminal portion of the leader peptide is shown in the case of precursors with presences longer than 40 amino acids. The precursors are divided into four groups, according to the indicated R-2, R-3, R-none, and R-10 cleavage-site motifs. For each sequence, the name of the protein and the Swiss Protein accession code are indicated below.

### A. R-2 precursors:

- ATPD (ATP synthase delta chain, AC P05626)
- COX8 (cytochrome c oxidase subunit VIII, AC P04039)
- COX6 (cytochrome c oxidase subunit 6, P00427)
- COX6A (ATP synthase A chain, P07251)
- F_{p} or ATPB (ATP synthase beta chain, P00830)
- ADH3 (alcohol dehydrogenase III, P07246)
- DHA1 (aldolase dehydrogenase precursor 1, P22821)
- DHA2 (aldolase dehydrogenase precursor 2, P23827)
- MPP1 (mitochondrial processing peptidase alpha subunit, P11914)
- MPP2 (mitochondrial processing peptidase beta subunit, P12834)
- ND1 (nucleotide-insensitive NADH-ubiquinone oxidoreductase, P23240)
- ODFX (pyruvate dehydrogenase E1 component, P16451)
- RM40 (60S ribosomal protein L4, P36517)
- RM31 (60S ribosomal protein L37, P36536)
- RM41 (60S ribosomal protein L4I, P32387)
- SYFA (phenylalanyl-tRNA synthetase alpha chain, P08425)
- SYH (histidyl-tRNA synthetase, P07086)

### B. R-3 precursors:

- ATPG (ATP synthase gamma chain, P38077)
- CISY (citrate synthase, P00890)
- COX6 (cytochrome c oxidase subunit 6, P00427)
- IATP (ATPase inhibitor, P01097)
- IDH1 (isocitrate dehydrogenase subunit 1, P28834)
- IDH2 (isocitrate dehydrogenase subunit 2, P28241)
- ODP1 (pyruvate dehydrogenase E1 component, P16451)
- ODFX (pyruvate dehydrogenase E2 component, P16451)
- RPM2 (ribonuclease P protein component, Q02773)
- STF1 (ATPase stabilizing factor, P01098)
- SODM (superoxide dismutase, P00447)

### C. R-none precursors:

- ABF2 (AR2-binding factor 2, Q02486)
- ATPA (ATP synthase alpha chain, P07251)
- F_{p} or ATPB (ATP synthase beta chain, P00830)
- ADH3 (alcohol dehydrogenase III, P07246)
- MPP1 (mitochondrial processing peptidase alpha subunit, P11914)
- MPP2 (mitochondrial processing peptidase beta subunit, P12834)
- ND1 (nucleotide-insensitive NADH-ubiquinone oxidoreductase, P23240)
- ODFX (pyruvate dehydrogenase E1 component, P16451)
- RM40 (60S ribosomal protein L4, P36517)
- RM31 (60S ribosomal protein L37, P36536)

### D. R-10 precursors:

- CoxIV (cytochrome c oxidase subunit IV, P04037)
- DHSA (succinate dehydrogenase flavoprotein subunit, P00711)
- DLDH (dehydrogenase deamidase, P09624)
- FeS or UCR1 (ubiquinol-cytochrome c reductase iron-sulfur subunit, P08067)
- HEMZ (ferrichelatase, P16622)
- IDH (NADH-dependent isocitrate dehydrogenase, P21954)
- MDHM (malate dehydrogenase, P17505)
- PT22 (PET122, P10355)
- RIM1 (single-stranded DNA-binding protein, P32445)
- RM20 (60S ribosomal protein L20, P32387)
- MP52S (40S ribosomal protein S28, P21771)

### E. Potential R-10 precursors for which the mature N terminus is not known:

- ABC1 (ABC1 protein, P27697)
- ACON (aconitate hydratase, P19414)
- ARGD (acetylornithine aminotransferase, P13044)
- ATPG (ATP synthase gamma chain, P38077)
- COQ2 (p-hydroxybenzoate-polyenyltransferase, P32378)
- EFGM (elongation factor G, P25039)
- UPF2 (eIF2B, P02992)
- MPL (protein import protein 1, Q01852)
- MRS2 (RNA splicing protein MRS2, Q01926)
- MSS1 (GTPase-ribozyme 1, P32559)
- PHR (DNA photolyase, P05066)
- PIF (DNA repair and recombination protein PIF1, P07271)
- PT11 (PET111 protein, P08468)
- RF1M (peptide chain release factor, P03770)
- RM6 (60S ribosomal protein L6, P32904)
- RM40 (60S ribosomal protein L4, P32387)
predominance of small hydroxylated amino acids, similar to the amino acid composition of authentic octapeptides. Thus, it is likely that many of these 18 octapeptides represent actual YMIP cleavage sites. Known and predicted R-10 precursors comprise about 22% of the 133 sequences in our compilation, which suggests that YMIP is involved in the biogenesis of a significant fraction of mitochondrial proteins.

YMIP Activity Is Required for Processing of R-10 Precursors in Vivo—Two of the yeast precursors in our compilation, pCoxIV and pFe/S, have been previously shown to be processed in two sequential steps via formation of an octapeptide-containing intermediate (11, 12). Additionally, we have shown previously that YMIP is required for maturation of pCoxIV and pFe/S in vivo (3). To our knowledge, two-step processing has not been previously reported for any of the remaining yeast R-10 precursors identified in this study; therefore, we have analyzed the proteolytic processing of in vitro translated pCoxIV and other yeast precursors predicted to be cleaved by YMIP. The previously cloned genes encoding precursors for CoxIV (26), DHSA (27), MRPS28 (30), DLDH (28), and CYPC (29) were used in in vitro transcription/translation reactions to produce radiolabeled polypeptides, which were then processed in vitro using mitochondrial matrix fractions prepared from either wild-type or mip1Δ yeast. The latter strain is totally deficient in MIP activity but contains levels of MPP activity which are comparable to wild-type (3).

The yeast pFβ is processed to the mature form by MPP in vivo (33) and in vitro (1), and was therefore used as a model for one-step processing. Upon incubation with a matrix fraction from wild-type yeast, pFβ was processed to the mature form (Fig. 1, lanes 2 and 4); mature Fβ was similarly produced when the precursor was incubated with a matrix fraction from mip1Δ yeast (lanes 3 and 5). A very different pattern of processing was observed with pCoxIV, which was processed to predominantly mature form with small amounts of intermediate form (iCoxIV) by wild-type matrix (lanes 7 and 9), whereas only iCoxIV was produced upon incubation of pCoxIV with mip1Δ matrix (lanes 8 and 10). We have shown previously that this processing pattern is characteristic of precursors cleaved in two steps by MPP and MIP (1, 7). The processing patterns of pDHSA (lanes 11–15) and pMRPS28 (lanes 16–20) were very similar to that of pCoxIV: mature forms were produced upon incubation of these precursors with matrix from wild-type yeast (pDHSA, lanes 12 and 14; pMRPS28, lanes 17 and 19), while intermediate-size species accumulated upon incubation with mip1Δ matrix (pDHSA, lanes 13 and 15; pMRPS28, lanes 18 and 20), indicating that, similar to pCoxIV, these precursors require YMIP activity for maturation.

Different processing patterns were observed with pDLDH and pCYPC. In addition to a typical R-10 motif, these precursors contain an arginine residue at −3 or −2, respectively; thus, they may be cleaved in two steps or one step, depending on the preferred MPP cleavage site. Incubation of pDLDH with wild-type versus mip1Δ matrix yielded two protein species of roughly similar electrophoretic mobility, although the polypeptide formed in the presence of mip1Δ matrix (lanes 23 and 25) was slightly larger than mature DLDH (lanes 22 and 24). This pattern was clearly different from the one-step processing of pFβ (lanes 1–5). Further, the same pattern was reproduced in several different sets of processing reactions, and was also observed with mitochondrial import assays (see below). Thus, although a single protein band was detected upon incubation of pDLDH with wild-type matrix (lanes 22 and 24), an intermediate-size protein was formed by mip1Δ matrix (lanes 23 and 25), strongly suggesting that pDLDH requires YMIP activity for maturation and that the presence of an arginine residue at −3 from the mature amino terminus of this precursor is not sufficient to direct cleavage by MPP. On the other hand, pCYPC was processed identically by wild-type (lanes 27 and 29) and mip1Δ matrix (lanes 28 and 30). This pattern was very similar to the one-step processing of pFβ (lanes 1–5) and clearly distinct from the processing patterns of all other R-10 precursors analyzed. This indicates that pCYPC does not require YMIP activity for maturation, presumably because the R-2 motif can direct cleavage of this precursor by MPP alone, an interesting result given that CYPC is cleaved in two steps in N. crassa (14). Similarly, pFe/S is cleaved in two steps in yeast (12) and N. crassa (13), but in one step in mammals (35). Therefore, while the overall function of MIP is conserved in eukaryotes (10), its requirement for maturation of particular precursors may be different depending on the organism.

YMIP Activity Is Required for Maturation of R-10 Precursors in Intact Mitochondria—Import reactions were carried out to assess whether the R-10 precursors require YMIP for normal biogenesis in intact mitochondria. Mitochondria isolated from mip1Δ yeast could not be used in this experiment, however, because these organelles were unable to import radiolabeled precursors efficiently (not shown). As YMIP inactivation causes multiple defects of respiratory chain complexes (3) and loss of functional mtDNA (see below), mip1Δ mitochondria must present a severe energy impairment which affects their ability to import precursor proteins in vitro. This is consistent with previous reports that import into respiration-deficient mitochondria can be performed in vitro only under specialized conditions (25). To overcome this problem, we constructed a mip1 temperature sensitive (ts) mutant which contains a chromosomally disrupted copy of wild-type MIP1 and is complemented by a MIP1-c-myc allele on a centromeric vector. The encoded YMIP-
c-myc protein contains a single amino acid substitution, glycine for leucine, at residue 578 of the YMIP sequence. G578 is found 16 residues C-terminal from the second histidine in the motif F-H-E-X-G-(X)2-H-(X)2-G-(X)2-D-(X)2-E-X-P-(X)2-E, which is shared by all known MIP and thimet oligopeptidase sequences and probably represents the active site of these enzymes (10). Conservative replacements of single amino acids in this motif caused complete loss of YMIP function in most cases. On the other hand, the mip1(G578L) mutant expressed wild-type levels of YMIP(G578L) and was able to grow slowly on YPEG at 25 °C but not at 37 °C (not shown), indicating that partial YMIP function is retained by this mutant at the permissive temperature.

To carry out import assays using mitochondria isolated from mip1(G578L) yeast, we established nonpermissive conditions which lead to only incomplete inactivation of YMIP(G578L), such that the respiratory function and thereby the import competence of these organelles is maintained. For this purpose, mip1(G578L) yeast were grown at 25 °C and shifted to the nonpermissive temperature for only 30 min during the preparation of spheroplasts; mitochondria were similarly prepared from wild-type yeast.

Isolated mitochondria were initially tested for their ability to import pF1β (Fig. 2, lanes 1-5). Similar to wild-type mitochondria (lane 2), mip1(G578L) mitochondria were able to process pF1β to the mature form (lane 3). When mitochondria were treated with trypsin upon import and reisolated by centrifugation, significant amounts of mature protein were associated with the mitochondrial pellets (wild-type, lane 4; mip1G578L, lane 5), protected from the externally added trypsin, while only residual amounts of precursor were found in the corresponding supernatants (not shown). Precursor and mature F1β were degraded when trypsin treatment of wild-type and mip1G578L mitochondria was carried out in the presence of the membrane detergent Triton X-100 (not shown). These results demonstrate that mip1(G578L) mitochondria are similar to wild-type mitochondria in their ability to import pF1β and to process this precursor to the mature form.

Similar to pF1β, pCoxIV was efficiently processed by wild-type mitochondria to the mature form (lane 7), which was protected from externally added trypsin (lane 9). In contrast, iCoxIV was predominantly accumulated by mip1(G578L) mitochondria (lane 8). However, upon trypsin treatment and reisolation of the mitochondria by centrifugation, only small amounts of mature CoxIV and traces of iCoxIV were detected (lane 10). Similar to pCoxIV, both pDHSA and pMRPS28 were processed to predominantly mature form upon import into wild-type yeast mitochondria (pDHSA, lane 12; pMRPS28, lane 17), while intermediate-size polypeptides were accumulated in mip1(G578L) mitochondria (pDHSA, lane 13; pMRPS28, lane 18). Small quantities of iDHSA (lane 15) and larger intermediates of MRPS28 (lane 20) were trypsin-protected, while all proteins were fully degraded when trypsin treatment was carried out in the presence of Triton X-100 (not shown).

These results are consistent with the pattern of processing shown by pCoxIV, pDHSA, and pMRPS28 upon incubation with wild-type and mip1Δ mitochondrial matrix (Fig. 1) and further indicate that YMIP activity is required by these precursors for normal biogenesis. Two-step processing of pDHSA is consistent with our previous observation that, in addition to complete defects of succinate cytochrome c reductase and cytochrome c oxidase, mip1Δ mitochondria also present a 90% reduction of succinate dehydrogenase activity (3). The fact that only very small amounts of iCoxIV and iDHSA could be recovered in trypsin-treated mitochondria may indicate that these intermediates have been only partially translocated by mip1Δ mitochondria; alternatively, iCoxIV and IDHSA may be rapidly degraded inside the mitochondrion. The latter possibility is supported by the observation that iCoxIV, but not iFe/S, is rapidly degraded in mip1Δ mitochondria in vivo (3), suggesting that a defect in YMIP activity may have different effects on different substrates.

Import of pDLDH by mip1(G578L) mitochondria yielded an intermediate-size protein (lane 23) which was protected from externally added trypsin (lane 25), while a smaller band, mature DLDH, was detected upon import of this precursor by wild-type mitochondria (lanes 22 and 24). This processing pattern was similar to the one obtained upon incubation of pDLDH with matrix fractions from mip1Δ and wild-type yeast, respectively, further supporting the conclusion that this precursor requires YMIP for normal biogenesis. To optimize the separation of intermediate- size protein, we used different electrophoretic conditions for each of the precursors an-

4 R. A. Rollins and G. Isaya, unpublished results.
alyzed in this study; however, the processing pattern of pDL DH indicates that a difference of only eight amino acids between a particular intermediate and the corresponding mature protein may not always be sufficient to separate these two species by standard SDS-PAGE. In such cases, matrix fractions and/or isolated mitochondria that are totally or partially deficient in MIP activity are required for accumulation and detection of intermediate-size species. These problems may explain why two-step processing has seldom been reported despite the fact that a number of R-10 precursors are known.

YMIP Cleave Components of the Yeast Mitochondrial Genetic Machinery—One of the R-10 precursors analyzed above, pMRPS28, is an essential component of the mitochondrial ribosome (30). To further investigate the role of YMIP in the biogenesis of mitochondrial genetic components, the single-stranded DNA-binding protein RIM1 (32) and elongation factor TuA, yeast tufM is predicted to contain an amino-terminal leader peptide of 37 amino acids, with a potential R-10 cleavage site displaced one residue to the mature-size form by in vitro processing. The tufM precursor was processed to an intermediate-size polypeptide which is not inside the mitochondria and is thus degraded by externally added trypsin.

Similar to pCoxIV, pRIM1 was incubated with wild-type and mip1Δ yeast mitochondria, as described above. The tufM precursor was processed to an intermediate-size form by mip1Δ mitochondria (Fig. 2, lane 28), and this protein was protected from externally added trypsin (lane 30); a slightly smaller protein, presumably mature tufM, was detected upon incubation of tufM with wild-type mitochondria (lane 27) and was also protected from trypsin (lane 29). This processing pattern is different from the one-step processing of pFZβ (lanes 1–5) and very similar to the pattern observed for pDL DH.

The in vitro translated tufM and pRIM1 were processed to predominantly mature form upon import into wild-type yeast mitochondria (Fig. 2, lane 32), and mature RIM1 was protected from externally added trypsin (lane 34). An intermediate-size polypeptide was accumulated in mip1Δ yeast mitochondria (lane 33) and was protected from externally added trypsin (lane 35), indicating that pRIM1 is processed in two steps. Discrete amounts of a mature-size protein were detected in lane 33; however, only traces of this protein were detected in the mitochondrial pellet after trypsin treatment (lane 35). Because a mature-size band was detected in the total translation reaction in the absence of mitochondria (lane 31), we conclude that most of the mature-size protein band detected in lane 33 does not represent a bona fide mature RIM1 species, but rather, a nonspecific translation product which is not inside the mitochondria and is thus degraded by externally added trypsin.

These data indicate that at least three components of the yeast mitochondrial genetic machinery, MRPS28, RIM1, and tufM, require YMIP activity for normal biogenesis.

Loss of YMIP Function Has a Secondary Effect on mtDNA Stability—Chromosomal disruption of the genes encoding MRPS28 (30), RIM1 (32), and tufM (20) has been previously shown to result in respiration-deficient phenotypes with loss of functional mtDNA. If octapeptide cleavage is required for normal function of these and, perhaps, other components of the mitochondrial genetic system, loss of YMIP function should indirectly cause mitochondrial genome instability. We showed previously that the mip1Δ phenotype can be rescued if an heterozygous disruption strain is transformed with a centromeric vector carrying MIP1 prior to sporulation (3). In contrast, haploid mip1Δ cells could not be rescued by transformation with MIP1 (not shown), suggesting that YMIP inactivation had resulted in some irreversible change in the mitochondria of these cells, presumably loss of intact mitochondrial genomes. The integrity of mtDNA in mip1Δ cells was then analyzed by genetic methods.

Two different isogenic sets of strains were used in genetic crosses: mip1Δ Y34, wild-type Y193, and yd57w A Y191; and mip1Δ Y6040 and Y6043, and wild-type Y6041 and Y6042 (Table I). The mip1Δ mutants Y34, Y6040, and Y6043 failed to complement a ρ0 tester, which is devoid of mtDNA, as well as three mit+ testers, which contain point mutations in the mitochondrial genes COXI (i.e. OX13), COXIII (i.e. OX12), and COB1, respectively (Table III). On the other hand, zygotes from a cross of a ρ+ tester, carrying normal mtDNA, with Y34 gave rise to 93% ρ+ diploid strains (not shown). These results are consistent with important deletions, if not complete loss, of mtDNA in mip1Δ cells. In contrast, the ρ+ and mit+ strains were complemented by the yd57w A mutant and the wild-type strains (Table III), indicating that the loss of functional mtDNA in mip1Δ mutants is independent of the genetic background of the parental strains.

The Known YMIP Substrates Are Functionally Related—Yeast mitochondrial proteins which mediate global protein import are essential for cell viability (19). Although the putative YMIP substrates seem to represent a significant fraction of imported mitochondrial proteins, inactivation of YMIP causes loss of mitochondrial oxidative phosphorylation without affecting viability (3). Thus, the actual fraction of mitochondrial proteins cleaved by YMIP may be smaller than we have predicted. Alternatively, proteins that are normally cleaved by YMIP may be at least partially functional in mip1Δ yeast. A third possibility is that only a specific subset of precursors contain octapeptides cleaved by YMIP, such that inactivation of the peptidase results in loss of respiratory function without affecting other metabolic pathways. The available evidence supports the last idea. The 11 R-10 precursors in Table I, include two distinct but functionally related sets of proteins. The first are components required for respiratory function: respiratory chain subunits, CoxIV (26), Fe/S (36), DHSA (27), tricarboxylic acid cycle enzyme components, DL DH (28), IDH (37), MDHM (38), and ferrochelatase, HEMZ (39), the enzyme which catalyzes the final step in the heme biosynthetic pathway. Genetic inactivation was previously carried out for six of these components and resulted in respiration-deficient phenotypes with intact mtDNA (26, 28, 36, 37, 39, 56). The second set includes essential components of the mitochondrial protein translational machinery, MRPS28 (30), PET122 (40), and RM20 (41), and a protein essential for mtDNA replication, RIM1 (32). Genetic inactivation of these components results in respiration-deficient phenotypes with loss of mtDNA. If the same type of analysis is extended to the remaining 18 potential R-10 precursors (Table IIE) similar results are obtained in 16 cases, including components required for respiratory function,
Because the number of yeast mitochondrial proteins identified to date is still relatively small, these observations do not exclude the possibility that proteins involved in other metabolic functions may be cleaved by YMIP. However, the YMIP substrates identified to date are totally consistent with the observed phenotype of mip1Δ yeast. Thus, further analysis of these substrates in mip1Δ mutants should help clarify the role of two-step processing in mitochondrial and mtDNA function.

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