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Two Related Genes Encoding Extremely Hydrophobic Proteins Suppress a Lethal Mutation in the Yeast Mitochondrial Processing Enhancing Protein*

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The processing enhancing protein of mitochondria (PEP) is an essential component that has been shown to participate in proteolytic removal of NH_2-terminal signal peptides from precursor proteins imported into the mitochondrial matrix. Using a yeast strain bearing a PEP mutation that renders it temperature-sensitive, an approach of genetic suppression was taken in order to identify additional components that could be involved with protein import: high copy plasmids comprising a yeast genomic library were tested for ability to suppress the 37 °C growth defect. Two plasmids were isolated, pSMF1 and pSMF2, which suppressed the growth defect nearly as well as the cloned PEP gene itself. Sequence analysis of the rescuing genes predicted extremely hydrophobic proteins with sizes of 63 and 60 kDa, respectively. Remarkably, the predicted SMF1 and SMF2 products are 49% identical to each other overall. To test the requirement for SMF1 and SMF2, the chromosomal genes were disrupted. Individual disruption was without effect, but cells in which both genes were disrupted grew poorly. When mitochondria were prepared from the double disruption strain grown in a nonfermentable carbon source, they were morphologically normal but defective for translocation of radiolabeled precursor proteins. SMF1 protein was provisionally localized to the mitochondrial membranes using epitope tagging. We suggest that SMF1 and SMF2 are mitochondrial membrane proteins that influence PEP-dependent protein import, possibly at the step of protein translocation.

Most mitochondrial proteins are synthesized in the cytosol as larger precursors, recognized by outer membrane components, and imported through the mitochondrial membranes. Precursors transit the membranes in unfolded conformations, and are then processed to mature size and refolded and assembled into active forms (1–3). A number of components have been identified as mediating specific events in the import pathway: several outer membrane proteins including 19-, 72-, and 38-kDa species form a complex involved in recognition and membrane insertion of precursors (4–6); cytosolic Hsp70 acts to maintain precursors in unfolded conformation (7, 8); Hsp70 in the mitochondrial matrix participates in translocation (9, 10); the processing enhancing protein (PEP)[1] and matrix processing peptidase (MPP) cooperate in proteolytic removal of signal peptides (11–16); and Hsp60 mediates refolding/oligomeric assembly (17–19). However, other components are likely to be involved, particularly in the step of translocation, where, *e.g.* no transmembrane components have been identified to date. Here, we describe an attempt to identify such components using an approach of genetic suppression beginning with the component, PEP, whose role in proteolytic processing is carried out either during or immediately after translocation.

Genetic studies in *Saccharomyces cerevisiae* revealed that both PEP and MPP are essential for cell viability on all carbon sources (12–14), as are other apparently mainstream components of the protein import pathway, including cytosolic and matrix-localized Hsp70s (7, 20, 21), the 42-kDa outer membrane protein (ISP42) (22), and Hsp60 (17, 23). PEP and MPP are hydrophilic polypeptides, 48- and 53-kDa, respectively, that are related to each other, sharing 35% of their amino acids (12). They have been isolated from yeast as a functionally active heterodimer (14, 16). In contrast, the homologues from *Neurospora crassa* purified as monomers (11). MPP by itself was found to exhibit a small amount of processing activity *in vitro*; addition of PEP enhanced the peptidase activity of MPP by at least 20-fold, but by itself, PEP lacked processing activity (11). Significantly, in *N. crassa*, PEP is found not only as a soluble component of the matrix, but it also has been identified as subunit I of the cytochrome reductase complex, apparently playing a second functional role as a structural component of this complex, localized to the inner membrane (24). In *S. cerevisiae* this role is mediated by Cor1, a protein distinct from PEP, that is nonessential (25).

The position of PEP function in the import pathway, assisting a processing step either coincident with or immediately following translocation, made PEP an attractive target for a strategy of genetic suppression that might identify additional components involved with protein import. Using an approach of high copy plasmid suppression, we isolated two genes able to suppress a PEP-deficient growth defect, and found that they encode extremely hydrophobic proteins. Remarkably, the predicted proteins prove to be 49% identical to

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[1] The abbreviations used are: PEP, processing enhancing protein; MPP, matrix processing peptidase; mif, mitochondrial import function; HA, hemaglutinin; ORF, open reading frame.

TABLE I
Yeast strains

Strain	Genotype	Source of reference
MCa1.3	MATa his4-519 ura3-52 leu2-3,-112 arg3	Derivative of MC3α; (12)
α429C	MATα mif1-1 his4-519 ura3-52 leu2-3,-112 arg3	This laboratory (12)
α5.9C	MATα mif1-2 his4-519 ura3-52 leu2-3,-112 arg3	This laboratory (12)
NY605	MATa ura3-52 leu2-3,-112	Peter Novick[a]
NY603	MATα ura3-52 leu2-3-112 pep4::URA3	Peter Novick
NY648	MATa/MATα ura3-52/ura3-52 leu2-3,-112/leu2-3,-112 gal2/gal2	Peter Novick
AW1-2	MATa/MATα ura3-52/ura3-52 leu2-3,-112/leu2-3,112	RP16-2Cα (12) × NY605
MIF1KO	MATa/MATα ura3-52/ura3-52 leu2-3,-112/leu2-3,-112 MIF1/MIF1::LEU2	AW1-2::pMIF1KO
B35.5	MATα mif2-1 his4-519 ura3-52 leu2-3,-112 arg3	This laboratory
SMF1KO(1A)	MATα ura3-52 leu2-3,-112 SMF1::LEU2 gal2	NY648::pSMF1KO
SMF2KO(1A)	MATa ura3-52 leu2-3,-112 gal2 SMF2::LEU2	NY648::pSMF2KO
SMF1/2KO(2D)	MATa ura3-52 leu2-3,-112 gal2 SMF1::LEU2 SMF2::LEU2	Derived from SMF1KO × SMF2KO

[a] Dept. of Cell Biology, Yale University.

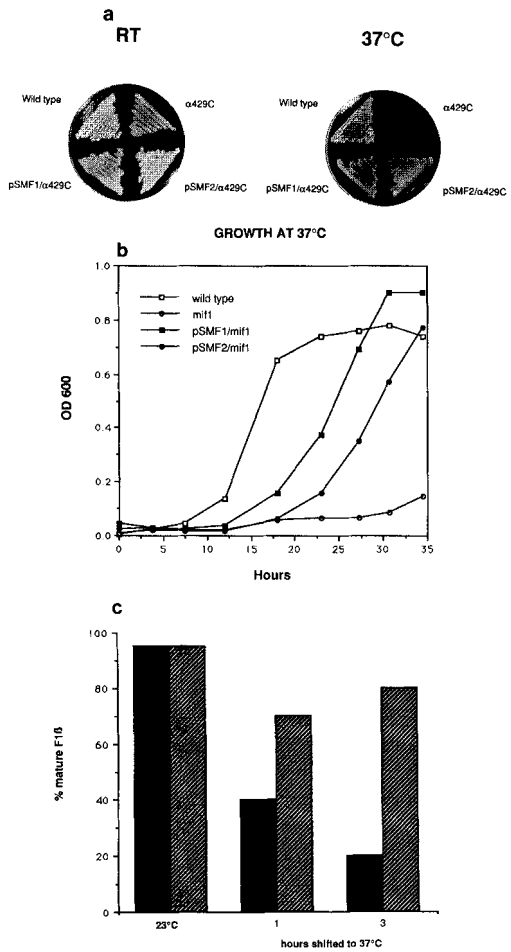

FIG. 1. **Suppression of temperature-sensitive growth of mif1 cells.** *a*, streaks on YPD plates of wild type (MCa1.3), mif1 (α429C), pSMF1/mif1, and pSMF2/mif1 strains. *b*, liquid growth of wild-type, mif1, and plasmid-bearing mif1 strains. Saturated cultures grown at 23 °C in minimal medium supplemented with the appropriate amino acids were diluted 1:500 into 50 ml of fresh medium and shifted to 37 °C. *c*, percentage conversion of F1β-ATPase precursor to mature form in mif1 (α429C) and pSMF1/mif1 during 30-min radiolabeling at 23 °C or after shift to 37 °C. The fluorogram of immunoprecipitated F1β-ATPase was scanned by laser densitometry to determine percentage of F1β species present as mature form.

each other overall. We present evidence that suggests that this two-member family of proteins localizes to the mitochondrial membranes and that the proteins influence the process of PEP-dependent protein import, possibly at the level of translocation.

MATERIALS AND METHODS

Yeast Strains—Strains of *S. cerevisiae* used in this study are listed in Table I. The temperature-sensitive PEP-defective mif1 strain employed, α429, and the temperature-sensitive MPP-defective mif2 strain used, B35.5, were isolated using a genetic screen for mutants impaired in mitochondrial import function (mif) (12, 17). Cultures were grown at 23 or 30 °C in either rich medium (YPD or YPEG: 1% yeast extract, 2% bactopeptone, 2% dextrose, or 2% ethanol/3% glycerol) or synthetic minimal medium (MM: 0.67% yeast nitrogen base, 2% dextrose, or 2% ethanol/3% glycerol) supplemented with required amino acids (Sigma) at 20 μg/ml. pSMF1/α429 and pSMF2/α429 were isolated following spheroplast transformation of the α429 strain with DNA prepared from a high copy (2u) yeast library (26). For construction of knockout strains, linear DNA segments each containing a LEU2 marker gene replacing internal portions of the respective coding domains were transformed into the diploid yeast strain NY648. In particular, the MIF1KO strain was constructed by transformation with a 4.1-kb *Pst*I fragment in which coding sequence within the MIF1 gene (1.0-kb *Xba*I-*Bst*EII fragment) had been deleted and replaced with a 2.2-kb DNA fragment bearing the LEU2 marker gene. The SMF1KO and SMF2KO strains were constructed similarly by transformation of NY648 with linear DNA molecules in which an internal region of SMF coding sequence had been removed (0.5-kb *Hin*dIII-*Sty*I deletion for SMF1 and 0.25-kb *Sca*I-*Eco*RV deletion for SMF2) and replaced with the 2.2-kb LEU2 gene. For the SMF1 chromosomal deletion, a 3.5-kb *Nhe*I-*Hin*dIII fragment was used for transformation. For chromosomal deletion of SMF2, a 2.7-kb *Hin*dIII-*Apa*LI fragment was transformed. A haploid double-knockout strain containing disruptions of both the SMF1 and SMF2 genes was generated by crossing the respective single knockout strains, sporulating, and analyzing LEU+ spores by Southern blot hybridization for a pattern reflecting the presence of LEU markers at both loci.

DNA Manipulations—Recombinant DNA techniques including gel electrophoresis, fragment isolation, enzymatic modification of DNA, bacterial transformation, and plasmid purification, were performed essentially as described (27). For DNA constructions employing the polymerase chain reaction, the manufacturer's recommended protocol was followed (Perkin-Elmer Cetus). DNA sequence analysis was carried out using the modified dideoxy termination method of Tabor and Richardson (28) employing Sequenase (version 1.0 and 2.0) reagents and protocols supplied by United States Biochemical Corp. Double stranded template DNA was purified by alkaline lysis (29). Clones for sequencing were obtained by subcloning fragments of the SMF1 and SMF2 genes into polylinker sites in pBluescript (Strata-

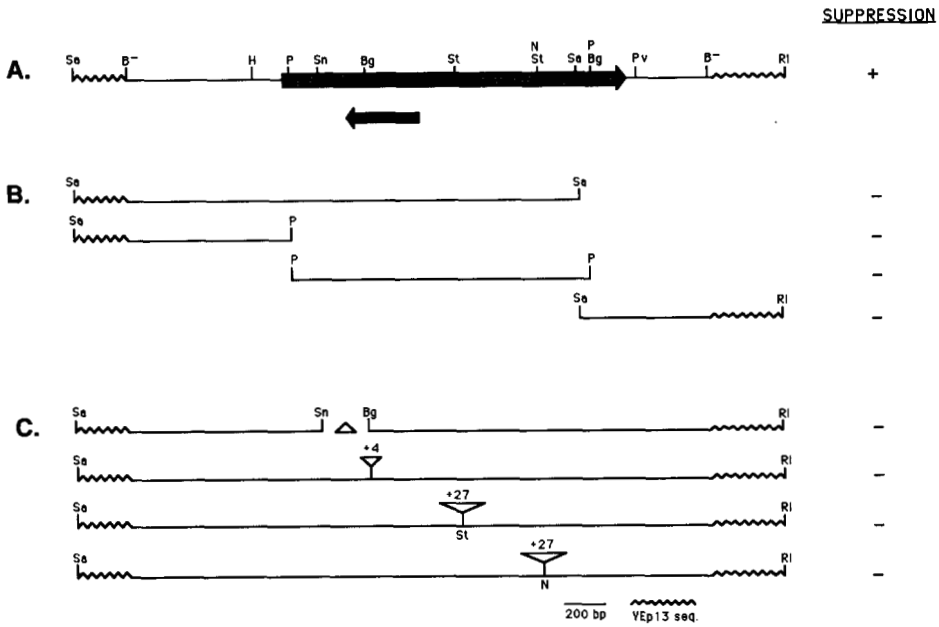

FIG. 2. **Delineation of SMF1 gene.** *A*, restriction map of the genomic insert in plasmid pSMF1. The positions of the two open reading frames deduced from the DNA sequence, and the direction of transcription, are indicated as a *checkered arrow* (large ORF) and a *solid arrow* (small ORF). *B*, lack of suppression by fragments of the SMF1 insert subcloned into a high copy yeast vector, YEp24BS. *C*, lack of suppression by insertion and deletion derivatives of pSMF1. Positions of the deletion mutation and insertion mutations are shown. The size of nucleotide insertions is indicated. Suppression was measured by transforming plasmid constructs into the mif1 mutant, α429, and directly plating on YPD medium at 37 °C. *Sa*, *Sal*I; *B*−, loss of *Bam*HI site; *H*, *Hpa*I; *P*, *Pst*I; *Sn*, *Sna*BI; *Bg*, *Bgl*II; *St*, *Sty*I; *n*, *Nco*I; *Pv*, *Pvu*II; *RI*, *Eco*RI.

gene); additional subclones were generated by random subcloning of small *Sau*3A or *Hae*III fragments. Data base searches and predictions of amino acid composition were compiled using University of Wisconsin Genetics Computer Group (UWGCG) software (30) on the Yale University VAX system computer.

Isolation of Mitochondria and in Vitro Import—Mitochondria were prepared as described (31) except that cell walls were digested with zymolyase 100T (ICN) at a concentration of 1 mg/g wet weight of cells for a 20–30-min incubation period. In addition, sorbitol was used in place of mannitol and 1 mM phenylmethylsulfonyl fluoride was included in the Dounce homogenization step. Protein concentrations were determined by the Bio-Rad protein assay. Mitochondrial preparations were stored frozen at −70 °C in 0.25 M sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2. Import of radiolabeled precursor proteins and treatment of samples following import were performed as described (19) except that import reactions contained 0.22 M sucrose and 0.5 mg/ml mitochondrial protein.

Cell Fractionation and Protein Analysis—Total cellular proteins were prepared by two different methods: 1) rapid lysis procedure: 1–5 OD units of cells were pelleted, washed with dH_2O, resuspended in 1 ml of dH_2O, and lysed by addition of 100 μl of 2 N NaOH and 50 μl of 100% trichloroacetic acid. Insoluble material was pelleted by centrifugation (15 min in a Microfuge at 4 °C). The pellet was washed twice with ice-cold acetone and resuspended in 50-μl sample buffer (32). 2) spheroplast procedure: 1–5 OD units of cells were pelleted in 1.5-ml Microfuge tubes, washed with 1 ml of dH_2O, resuspended in 1 ml of 1 M sorbitol, 0.1 M sodium citrate, pH 5.8, 10 mM EDTA (SCE), and 10 μl of β-mercaptoethanol and 75 μl of zymolyase 100T (3 mg/ml in SCE) were added followed by incubation carried out at 23 or 37 °C for 20–30 min. Spheroplasts were pelleted at low speed (5,000–10,000 rpm in the Microfuge) and washed with 1 ml of 1.2 M sorbitol. The cell pellet was resuspended in 100 μl of Laemmli sample buffer and an equal volume of acid-washed glass beads (0.4-mm diameter, Sigma). Cells were lysed by high speed vortexing for a total of 30 s (alternating 10-s vortexing with 10 s on ice). Samples were boiled for 5 min, and proteins were resolved by SDS-polyacrylamide gel electrophoresis.

Western blotting was carried out by the method of Towbin *et al.* (33). Nonradioactive enhanced chemiluminescence (Amersham) was used. For recognition of the HA epitope, a monoclonal antibody, 12CA5, was employed, purchased from Berkeley Antibody Co. (Babco).

Epitope Tagging—The coding sequence for a nine-amino acid epitope (YPYDVPDYA) from the influenza virus HA protein (34) was joined through restriction sites, in frame, at various positions in the SMF1 coding sequence. The SMF1HA fusion gene was expressed from a high copy plasmid vector, YEp24.

RESULTS

Suppression of the Lethal Growth Defect of α429 Cells by High Copy Yeast DNA Sequences—Transformation of the PEP mutant α429 was carried out with a high copy 2u (YEP13) genomic plasmid library (26), and the cells were plated at room temperature. 6,000 transformants (approximately 3–5 genome equivalents) were replica-plated at 37 °C to screen for suppression. A total of 34 individual colonies were isolated. DNA was harvested and used to transform *Escherichia coli* DH5α cells to recover the 2u plasmids, which were in turn retransformed into α429 cells to confirm ability to rescue the 37 °C growth defect. Four different groups of plasmid were able to rescue α429 as judged by restriction analysis. One of these carried the MIF1 gene. Three others were distinct and were named pSMF1, pSMF2, and pSMF3, respectively (for suppressor of mif1). pSMF1 and pSMF2 rescued the growth of α429 cells at 37 °C in both solid (Fig. 1*a*) and liquid medium (Fig. 1*b*). In liquid medium a slightly longer lag period was observed than for wild-type. To assess whether rescue of growth was associated with correction of the defect of mitochondrial protein biogenesis associated with PEP deficiency, we inspected the suppressed cells for reversal of the feature of accumulation of mitochondrial precursor proteins. α429 and pSMF1/α429 cells were radiolabeled at either 23 or 37 °C, and the extent of conversion of the subunit precursor of F1β-ATPase to its mature form was measured (Fig. 1*c*). In both cell types, at 23 °C virtually all of the immunoprecipitable F1β was found in the mature form. By contrast, in mif1 cells shifted to 37 °C, less than half of the precipitable F1β was found as mature form. In the pSMF1

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-279  GAAACCAGCTTGCTTAGATGTTAAATCATCGGAATCAATCCTGTTCGCATTCCTGTGCCGAAAATATTTCGACGTACTACGTGGACCCTA  -190

-189  GCGTCCCTAAATGCAACAGTTTTTTCTAATTTAGGATTTGTTAACTCCGCCGGGGGTGATTTGATGCTGCAATATAAATATACATAGAATA  -100

-181  ATATTTGGCTGCTCATCATTGTGCGAGCATATTAATATTTCATGAATAGGTAGAAAGATATATATCAGCAGAAAAACTAACTTTCTCAAT  -10

 -9   TAGGTCAAAATGGTGAACGTTGGTCCTTCTCATGCTGCAGTTGCTGTGGATGCTAGCGAAGCCCGCAAAAGAAATATTTCAGAAGAAGTA  81
  1           M  V  N  V  G  P  S  H  A  A  V  A  V  D  A  S  E  A  R  K  R  N  I  S  E  E  V    27

 82   TTCGAACTGAGGGACAAGAAAGATTCTACAGTGGTAATTGAGGGTGAAGCCCCGGTAAGAACTTTTACCAGTAGCTCTAGTAACCATGAA  171
 28   F  E  L  R  D  K  K  D  S  T  V  V  I  E  G  E  A  P  V  R  T  F  T  S  S  S  S  N  H  E    58

172   AGAGAGGATACGTATGTTTCTAAAAGGCAGGTAATGAGAGATATTTTTGCTAAATACTTGAAGTTCATTGGACCTGGATTGATGGTTAGT  261
 59   R  E  D  T  Y  V  S  K  R  Q  V  M  R  D  I  F  A  K  Y  L  K  F  I  G  P  G  L  M  V  S    88

262   GTGGCTTACATCGATCCCGGTAATTACTCTACTGCCGTCGATGCAGGTGCCTCTAATCAATTTTCCCTACTTTGTATCATTTTGTTATCA  351
 89   V  A  Y  I  D  P  G  N  Y  S  T  A  V  D  A  G  A  S  N  Q  F  S  L  L  C  I  I  L  L  S   118

352   AACTTTATTGCCATATTTCTGCAATGTCTGTGTATCAAGTTGGGTTCCGTTACGGGACTAGATCTAAGTCGAGCTTGCAGAGAGTACTTA  441
211   N  F  I  A  I  F  L  Q  C  L  C  I  K  L  G  S  V  T  G  L  D  L  S  R  A  C  R  E  Y  L   148

442   CCACGGTGGCTCAACTGGACATTGTATTTCTTTGCAGAATGTGCCGTTATAGCCACCGATATAGCTGAAGTGATTGGTACAGCGATTGCC  531
149   P  R  W  L  N  W  T  L  Y  F  F  A  E  C  A  V  I  A  T  D  I  A  E  V  I  G  T  A  I  A   178

532   TTGAATATCCTGATCAAAGTGCCCCTTCCAGCGGGCGTGGCCATTACTGTTGTGGATGTGTTTTTGATTATGTTTACATATAAACCTGGT  621
179   L  N  I  L  I  K  V  P  L  P  A  G  V  A  I  T  V  V  D  V  F  L  I  M  F  T  Y  K  P  G   208

622   GCGTCATCAATTAGGTTCATTAGAATATTTGAATGTTTTGTTGCAGTATTAGTTGTTGGCGTGTGCATTTGTTTCGCAATAGAATTGGCT  711
209   A  S  S  I  R  F  I  R  I  F  E  C  F  V  A  V  L  V  V  G  V  C  I  C  F  A  I  E  L  A   238

712   TATATCCCGAAGAGTACGTCCGTTAAACAAGTGTTCAGAGGATTTGTGCCATCTGCCCAAATGTTTGACCATAATGGTATTTATACCGCT  801
239   Y  I  P  K  S  T  S  V  K  Q  V  F  R  G  F  V  P  S  A  Q  M  F  D  H  N  G  I  Y  T  A   268

802   ATTTCCATCTTAGGTGCTACTGTTATGCCACATTCGTTGTTTTTGGGTTCCGCTTTAGTGCAGCCAAGGCTTTTAGATTATGACGTTAAA  891
269   I  S  I  L  G  A  T  V  M  P  H  S  L  F  L  G  S  A  L  V  Q  P  R  L  L  D  Y  D  V  K   298

892   CACGGTAATTATACTGTTTCTGATGAACAAGATAAAGTGAAAAAATCTAAATCCACTGAAGAGATTATGGAAGAAAAATATTTTAATTAT  981
299   H  G  N  Y  T  V  S  D  E  Q  D  K  V  K  K  S  K  S  T  E  E  I  M  E  E  K  Y  F  N  Y   328

982   AGACCCACGAACGCTGCTATCAAATATTGCATGAAATATTCTATGGTCGAATTAAGCATAACTCTCTTCACCCTAGCGCTTTTCGTCAAT  1071
329   R  P  T  N  A  A  I  K  Y  C  M  K  Y  S  M  V  E  L  S  I  T  L  F  T  L  A  L  F  V  N   358

1072  TGTGCCATCCTAGTTGTTGCGGGCTCCACTCTATATAACTCACCAGAAGCAGATGGGGCAGATTTGTTTACTATTCATGAATTATTATCA  1161
359   C  A  I  L  V  V  A  G  S  T  L  Y  N  S  P  E  A  D  G  A  D  L  F  T  I  H  E  L  L  S   388

1162  AGAAATCTGGCACCCGCAGCAGGTACGATTTTCATGCTCGCACTTTTATTAAGTGGTCAATCCGCAGGTGTAGTGTGTACTATGGCGGGT  1251
389   R  N  L  A  P  A  A  G  T  I  F  M  L  A  L  L  L  S  G  Q  S  A  G  V  V  C  T  M  A  G   418

1252  CAAATTGTAAGTGAGGGTCATATTAATTGGAAGTTGCAGCCATGGCAAAGAAGATTGGCCACTAGATGTATTTCGATAATCCCTTGTTTG  1341
419   Q  I  V  S  E  G  H  I  N  W  K  L  Q  P  W  Q  R  R  L  A  T  R  C  I  S  I  I  P  C  L   448

1342  GTCATCTCTATCTGTATCGGTAGAGAAGCTTTATCAAAGGCCTTAAATGCTTCCCAAGTTGTTTTATCCATAGTTCTGCCATTTTTGGTA  1431
449   V  I  S  I  C  I  G  R  E  A  L  S  K  A  L  N  A  S  Q  V  V  L  S  I  V  L  P  F  L  V   478

1432  GCACCTTTAATTTTCTTCACATGTAAAAAATCAATCATGAAAACCGAAATTACCGTCGACCATACTGAAGAAGATAGCCATAACCATCAA  1521
479   A  P  L  I  F  F  T  C  K  K  S  I  M  K  T  E  I  T  V  D  H  T  E  E  D  S  H  N  H  Q   508

1522  AATAACAACGATAGATCTGCAGGTAGCGTAATCGAGCAAGATGGTTCTAGTGGCATGGAGATAGAAAATGGAAAAGATGTCAAAATCGTT  1611
509   N  N  N  D  R  S  A  G  S  V  I  E  Q  D  G  S  S  G  M  E  I  E  N  G  K  D  V  K  I  V   538

1612  TATATGGCAAACAATTGGATTATCACTGTTATTGCTATAATTGTGTGGCTTTTCTTATCTTTACTGAACGTTTATGCCATTGTTCAATTA  1701
539   Y  M  A  N  N  W  I  I  T  V  I  A  I  I  V  W  L  F  L  S  L  L  N  V  Y  A  I  V  Q  L   568

1702  GGCATGTCTCATGGTGATATCAGTTAATGTCTGCCTCTTACATTCTTGCGCACAATAACATTAAGATTATTTGTAAGCTTTATGACAGCT  1791
569   G  M  S  H  G  D  I  S  ***                                                                  575

1792  G
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FIG. 3. **DNA sequence analysis of pSMF1 and deduced amino acid sequence of SMF1p.** The numbering of the nucleotide sequence begins as +1 at the first base of the initiation codon. The deduced amino acid sequence is shown below and predicts a protein product of 575 amino acids.

transformant, however, the extent of conversion to mature form was nearly doubled, to 70–80%. Thus the suppressing plasmid substantially reversed the phenotype of precursor accumulation characteristic of mif1 mutant cells.

To determine whether the SMF plasmids encoded products directly participating in proteolytic processing of precursor proteins, we tested whether they could rescue the lethal growth defect of MPP deficiency. Following transformation of the MPP-deficient strain, B35.5, at permissive temperature, transformants were plated at 37 °C. No growth was detected. This result is consistent with an additional observation that upon library transformation of B35.5, no other plasmids than that carrying a wild-type MIF2 gene were isolated.

The high copy plasmids, pSMF1 and pSMF2, were also tested for their ability to rescue cells devoid of PEP protein as a measure of whether suppression is mediated via bypass of PEP-mediated function. pSMF1 and pSMF2 plasmids bearing a URA3 gene were individually transformed into a diploid strain containing a wild-type allele of the MIF1 (PEP) gene and an allele disrupted via replacement with a LEU2 gene (MIF1KO). URA+ transformants were sporulated. No viable URA+LEU+ spores were detected, indicating that neither SMF1- nor SMF2-encoded products can replace the function of PEP. Apparently, PEP protein must be present albeit in a form altered by the temperature-sensitive mutation, in order for suppression to be observed by SMF1 or SMF2.

Identification and Sequence Analysis of Suppressing Genes within Plasmids pSMF1 and pSMF2—In order to identify the suppressing gene within pSMF1, fragments of the original 3.0-kb genomic insert were subcloned into a high copy yeast vector, YEP24BS, and tested for their ability to suppress the mif1 mutant, α429 (Fig. 2). None of the subcloned plasmids (Fig. 2*B*) was capable of rescuing the mif1 mutant, suggesting that the suppressing gene was likely to occupy a large portion of the insert. Also unable to rescue were plasmid derivatives deleted of 230 bp (between *Sna*BI and *Bgl*II) and a plasmid predicted to produce a shift of putative translational open reading frame at a filled-in *Bgl*II site (Fig. 2*C*).

Sequence analysis of the genomic insert of pSMF1 revealed two overlapping open reading frames (ORFs) encoded by opposite strands of the DNA (Fig. 2*A*). The larger ORF predicts a protein of 575 amino acids (calculated molecular mass of 63 kDa). The smaller ORF could encode a protein of

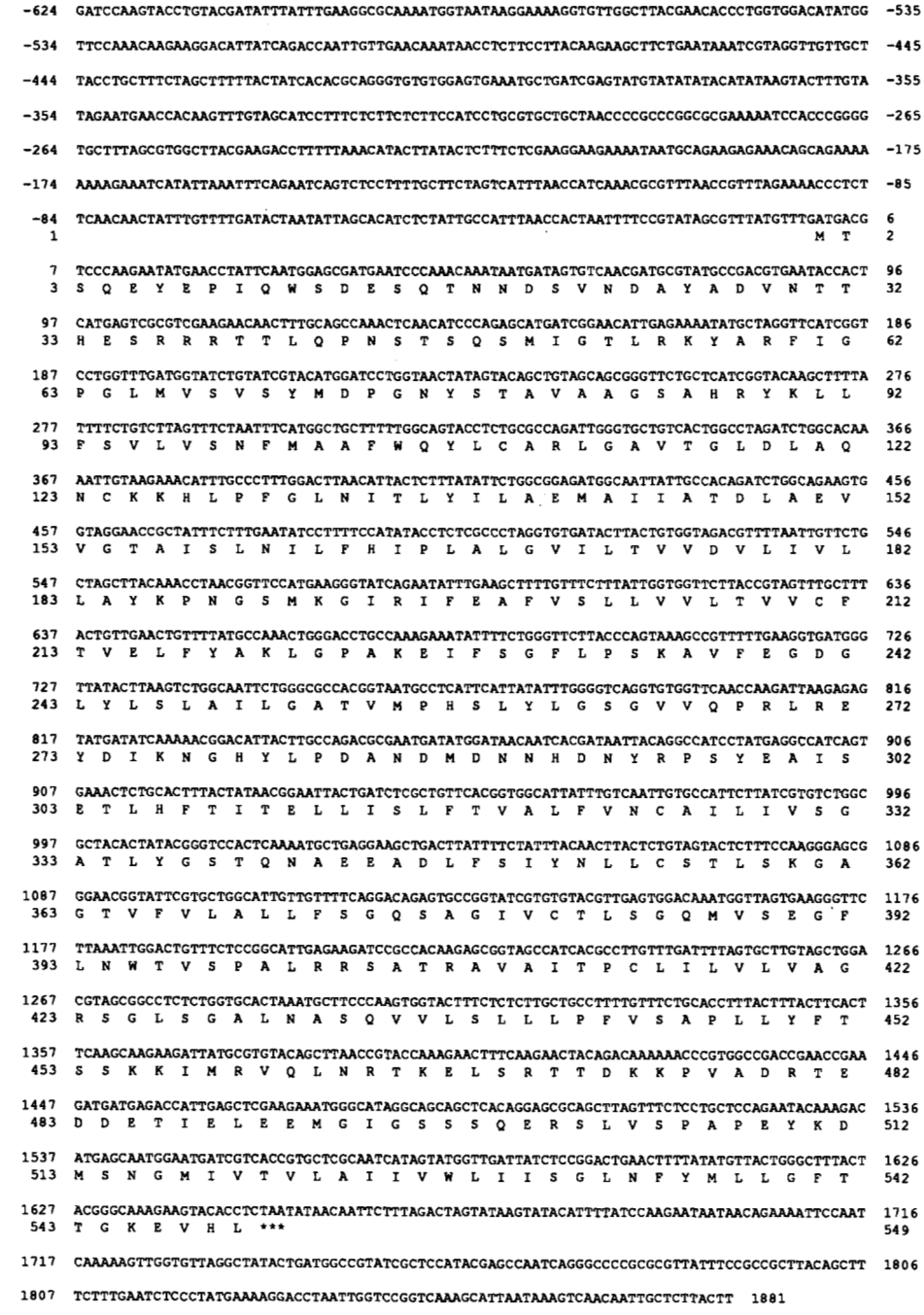

FIG. 4. **DNA sequence analysis of pSMF2 and deduced amino acid sequence of SMF2p.** The numbering of the nucleotide sequence begins as +1 at the first base of the initiation codon. The deduced amino acid sequence is shown below and predicts a protein product of 549 amino acids.

123 amino acids (13.5 kDa). Both ORFs appear to be transcribed in wild-type *S. cerevisiae* since blot analysis of poly A+ RNA carried out with an insert of pSMF1 as a probe revealed two species of approximately 2.1 and 0.6 kb (data not shown).

From the fact that subclones containing the small ORF were unable to rescue the mif1 mutant, we suspected that the small ORF was not responsible for phenotypic suppression. Consistent with this conclusion, two additional 27-base insertions in the 3′ coding domain of the longer ORF, lying 176 and 602 bases, respectively, away from the small ORF, resulted in loss of suppression (Fig. 2*C*). Thus, it is the longer open reading frame that apparently encodes the suppressing product, SMF1p.

The DNA sequence of the rescuing region and deduced amino acid sequence of SMF1p are displayed in Fig. 3. Comparisons of both DNA and predicted amino acid sequence against EMBL/GenBank, SwissProt, and the NBRF protein and nucleic acid data banks failed to reveal significant homology to any known sequence. In particular, direct sequence comparisons of SMF1 to the PEP and MPP genes and to the mitochondrial Hsp70 (SSC1) gene using the UWGCG software BESTFIT program, failed to show significant relationship. Remarkably, when the DNA and predicted amino acid sequence of SMF1p was compared with the respective sequences of the rescuing portion of pSMF2 (Fig. 4), a striking degree of identity was observed, 59% at the DNA level and 49% at the amino acid level (Fig. 5*a*). Apparently, SMF1 and SMF2 genes are members of a family, bearing structural relatedness but also functional similarity displayed by their shared ability to suppress the PEP defect of α429 cells.

Both proteins are predicted to contain an abundance of hydrophobic residues based on computer analysis of secondary structure using Kyte-Doolittle parameters. SMF1p and

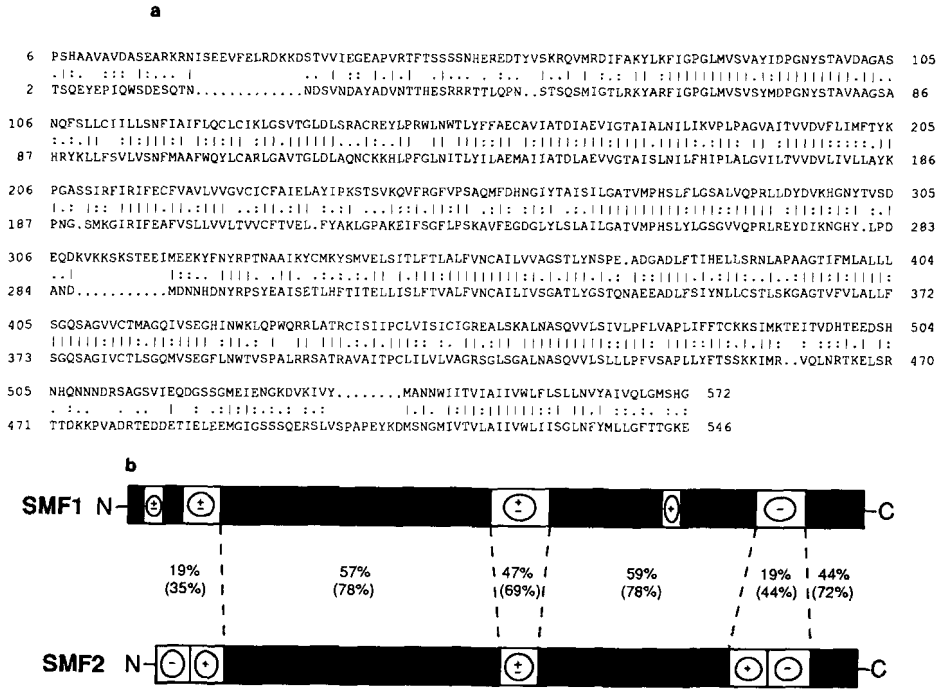

FIG. 5. **Comparison of SMF1p and SMF2p and arrangement of predicted hydrophobic domains.** *a*, protein sequence alignment between SMF1p and SMF2p was generated using UWGCG software BESTFIT program (30, 40). The SMF1p sequence is displayed above the SMF2p sequence. The numbering of the amino acid sequences is shown at the start and end of each line. Sequence identity is indicated by a *vertical line*. Sequence similarity was determined in this program using numerical values assigned each pair of amino acids based on their chemical similarity or evolutionary distance (values >0.5 are denoted by ":" and values between 0.1 and 0.5 are denoted by "."). *b*, hydrophobic domains (*shaded*) are defined as containing <13% charged amino acid residues; hydrophilic domains contain 25–40% charged amino acid residues. The percentage of identical amino acid residues between SMF1p and SMF2p within each domain is shown between the two bar diagrams, with the percentage of similar amino acids residues shown in parentheses. Regions containing charged residues are designated as + or − indicating the overall local charge.

SMF2p show a similar arrangement of extended hydrophobic stretches. As shown in Fig. 5*b*, both proteins are essentially comprised of three short hydrophilic patches, one near either terminus and one in the midportion, separated by long hydrophobic stretches. Strikingly, the highest degree of amino acid sequence identity localizes to the hydrophobic domains (78%), whereas the central hydrophilic domain is less conserved and the terminal domains are not significantly related.

Notably, neither predicted protein contains a recognizable NH_2-terminal mitochondrial targeting sequence that could direct the protein to the matrix space. Such targeting sequences of 20 to 60 amino acids are usually rich in basic residues while devoid of acidic residues. Rather, the NH_2 termini of both SMF products contain a substantial number of acidic residues. A mitochondrial matrix localization thus seems unlikely. Consistent with this is the hydrophobicity of these proteins, that makes it unlikely that they would reside outside of a membrane. Notably, proteins of the outer mitochondrial membrane and many integral membrane-spanning proteins of the mitochondrial inner membrane lack NH_2-terminal arginine-rich targeting sequences (35).

Disruption of SMF1 and SMF2 Genes—To determine whether SMF1 and/or SMF2 genes encode an essential function in *S. cerevisiae*, diploid strains were constructed in which one copy of either the SMF1 or the SMF2 gene was disrupted. Sporulation of the diploids and analysis of the resultant tetrads showed that deletion of either SMF1 or SMF2 alone in a haploid cell has no detectable effect on cell viability. This result was not surprising given the apparent structural and functional similarity of the two genes. A cross between the single disruption strains was thus carried out in order to produce spores disrupted at both loci. Indeed, following dissection of the tetrads from this cross, a population of feeble colonies was observed that was shown by Southern blot analysis to be disrupted at both SMF1 and SMF2 loci (Fig. 6*a*). The correlation between small colony growth and the presence of both disrupted genes was precise.

To further examine the growth defect of cells with the double disruption, growth was measured in liquid medium containing glycerol, a nonfermentable carbon source which requires mitochondrial function for its metabolism. Here, as on solid medium, the double disruption cells were not dead but grew more slowly than either wild-type or single disruption strains (Fig. 6*b*).

Effect of Double Disruption of SMF1 and SMF2 on Mitochondrial Protein Import—Having observed that overproduction of either SMF1 or SMF2 from a 2u plasmid suppresses the mitochondrial protein import defect of PEP deficiency, we assessed whether the simultaneous deletion of the SMF1 and SMF2 genes would affect protein import. Mitochondria were isolated from the double disruption strain grown in YPEG medium, and ability to import radiolabeled precursor proteins was examined (Fig. 7). Wild-type mitochondria were able to import and proteolytically process radiolabeled precursors of F1β-ATPase and Rieske Fe/S protein as judged by the amount of mature-sized protein that remained protected from exogenously added proteinase K (*+PK lanes*, righthand portion of Fig. 7). In contrast, while precursor proteins were found associated with mitochondria lacking SMF1 and SMF2 (SMF1/2 KO) (*−PK lanes*, lefthand portion Fig. 7), the proteins were completely susceptible to protease digestion (*+PK lanes*), indicating that they had not been translocated across the mitochondrial membranes. This apparently severe defect of translocation was observed with organelles isolated from the double disruption cells on each occasion when the cells had been grown in ethanol/glycerol. It was not, however,

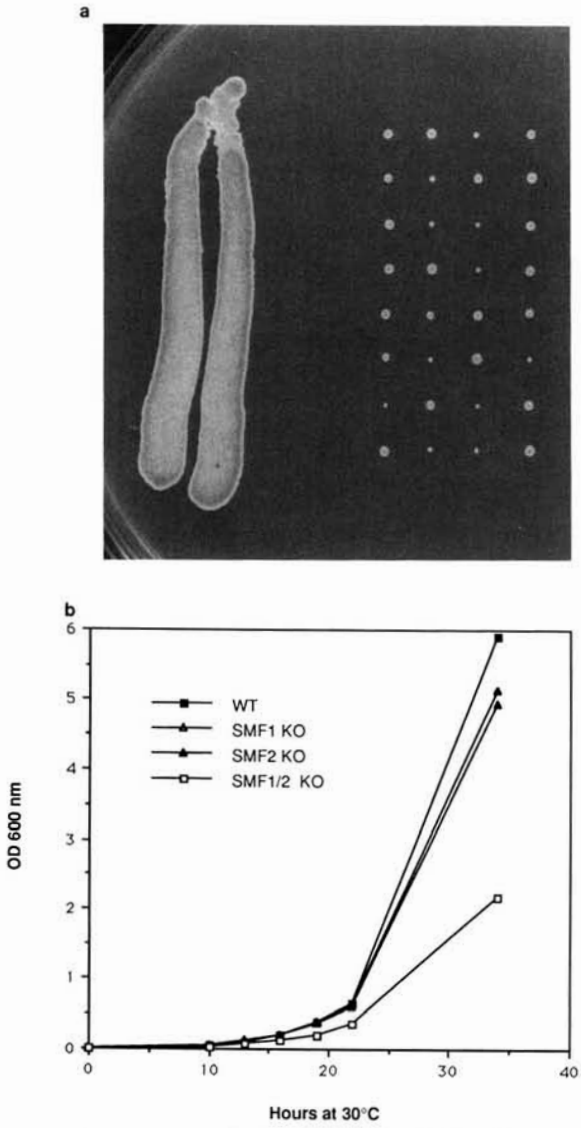

FIG. 6. **Growth of the SMF1/SMF2 double knockout strain.** *a*, YPD plate of tetrads dissected following sporulation of a diploid strain heterozygous for disruption of both SMF1 and SMF2. *b*, growth of SMF1/SMF2 double disruption haploid strain in liquid medium. Saturated liquid cultures of wild-type and SMF1/SMF2 double knockout grown in liquid YPEG were used to inoculate 50 ml of fresh YPEG medium.

observed when cells were grown in dextrose-containing YPD medium. Additionally, attempts to observe a translocation defect in intact cells using pulse-chase radiolabeling were unsuccessful in either growth medium. In contrast with the defective import of two matrix proteins by double disruption mitochondria, import of two proteins that do not enter the matrix, porin and cytochrome *c* heme lyase, was unaffected.[2] Given that neither of these latter proteins requires an electrochemical gradient for its import (36, 37), the double knockout could be considered to exert its effect via disruption of the gradient. Preliminary measurements, however, indicate that it is intact. The integrity of the organelles was also examined in the electron microscope; no discernible morphological abnormalities were observed, suggesting that the defect in import is not due to gross disruption of the integrity of the organelles.

Detection of the SMF1 Protein and Subcellular Localization—To further evaluate the involvement of SMF1 and SMF2 in mitochondrial protein import, it was desirable to address whether the proteins in fact localize to mitochondria. Initial attempts at production of antisera using both TrpE fusion proteins and synthetic peptides were unsuccessful. We thus turned to the epitope tag method of immunodetection. A nine-amino acid epitope from the influenza HA molecule was employed (34), recognizable by an available monoclonal antibody (12CA5). In-frame HA insertions were engineered into several restriction sites in the SMF1 coding sequence as shown in Fig. 8. Recombinant constructs were transformed into the mif1 mutant, α429, and tested for ability to suppress lethality at 37 °C. A single construct, *Bgl*II no. 2, containing an insertion of HA into the hydrophilic domain near the COOH terminus of SMF1p proved able to suppress.

To assess whether the SMF1HA protein in α429 cells localized to mitochondria, organelles were prepared, and solubilized extracts were fractionated and immunoblotted using anti-HA monoclonal antiserum (Fig. 9). As shown in *lane 1* in the SMF1HA transformant, a discrete immunoreactive species of apparent molecular size 70 kDa is observed. Also a smear of species of higher molecular size and several discrete species of smaller apparent size were observed. In contrast with this array of immunoreactive species, when proteins were similarly analyzed from the pSMF1/α429 strain, devoid of HA epitope, no immunologically detectable species were observed (*lane 3*). To examine whether the smaller species in the HA strain represented degradation products, we included a protease inhibitor cocktail in the Dounce homogenization step and all subsequent steps of the mitochondrial purification procedure. Rather than protecting a single species from degradation, all of the immunoreactive proteins appeared to be stabilized, since the overall intensity of the protein pattern was increased (*lane 2*). Since the HA antibody apparently does not react with endogenous mitochondrial proteins, the pattern seen in *lanes 1* and *2* must be directly related to the expression of the HA-tagged SMF1 protein. Such specificity of the anti-HA reaction was further established by analysis of proteins from influenza virus, a single reactive protein of the predicted size was observed (*lane 4*).

Several efforts were made to further evaluate the complex immunoreactive pattern seen in the SMF1HA strain by altering conditions of extraction, solubilization, and gel analysis. None of the manipulations had an effect, suggesting that the high molecular weight species do not result from aggregation of SMF1HA or oligomerization via disulfide bonds. Additionally, expression of SMF1HA in a strain deficient of the

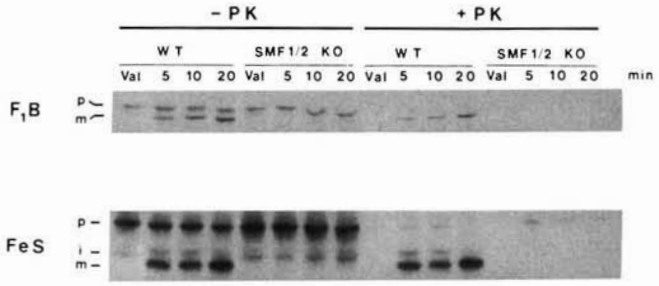

FIG. 7. ***In vitro* import into wild-type and SMF1/SMF2 double knockout mitochondria.** Import of [^{35}S]methionine-labeled precursors of F1β and Rieske Fe/S protein into MCa1.3 (*WT*) and SMF1/SMF2 double knockout mitochondria (*SMF1/2 KO*), was performed as described under "Materials and Methods." Proteins were separated by 8% SDS-polyacrylamide gel electrophoresis followed by fluorography using Amplify (Amersham). *p*, precursor (F1β, 56 kDa; FeS, 28 kDa); *i*, intermediate form (FeS, 26 kDa); *m*, mature form (F1β, 54 kDa; FeS, 25 kDa); *val*, valinomycin (1 μM), included as a negative control to block import.

[2] J. Martin, unpublished observation.

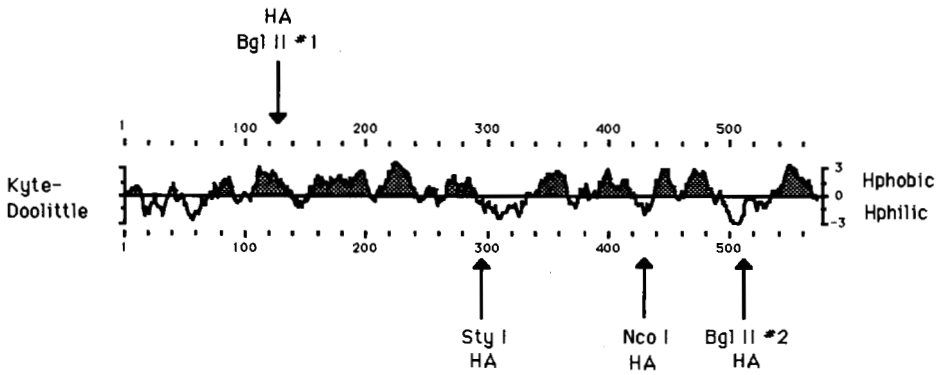

FIG. 8. **Positions of HA insertions into SMF1 coding sequence.** Positions of the restriction enzyme sites where an HA epitope was inserted is shown relative to a hydrophobicity plot of the SMF1 protein generated using the PEPPLOT program (UWGCG software) specified by Kyte-Doolittle parameters for predicting protein secondary structure. Hydrophobic domains, extending above the line, are *shaded*. Beneath the plot is a summary of analyses of the recombinant plasmids carrying HA insertions.

Recombinant	Number of HA inserts	Suppression of *mif1*
Bgl II #1	multiple	negative
Sty I	single	negative
Nco I	multiple	negative
	single	negative
Bgl II #2	multiple (fs)	negative
	single	positive

major vacuolar protease (NY603, pep4-) failed to eliminate the lower molecular size species. It appears, therefore, that the SMF1 pattern could be an inherent property of the SMF1 protein itself, perhaps related to its hydrophobic character.

DISCUSSION

We report here that a temperature-sensitive lethal mutation of the yeast mitochondrial processing enhancing protein (PEP) could be rescued by overexpression of two genes that are related to each other, SMF1 and SMF2. These genes encode extremely hydrophobic proteins that appear to localize to the mitochondrial membranes. Not only was cell growth at nonpermissive temperature rescued by multicopy plasmids carrying these genes, but to a somewhat lesser extent, growth was rescued by single copy CEN plasmids. In addition to correction of the growth defect of PEP deficiency, overexpression of the SMF genes produced correction of the biochemical defect—in both the rescued cells and mitochondria isolated from them—we observed near-normal processing of precursor proteins to their mature forms. The mechanism of genetic suppression mediated by overexpression of these genes remains unclear, but suppression required the presence of the PEP protein, albeit conditionally defective, because introduction of SMF1 and SMF2-bearing plasmids was unable to rescue the lethal phenotype of PEP deletion. Additionally, the SMF proteins themselves do not exhibit processing activity, as they could not rescue the growth or biochemical defects of MPP deficiency. Thus while the SMF proteins cannot replace either PEP or MPP, they can improve the function of a defective PEP. Their ability to suppress was not confined to the α429 allele, examined here, but extended also to the only other strong mif1 temperature-sensitive allele (α5.9) isolated from our original mutant screen.

The genetic information presented here does not address the question of whether the SMF products normally interact directly with PEP. Comment can be made, however, concerning localization of the SMF products. While the complex immunoreactive pattern of the epitope-tagged SMF1 remains to be elucidated, the pattern itself could be scored in cell fractionation experiments, permitting determination that the SMF1HA pattern associated prominently with the mitochondrial pellet. The immunoreactive species are not extractable with sodium carbonate, suggesting an integral membrane localization. Nevertheless, because mitochondrial fractions are significantly contaminated with endoplasmic reticulum, we cannot absolutely exclude endoplasmic reticulum localization, although a genetic experiment expressing SMF1HA in a temperature-sensitive mutant affecting protein secretion, sec62 (38), failed to reveal any effect on the HA-reactive pattern.

PEP in yeast resides in the matrix compartment, although a portion could possibly localize to the inner membrane as it does in *N. crassa*. How the overexpression of the SMF products with their putative mitochondrial membrane localization might serve either to stabilize mutant PEP or to improve interaction of mutant PEP with precursor proteins or with MPP, remains to be determined. How the SMF products function normally, in a setting of wild-type PEP, is also unclear. Evidently, the two SMF products are not needed for viability of yeast. While double knockout cells were slow to emerge from lag phase in liquid culture, they ultimately achieved a normal log phase growth pattern. It seems possible that additional, perhaps related, genes can perform the function of the SMF products.

Within the SMF proteins, it seems likely that the hydrophobic domains play a critical role because they are the regions most highly conserved between SMF1 and SMF2. These domains would seem likely to function at the level of a membrane. Considering the results of import experiments with the double knockout mitochondria, a role in protein translocation seems possible. This could be either a direct involvement, or perhaps, a structural role (see Ref. 39), sup-

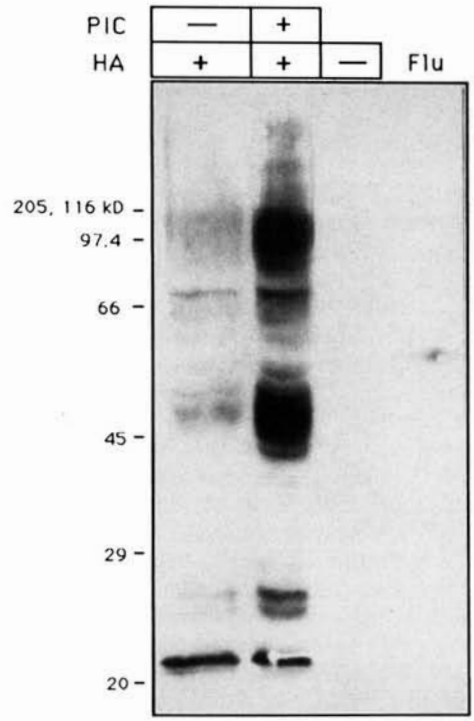

FIG. 9. **Immunodetection of the SMF1 protein.** Western blot of mitochondrial preparations from strains with and without an epitope-tagged SMF1 protein and influenza virus. Proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The filter was probed with a monoclonal antibody against the HA epitope (12CA5, Babco), and immunoreactive bands were visualized using Amersham's enhanced chemiluminescence reagents and protocol. *Lane 1*, 100 μg of mitochondrial protein from pSMF1HA/α429C strain; *lane 2*, 75 μg of mitochondrial protein from pSMF1HA/α429C strain isolated in the presence of 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture consisting of 5 μg each of leupeptin, pepstatin A, antipain, chymostatin, and aprotinin; *lane 3*, 100 μg of mitochondrial protein from pSMF1/α429C strain (no epitope added); *lane 4*, influenza virus protein preparation (acid-treated; kindly provided by Toon Stegman and Ari Helenius).

porting the action of other components. Further genetic and biochemical studies should more precisely define the function of these unusually hydrophobic components.

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