Mft52, an Acid-bristle Protein in the Cytosol That Delivers Precursor Proteins to Yeast Mitochondria*

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We have identified a novel protein, Mft52, in the cytosol of yeast cells. Mft52 has a two-domain structure that includes a receptor-like carboxyl-terminal “acid-bristle” domain, which binds basic, amphipathic mitochondrial targeting sequences. Native Mft52, purified from the cytosol of yeast cells, is found as a large particle eluting in the void volume of a Superose 6 gel filtration column. Fusion proteins, consisting of mitochondrial targeting sequences fused to nonmitochondrial passenger proteins, are targeted to mitochondria in wild-type yeast cells, but defects in the gene encoding Mft52 drastically reduce the delivery of these proteins to the mitochondria. We propose that Mft52 is a subunit of a particle that is part of a system of targeting factors and molecular chaperones mediating the earliest stages of protein targeting to the mitochondria.

Eukaryotic cells are compartmentalized. Ribosomes in the cytosol translate proteins bound for all intracellular compartments, and newly synthesized proteins have to be sorted and delivered to their correct subcellular destination. The fate of secretory proteins is determined from the time that the amino-terminal signal sequence emerges from the ribosome; a signal-recognition particle binds the hydrophobic signal sequence of the nascent secretory protein, designating the ribosome-nascent chain complex for delivery to the endoplasmic reticulum (1–4). Because almost all precursor proteins destined for the mitochondria also carry a targeting sequence at the extreme amino terminus, we have begun a search for cytosolic factors that might bind mitochondrial precursors at a similarly early stage of translation, promoting the delivery of nascent precursor proteins to the mitochondria.

At least two molecular chaperones are known to stimulate the import of precursor proteins into mitochondria: HSP70 and MSF (5–7) and MSF (8, 9). However, neither of these chaperones interacts eluting in the void volume of a Superose 6 gel filtration column. Fusion proteins, consisting of mitochondrial targeting sequences fused to nonmitochondrial passenger proteins, are targeted to mitochondria in wild-type yeast cells, but defects in the gene encoding Mft52 drastically reduce the delivery of these proteins to the mitochondria. We propose that Mft52 is a subunit of a particle that is part of a system of targeting factors and molecular chaperones mediating the earliest stages of protein targeting to the mitochondria.

However, we have identified a novel cytosolic protein, Mft52, that functions in the delivery of proteins to the mitochondria. In particular, Mft52 is required for the delivery of fusion proteins such as COXIV-DHFR, where the only targeting information is encoded at the extreme amino terminus of the precursor protein, the first part of the precursor to emerge from the ribosome. Mft52 has an “acid-bristle” domain, homologous to that found in the presequence-binding subunits of the import receptor on the mitochondrial surface (14, 15). We propose that Mft52 functions to usher nascent precursor proteins to the mitochondria.

EXPERIMENTAL PROCEDURES

Expression of Mft52 in Escherichia coli—The open reading frame encoding Mft52 was amplified by polymerase chain reaction using the primers 5′-GCG GGA TCC ATG CCT CGT TCA CAA AAA CAA ATA G-3′ and 5′-CAG CGG ATC CAA GCT TGC ATT ATA CGT GGT CAT TT-3′ and ligated into the plasmid pQE9 (Qiagen) to produce a hexahistidine-tagged form of Mft52. The hexahistidine-tagged protein was expressed in a low-copy strain of E. coli (a kind gift of Sabine Gratzer) and purified using Ni-NTA resin (Qiagen) according to the manufacturer’s instructions. Truncated Mft52 was engineered by polymerase chain reaction with the mutagenic primer 5′-AAA AGG CAG AGA TCA AGC TTG GAA GTC AAT ACT AT-3′ and cloned into the plasmid pGEN (a kind gift of Donald Smith) cut with XbaI and XhoI. This results in fusion of the Mft52 fragment from Leu157–Lys392 to GST.

Antibody Production—Monoclonal antibodies specific for Mft52 were produced from mice immunized with hexahistidine-tagged Mft52. In addition, sheep were injected with the peptide Mft52(Tyr57-His59) conjugated to diphtheria toxoid (Chiron Mimotopes) to generate epitope-specific antibodies. The anti-peptide antibodies specifically recognize both Mft52 and Tom20. Antibodies raised in rabbits to cytochrome b5, dihydrofolate reductase, and 80 S ribosomes were a kind gift from Jeff Schatz.

Tryptic Digest and Mass Spectrometry—Polyacrylamide gel slices containing approximately 5 µg of protein were reduced in the presence of protein substrates, binds to mitochondrial precursors that do not carry amino-terminal targeting sequences and binds poorly if at all to isolated targeting sequence peptides (9). By definition, chaperones such as HSP70 and MSF would be recruited to the nascent precursor protein after most of the polypeptide had been synthesized.

It is clear that precursor proteins could begin translocation across the mitochondrial membranes before translation is complete. Indeed, ribosomes can be visualized on the mitochondrial surface in situ (10) and have been shown to be actively translating mitochondrial precursor proteins (10, 11). It is also clear that the cotranslational import of precursor proteins in vitro is an efficient process, ensuring that every molecule of precursor made will be imported into the mitochondria (12, 13). What is not yet clear is how the ribosome, programmed to translate a mitochondrial precursor protein, could be recognized and then delivered to the surface of the mitochondria in vivo.

Here we identify a novel cytosolic protein, Mft52, that functions in the delivery of proteins to the mitochondria. In particular, Mft52 is required for the delivery of fusion proteins such as COXIV-DHFR, where the only targeting information is encoded at the extreme amino terminus of the precursor protein, the first part of the precursor to emerge from the ribosome. Mft52 has an “acid-bristle” domain, homologous to that found in the presequence-binding subunits of the import receptor on the mitochondrial surface (14, 15). We propose that Mft52 functions to usher nascent precursor proteins to the mitochondria.

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of dithiothreitol and alkylated with 4-vinylpyridine (16). The proteins were digested in situ with 0.2 μg of trypsin at 37 °C for 16 h. The tryptic fragments were extracted by sonication in 1% trifluoroacetic acid and then in 0.1% trifluoroacetic acid/60% acetonitrile. The extracts were pooled, lyophilized, dissolved in 4 mM ammonium acetate/0.1% formic acid, and chromatographed by reversed phase-high performance liquid chromatography. Analysis of the tryptic digest was by on-line electrospray mass spectrometry.

Synthetic Peptides and in Vitro Binding Assay—The synthetic peptide representing the targeting sequence from subunit IV of cytochrome oxidase (COXIV; MSLRQRSSRFKPAKTTYLCSRR) was synthesized commercially (Chiron Mimotopes). Synthetic peptides representing the mitochondrial targeting sequence of chaperonin 60 (CPN60; MLRLPTVLRQMRPVSRALAPHTRAC), a non-amphipathic analog of the COXIV sequence (SYNB2; MSLRQRSSRFKPAKTTYLCSRL), a highly-basic segment from ribosomal protein S6 (SGS22–249; AKRRRLSSLASTSKESSEQK) and the uncharged, amphipathic peptide SCC1-19M were kind gifts from Peter Høj, Jeff Schatz, Paul Jeno, and Katuyoshi Mihara, respectively. Purified hexahistidine-tagged Mft52 was diluted in binding buffer (50 mM sodium phosphate, pH 7.0) and bound to Ni-NTA resin by incubation on ice for 30 min. The immobilized Mft52 was incubated with the indicated amount of peptide in binding buffer (100 μl) for 30 min on ice. The resin was collected by centrifugation, washed twice in binding buffer, and analyzed by SDS-PAGE (17) and silver staining. To competitively inhibit precursor binding to Mft52, synthetic peptides (25 μM) were incubated with GST-Mft52 in 50 mM potassium phosphate (pH 7.4) for 30 min on ice, and in vitro translated [35S]-labeled Sub4-DHFR was added for a further 30 min. Unbound material was washed away with 50 mM potassium phosphate, pH 7.4, and the bound precursor was analyzed by SDS-PAGE and fluorography. The plasmid encoding Sub4-DHFR was a kind gift from Klaus Flanagan.

Deletion of the MFT1 Gene—To delete the MFT1 gene (18) from haploid yeast cells, the polymerase chain reaction-amplified DNA fragment in pQEMTFT1 was digested with PstI and EcoRV, and a PstI-XbaI (blunt) fragment of the LEU2 gene was ligated into the plasmid, deleting the sequence encoding Mft52 after the codon corresponding to Leu75. The LEU2/amft1 fragment was transformed into the yeast strain JK3–3d to generate the Δamft1 strain (YTHB2: leu3, ura3, trp1, 2 μm auxotrophic, 5 μM vanadyl complex, 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 5 μg/ml pepstatin, and 0.5 mM benzamidine (sodium salt)) to test that the hexahistidine-tagged form of Mft52 is functional. The EcoRI-HindIII fragment containing the modified open reading frame was subcloned from pQES9 into pBluescript (Stratagene), and then the EcoRI-ClaI fragment was subcloned into the yeast expression vector YPGE (19). Yeast cells (YTHB2) were transformed with the plasmid and tested for growth on rich media at 37 °C. Yeast cells carrying the YPGE–MPT1 plasmid overexpress Mft52 (3–5-fold as judged by immunoblot analysis) and grow as fast as wild-type cells at 37 °C.

Subcellular Fractionation—Yeast cells were grown in semisynthetic lactate media to mid-log phase and are converted to spheroplasts (20). To prepare the cytosolic fraction, spheroplasts were homogenized in a 40 mM Hapes buffer (pH 7.4, 0.6 M sorbitol, 5 μM vanadyl complex, 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin, 5 μg/ml pepstatin, and 0.5 mM benzamidine (sodium salt)) and centrifuged for 10 min at 10,000 rpm (Sorvall SS-34 rotor) and then for 30 min at 55,000 rpm (Beckman TL.100.3 rotor). The protein concentration of the cytosolic fraction was around 20 mg/ml. Mitochondria prepared from the 10,000 rpm pellet were purified on Nycodenz gradient centrifugation for 30 min at 55,000 rpm (Beckman TL100.3 rotor). The protein concentration of the mitochondrial fraction was around 20 mg/ml. Mitochondria were homogenized and separated by SDS-PAGE and immunoblot analysis. SDS-PAGE and immunoblot analysis in control runs, to rule out the presence of phosphohistidine in the extract, 0.1% Brij58 was added to the culture broth and centrifuged for 10 min at 10,000 rpm (Sorvall SS-34 rotor) and then the high-speed pellet (22) was separated by SDS-PAGE and immunoblot analysis. The plasmid encoding YEG-DHFR (pkSSE) was a gift from Jeff Schatz.

RESULTS

The MFT1 Gene Encodes Mft52, a Protein That Enhances Precursor Delivery to the Mitochondria—To isolate yeast mutants compromised in the targeting of proteins to the mitochondria, Emr et al. (23) devised an elegant selection system based on a toxic lacZ fusion protein. In wild-type yeast cells, efficient delivery of the F1β-lacZ fusion protein (composed of the amino-terminal targeting sequences of the β-subunit of the F1-ATPase fused to β-galactosidase) from the cytosol to the mitochondria allows cells to grow on rich media at 37 °C. Yeast cells carrying the YPGE–MFT1 plasmid overexpress Mft52 (3–5-fold as judged by immunoblot analysis) and grow as fast as wild-type cells at 37 °C.

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The cytosol fraction (0.5 ml) was loaded onto either a Superdex-75 column or a Superose 6 column equilibrated in 50 mM sodium phosphate, 50 mM sodium chloride, pH 7.0, and the eluant was monitored by absorbance at 280 nm. Fractions (1 ml) were collected and assayed by SDS-PAGE and immunoblot analysis. In control runs, to rule out the presence of phosphohistidine in the extract, 0.1% Brij58 was added to the culture broth.

Miscellaneous—DNAseq software (Hitachi) was used for sequence alignments and secondary structure predictions. The plasmid YCPFβ-lacZ (C251) was a gift from Scott Emr, and the plasmid YEGX-DHFR (pkSSE) was a gift from Jeff Schatz.

FIG. 1. Mft52 is a cytosolic protein required for the efficient delivery of fusion proteins to the mitochondria. A, wild-type (wt) or mutant (mft1) yeast cells were transformed with either pCZ1 (encoding F1β-lacZ) or pKSE (encoding COXIV-DHFR), and mitochondria were purified from the cells and analyzed by SDS-PAGE; immunoblotting used antibodies recognizing β-galactosidase (F1β-lacZ) and cytochrome b2 (cytb) or cytochrome b1 (cytb) and dihydrofolate reductase (COXIV-DHFR); B, yeast cells (lane 1) were homogenized and separated into mitochondria (lane 2), postmitochondrial supernatant (lane 3), high-speed pellet (lane 4), and cytosol (lane 5). The fractions were separated by SDS-PAGE, and immunoblots were probed with antibodies to mitochondrial cytochrome b2 (cytb), total ribosomal proteins (80S), and Mft52.
Mft52 Is a Component of a Cytosolic Particle—To determine the native molecular size of Mft52, we subjected a yeast cytosolic extract to gel-filtration chromatography. Native Mft52 is not a monomer, because it elutes in the void fraction of a Superdex-75 column (Fig. 2A). To better resolve the size of Mft52, we used a Superose 6 column. Fig. 2B shows only the earliest fractions from this column. As a marker for resolution of the column, the clathrin heavy chain, Chc1, is shown (identified by amino-terminal sequencing). Immunoblot analysis of all of the column fractions revealed that native Mft52 was recovered quantitatively in the void fraction of the Superose 6 column (i.e. 7.5–8.0 ml, corresponding to an apparent native molecular size greater than 5000 kDa), ahead of the clathrin triskelions. Note that the leading edge of the clathrin peak overlaps the void fraction, so that some Chc1 is seen in these fractions.

The huge apparent size of the native protein was surprising, given that the Mft52 subunit purified from E. coli chromatographs at 50–100 kDa as would be expected for a monomer or dimer (data not shown, see below). We conclude that native Mft52 is a subunit of a large particle in the cytosol of yeast cells.

Many of the other proteins present in the void fraction of the Superose 6 column appear to be subunits of one or more ribonucleoprotein particles. After pretreatment of the cytosolic extract with RNase, these proteins (in the range 10–45 kDa by SDS-PAGE) are removed from the void fraction (Fig. 2C). Mft52 is unaffected by RNase treatment, coeluting in the void fraction of the RNase-treated cytosol with proteins of subunit size 65, 90, and 170 kDa.

Sequence Homology of Mft52 to Acid-bristle Proteins—Sequence analysis revealed that Mft52 has two short regions of sequence similarity to the yeast proteins Tom20 and Tom22 (Fig. 3A), and antibodies raised to the peptide Mft52(T347-E365) cross-react with Tom20 on Western blots (data not shown). Tom20 and Tom22 are partner subunits of the import receptor, and both have been shown to be in direct contact with the basic, amphipathic targeting sequence of mitochondrial precursor proteins bound at the mitochondrial surface (25–27). The acid-bristle sequences in Tom20 and Tom22 define the sites responsible for recognition and binding of targeting sequences (15). The sequence segments shared between Tom20/22 and Mft52 correspond precisely to the acid-bristle sequence segments. In Mft52, the two acid-bristle sequences are predicted to sit in tandem in a carboxyl-terminal, four-helix domain (data not shown).

Domain Structure of Mft52—To produce sufficient Mft52 to investigate the predicted domain structure, we added a hexahistidine tag and overexpressed the protein in E. coli. The hexahistidine-tagged form of Mft52 was functional, because it complemented the temperature-sensitive defects of \( \Delta m t1 \) yeast cells (data not shown, see "Materials and Methods"). Recombinant Mft52 was purified from the soluble fraction of E. coli cells, but a substantial proportion was always recovered as a 34-kDa fragment (Fig. 3B). The 34-kDa form was identified as an amino-terminal fragment of Mft52 by amino-terminal sequence analysis. Thus, in addition to the acid-bristle domain, Mft52 has an amino-terminal, protease-resistant domain, which can be purified from E. coli.

To identify the protease-sensitive region that defines this domain, we prepared a complete trypsin cleavage map of both the intact (52 kDa) and fragmented (34 kDa) forms of Mft52. Mass spectrometry of the complete trypsin digest revealed that the most carboxyl-terminal peptide that could be recovered from the 34-kDa fragment corresponded to the sequence ending at Arg\(^{287} \). This suggests that in Mft52, Met\(^1 \)–Arg\(^{287} \).
The acid-bristle sequences in Tom22 and Tom20 include those disrupted in the control SCC peptide (Fig. 4). To further define the requirements for peptide binding, we tested the ability of Mft52 to bind mitochondrial targeting sequences. To test this directly, we incubated purified Mft52 with synthetic peptides representing the acidic domain of COXIV-DHFR and similar fusion proteins, and quantified the competition by PhosphorImager analysis.

**DISCUSSION**

**Targeting of Proteins to the Mitochondria: the MFT Genes**

The process of mitochondrial protein import has been the subject of intense investigation. Details on the mechanisms by which precursor proteins can be recognized by the import receptor on the mitochondrial surface and translocated across the mitochondrial membranes are becoming more and more clear (35–38). But how does a nascent mitochondrial precursor find its way from a ribosome to the surface of its target organelle? It seems unlikely that this is achieved exclusively by diffusion through the cytosol after translation is complete. We are attempting to define components of the protein targeting machinery of the cell that can direct nascent precursor proteins toward the mitochondria.

Many precursor proteins are assisted in their delivery to the mitochondria by molecular chaperones such as MSF and HSP70. However, a second class of precursors, typified by COXIV-DHFR and similar fusion proteins, do not use these chaperones, suggesting that an ATP-independent targeting factor recognizes the targeting sequence of these fusion proteins (28, 39). Two subunits of the import receptor, Tom20 and Tom22, provide acidic domains responsible for recognition and binding of the basic, amphipathic targeting sequence of both "classes" of mitochondrial precursor protein. Thus, fusion proteins like COXIV-DHFR provide a specific means to identify those cytosolic targeting factors that interact directly with the mitochondrial targeting sequence.

We have shown that in mft1 mutant yeast cells, COXIV-DHFR fails to be delivered to the mitochondria, and we have...
identified and characterized the cytosolic Mft52 protein encoded by the MFT1 gene. Mft52 has two segments of homology to the acid stiffles of the import receptor subunits Tom20 and Tom22. The Tom20 and Tom22 acid-stiffles are important in the binding of targeting sequences at the surface of the mitochondria. Indeed, a nascent Mft52 particle acts subsequently, specifically binding mito- 

reticulum, mitochondria, or cytosol (46). We propose that the first targeting component to access the emerging nascent chain their delivery to the mitochondria. 

Perhaps in the mature region of the precursor, which allow the terminal presequence, “natural” mitochondrial precursor pro-

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