A Toxic Fusion Protein Accumulating between the Mitochondrial Membranes Inhibits Protein Assembly in Vivo*

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When overexpressed in Saccharomyces cerevisiae, β-galactosidase fusion proteins directed to the mitochondria are toxic, preventing growth of yeast cells on non-fermentable carbon sources (Emr, S. D., Vassarotti, A., Garrett, J., Geller, B. L., Takeda, M., and Douglas, M. G. (1986) J. Cell Biol. 102, 523–533). We show that such fusion proteins interfere with the assembly of respiratory complexes in the mitochondrial inner membrane, without blocking protein translocation. The gene YME1, encoding an ATP-dependent metalloprotease of the mitochondrial inner membrane, acts as a suppressor of this defect; a 3-fold overexpression of Yme1p is sufficient to restore respiratory complex assembly and mitochondrial function. Detailed knowledge of the topology and effect of the toxic β-galactosidase fusion proteins will permit the identification and characterization of components that control protein sorting and protein assembly within the mitochondrial inner membrane.

A classic strategy devised, reviewed by Beckwith and coworkers (2), in characterizing the bacterial protein secretion (sec) machinery involved jamming LacZ fusion proteins into the bacterial cytoplasmic membrane. Fusion proteins consisting of a large fragment of MalE fused to the amino terminus of LacZ become lodged in the cytoplasmic membrane, and prolonged induction of the fusion protein leads to a fatal accumulation of natural secretory proteins in wild-type bacterial cells (3). The toxic fusion protein provided a tool for selecting bacterial mutants with compromised secretion: some sec mutants are so impaired in fusion protein targeting that the mutant cells survive the induction conditions and grow. These Escherichia coli mutants have been used to define several of the components of the bacterial protein secretion machinery: SecA, SecB, SecD, and PrfP (4–7).

This genetic approach was adapted by Emr et al. (1) to isolate yeast mutants that helped identify components of the transport machinery for protein import into mitochondria (8, 9). A toxic LacZ fusion protein, consisting of two-thirds of the F1-ATPase β-subunit fused to the amino terminus of LacZ (Fβ-LacZ)1 was translocated into mitochondria but remained associated with the inner membrane. Fβ-LacZ expressed in yeast disrupted mitochondrial function, and cells were respiratory-deficient and unable to grow on the non-fermentable carbon source glycerol (1). Yeast mutants that regained respiration-dependent growth defined four MFT genes (9). The first of these MFT genes encodes Mft52p, a cytosolic factor required for accumulation of Fβ-LacZ in the mitochondria (9–11).

Because ATP synthesis is measurably decreased in yeast cells expressing Fβ-LacZ, it seemed likely that the growth defect of these cells might be explained by an incorporation of Fβ-LacZ into the F0,F1-ATPase complex (8). Here we show that Fβ-LacZ is not integrated into the ATPase complex; Fβ-LacZ initiates translocation across the mitochondrial inner membrane but then aborts, and the fusion protein becomes lodged in the intermembrane space. As a result, the assembly of multisubunit complexes like cytochrome oxidase and the F0,F1-ATPase complex is dramatically decreased. YME1, encoding an ATP-dependent metalloprotease, acts as a multicopy suppressor of these defects. The toxic Fβ-LacZ fusion protein will be a useful tool to identify and characterize further components of the protein import, sorting, and assembly machineries in the mitochondrial inner membrane.

EXPERIMENTAL PROCEDURES

Yeast Strains—The yeast strain SEY2102 (MATα, ura3, leu2, his4, suc2, gal2) and plasmid CjCz1 were kind gifts from Scott Emr and Jennie Garrett (1). PCR-mediated gene disruption was used to construct the yme1 mutant strain (12). A fragment of DNA was amplified from the plasmid pRS404, using the oligonucleotide primers 5'-CGA AGA CGT GAT AGA TGA ACG TTT CAA AAA TAC TTG TGT CGC 3' and 5'-CCG GCT TGA GAC AAA TCA CCG ATA GCG TTA GAC ACC GAC 3' and 5'-CCG GCT TGA GAC AAA TCA CCG ATA GCG TTA GAC ACC GAC 3'. Yeast cells transformed with this plasmid (pRS404) were selected on minimal media containing 5-fluoroorotic acid (5-FOA) and unable to grow on the non-fermentable carbon source glyceral (1). Yeast mutants that regained respiration-dependent growth defined four MFT genes (9). The first of these MFT genes encodes Mft52p, a cytosolic factor required for accumulation of Fβ-LacZ in the mitochondria (9–11).

To construct the plasmids YEPδβ and YCPδβ, the DNA fragment encoding the Fβ-LacZ fusion was excised from CjCz1 with EcoRI and SalI and cloned into YEplac181 or YCplac111, respectively. To truncate the coding sequence of Fβ, the plasmid CjCz1 was digested with BamHI and treated with the Klenow fragment of DNA polymerase. A blunt-ended fragment of DNA was cloned into this site to truncate the coding sequence of Fβ with the amino acid sequence QFE-LARGFP(stop). Yeast cells transformed with this plasmid (pCβstop) grow at wild-type rates on glycerol-containing medium. The plasmid pPT48 carrying the YME1 gene under the control of the GAL promoter was a kind gift from Peter Thorness (13). Yeast transformations were according to Gietz et al. (14).

Preparation of Mitochondria and in Vitro Import Assays—Although SEY2102 cells expressing the Fβ-LacZ fusion protein fail to grow on glycerol, they can be forced to grow on synthetic medium with lactate as a carbon source (data not shown).2 Yeast mitochondria were prepared from cells grown on lactate-containing medium, stored frozen, and used in trypsin-accessibility assays and assays for import of 35S-labeled precursors as described previously (15).

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‡ The abbreviations used are: Fβ-LacZ, fusion between two-thirds of the F1-ATPase β-subunit to the amino terminus of β-galactosidase; PAG, polyacylamide gel electrophoresis; Su9-DHFR, fusion between two-thirds of subunit 9 of F1-ATPase to the amino terminus of dihydrofolate reductase; TOM complex, translocase in the mitochondrial outer membrane; TIM complex, translocase in the mitochondrial inner membrane; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

2 T. Beilharz and T. Lithgow, unpublished data.
**RESULTS**

*F*₁β-*LacZ Is Not Misassembled into the Mitochondrial ATPase or Jammed in the Protein Translocation Channel*—Initial characterization of yeast cells expressing *F*₁β-*LacZ* demonstrated a respiratory-dependent growth defect and a tight association of the fusion protein with the inner mitochondrial membrane; suggesting that *F*₁β-*LacZ* might be integrated into the mitochondrial *F*₁*F*₀-ATPase or the TIM23/17 (Translocase in the Inner Mitochondrial membrane) complex (8). However, this appears not to be the case. Mitochondria were prepared from either wild-type yeast cells or from cells transformed to express and accumulate *F*₁β-*LacZ* (Fig. 1A, lane 2, see also Fig. 3). The subunit stoichiometry of the *F*₁ portion of the *F*₁*F*₀-ATPase was not perturbed as judged by immunoblotting with antibodies recognizing the α, β, and γ subunits (Fig. 1B), and the rate of import of the model precursor protein Su9-DHFR, which is translocated through the TIM23/17 channel into the matrix, is virtually identical in mitochondria carrying the fusion protein as compared with control mitochondria (Fig. 1C). The genuine *F*₁α, *F*₁β, and *F*₁γ subunits were present in control mitochondria as compared with mitochondria carrying *F*₁β-*LacZ* (Fig. 1B), we checked the proportion of the *F*₁α, *F*₁β, and *F*₁γ subunits were present in control mitochondria as compared with mitochondria carrying *F*₁β-*LacZ* (Fig. 1B). We found that the presence of *F*₁β-*LacZ* led to a reduction in fully assembled cytochrome *c* oxidase complex (complex IV). Analyzed by SDS-PAGE, similar levels of both *F*₁β and *Cox2p* subunits are present in each mitochondrial sample (Fig. 2, bottom panel). The i-AAA complex, an integral complex in the mitochondrial inner membrane containing the AAA-protein *Yme1p* (18), serves as a useful internal control for loading of Blue native PAGE, as the level of i-AAA complex was unaffected in mitochondria carrying *F*₁β-*LacZ* (Fig. 2).

Waller streaks in the inner membrane remained intact under these conditions, as shown by the protection of the matrix-located α-ketoglutarate dehydrogenase. Only if the inner membrane is breached with Triton X-100 is the enzyme accessible to trypsin (lane 5). In the case of B. subtilis, a soluble protein in the intermembrane space, the enzyme is degraded. While present in the intermembrane space, *F*₁β-*LacZ* was not readily solubilizable. After osmotic shock and resolation of the remaining mitoplasts by centrifugation, cytochrome *b*₂, a soluble protein in the intermembrane space, was released into the supernatant. However, extraction of *F*₁β-*LacZ* required treatment with sodium carbonate (Fig. 3B, alkali), suggesting that the fusion protein was associated peripherally with a membrane surface within the intermembrane space. Since the fusion protein is thought to be linked to the inner membrane with the hydrophobic *β*-LacZ domain, it is reasonable to assume that the fusion protein is associated with the outer surface of the intermembrane space. The genuine *F*₁β precursor is synthesized in the cytosol with...
phenotype reminiscent of the loss-of-function phenotypes of Δyme1 mutants. First, both Δyme1 mutants and wild-type cells overexpressing F1β-LacZ show loss of growth on glycerol as a carbon source. Second, both are cold-sensitive for growth on glucose media (Ref. 13; data not shown). Third, like wild-type cells overexpressing F1β-LacZ, yeast mutants lacking Yme1p show diminished assembly of Cox2p into the cytochrome oxidase complex. Might the F1β-LacZ fusion protein exert its toxic effect by titrating the function of Yme1p in the intermembrane space?

Overexpression of YME1 relieves the phenotype of cells expressing the F1β-LacZ fusion protein, allowing the cells to grow at near wild-type rates on media with glycerol as the carbon source (Fig. 4A). Overexpression of the related genes RCA1 or AFG3 did not restore growth on media with glycerol as a carbon source. In the absence of the fusion protein, levels of Yme1p are tightly regulated, so overexpression of YME1 does not lead to an increase in the steady-state level of Yme1p in control mitochondria (Ref. 13; data not shown). However, overexpression of YME1 in cells carrying F1β-LacZ fusion protein leads to a 3-fold increase in the steady-state level of Yme1p (Fig. 4B, lane 3).

There are three possible mechanisms by which overexpression of Yme1p might relieve the mitochondrial defects of yeast cells carrying F1β-LacZ. First, F1β-LacZ might be degraded as a result of increased proteolysis mediated by Yme1p. Second, F1β-LacZ might be forced across the inner membrane into the matrix of the mitochondria in a Yme1p-dependent manner. Third, F1β-LacZ might be maintained in the intermembrane space by a chaperone-like activity of Yme1p. We found that overexpression of YME1 did not lead to degradation of F1β-LacZ (Fig. 4B, lane 3), rather the fusion protein remained in the intermembrane space, accessible to exogenously added trypsin when the mitochondrial outer membrane was ruptured by osmotic shock.2

Without affecting the relative levels of F1β and F1β-LacZ (Fig. 4C, lanes 2 and 3), the increased level of Yme1p is sufficient to complement the assembly of β-subunits into the F1F0-ATPase complex (Fig. 4C, bottom panel) or Cox2p into cytochrome oxidase.2

DISCUSSION

Large F1β-LacZ Fusion Proteins Remain Associated with the i-AAA Complex—The F1β-LacZ fusion protein has been previously shown to remain associated with the mitochondrial inner membrane (1). In this study, we demonstrate that F1β-LacZ can complete translocation through the TOM complex in the outer membrane; however, it fails to complete translocation across the inner membrane. The amino terminus of the F1β-LacZ precursor is translocated far enough into the mitochondrial matrix to be processed correctly by the matrix-located processing peptidase. However, translocation of F1β-LacZ was not complete, and the mature protein remained in the intermembrane space.

Overexpression of YME1 Suppresses Growth Defects in Cells Expressing F1β-LacZ—The assembly of protein subunits encoded from the nuclear and mitochondrial genomes may depend on a set of ATP-dependent proteases located within the mitochondrial inner membrane. These proteases possess a chaperone-like function that can be utilized in the degradation as well as the assembly of mitochondrial proteins (21). Yeast mitochondria have at least three members present in the mitochondrial inner membrane: Afg3p/Yta10p, Rca1p/Yta12p, and Yme1p (14, 18, 22–26). Only Yme1p is oriented with its ATPase and protease sites in the intermembrane space (26), and overexpression of the F1β-LacZ fusion protein yields a
F1β-LacZ Inhibits Protein Assembly in Mitochondria

... growth was compared on synthetic media with glycerol as a carbon source. ... protein conformation responsible for aborting translocation across the inner membrane. More detailed experiments are required to address this point.

Yme1p Is a Chaperone Maintaining Mitochondrial Inner Membrane Function—Yme1p belongs to the AAA family of ATPases associated with diverse cellular activities. A bacterial homolog, FtsH, has been extensively characterized by Ito and coworkers (27). FtsH is an ATP-dependent metalloprotease anchored in the cytoplasmic membrane where it performs two functions: it degrades incompletely assembled membrane proteins, and it influences the recognition of the stop-transfer sequences of translocating polypeptides. In ftsH mutant cells, some proteins fail to be translocated into the periplasm, and overexpression of FtsH leads to aberrant translocation of some integral proteins across the membrane (27).

Like FtsH, Yme1p is also an ATP-dependent metalloprotease that is responsible for the degradation of unassembled Cox2 subunits (23, 26, 29, 30), and yme1 cells have defects in the assembly of cytochrome oxidase. We have found that overexpression of Yme1p suppresses the defects of yeast cells expressing F1β-LacZ. The ability of Yme1p to overcome the toxic effects of F1β-LacZ expression is not because of increased degradation of the fusion protein nor by promoting translocation of F1β-LacZ across the inner membrane. Rather, overproduction of Yme1p promotes the assembly of inner membrane protein complexes in mitochondria carrying the fusion protein. We propose that F1β-LacZ titrates the available Yme1p. In wild-type yeast cells, expression of Yme1p is tightly regulated: overexpression of the YME1 gene does not lead to increased levels of Yme1p (13). We have found, however, that in the presence of the artificial substrate F1β-LacZ, levels of Yme1p can be increased 3-fold by overexpression of YME1, which is sufficient to restore mitochondrial function.

By analogy with FtsH, Yme1p might be directly involved in the translocation arrest and release of F1β-LacZ. Certainly, one component of the translocation machinery is determining that the fusion protein should be released in the intermembrane space. F1β-LacZ will be a useful substrate to test the involvement of Yme1p and other components in the translocation of precursor proteins across the inner mitochondrial membrane.

It is possible that other genes encoding putative chaperones could also function as multicopy suppressors of the defects of yeast cells in which F1β-LacZ is jammed in the intermembrane space. In addition, components of the translocation machinery might contribute to toxicity because F1β-LacZ fails to be sorted into the matrix. If so, conditional yeast mutants, akin to those described from Escherichia coli, might define the sorting machinery in the inner membrane. By analogy with its success in terms of defining the bacterial sec machinery, our understanding of how F1β-LacZ fusion proteins work provides an important tool for characterizing in molecular detail the protein sorting and assembly machinery within the mitochondrial inner membrane.

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