Zim17, a Novel Zinc Finger Protein Essential for Protein Import into Mitochondria*

Received for publication, August 11, 2004, and in revised form, September 10, 2004
Published, JBC Papers in Press, September 21, 2004, DOI 10.1074/jbc.M409194200

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Translocation of precursor proteins across the mitochondrial membranes requires the coordinated action of multisubunit translocaases in the outer and inner membrane, and the driving force for translocation across the inner membrane is provided by the matrix-located heat shock protein 70 (mtHsp70). The central components of the protein import machinery are essential. Here we describe Zim17, an essential protein with a zinc finger motif involved in protein import into mitochondria. Comparative genomics suggested a correction to the open reading frame of YNL310c, the gene encoding Zim17 in Saccharomyces cerevisiae. The revised open reading frame codes for a classic mitochondrial targeting signal, which is processed from Zim17 in the mitochondrial matrix. Loss of Zim17 selectively diminishes import of proteins into the matrix of mitochondria, but this loss of Zim17 is partially suppressed by overexpression of the J-protein Pam18/Tim14. We propose that Zim17 functions as an example of a “fractured” J-protein, where a protein like Zim17 contributes a zinc finger domain to Type III J-proteins, in toto providing for substrate loading onto Hsp70.

Molecular chaperones of the 70-kDa heat shock protein (Hsp70) family bind short, unfolded, hydrophobic segments of substrate polypeptides (1). The Hsp70 has both a substrate-binding domain and an ATPase domain, and substrate-binding activity is coupled to ATP hydrolysis (2, 3). A member of the J-protein family usually works as a coupling factor, stimulating ATPase activity of the Hsp70 once the Hsp70 has substrate occupying its substrate-binding domain (3). J-proteins are characterized by a small J-domain that docks to and stimulates the ATPase domain of Hsp70, and a combination of zinc finger and carboxyl-terminal domains that together assist binding non-native structures in a substrate polypeptide (4–6). In combination, the J-protein can load substrate into the binding pocket of Hsp70 and stimulate the hydrolysis of ATP for productive chaperone action.

The import of mitochondrial proteins requires the activity of Hsp70s at several stages (7–9). Most mitochondrial proteins are synthesized in the cytosol, and these relatively hydrophobic polypeptides must remain unfolded (or be actively unfolded) to cross the mitochondrial membranes and only subsequently fold to their final structure (9–11). In the later stages of the mitochondrial protein import pathway, Hsp70 activity is localized at the TIM23 complex (one of the translocaases in the inner mitochondrial membrane) where cycles of ATP hydrolysis by the chaperone are used to effectively drive translocation of the substrate across the membrane. Precursor proteins encounter the mitochondrial Hsp70, mtHsp70 (also called Sec1), immediately upon entry of the terminus of the unfolded polypeptide in the matrix (12, 13); mtHsp70 provides both the driving force for translocation of polypeptides through the TIM23 complex and works in the matrix to fold protein substrates once imported (9, 14).

J-proteins in the mitochondrial matrix can regulate the activity of mtHsp70. One of these, Mdj1, has all the sequence features of a J-protein, a J-domain to stimulate ATPase activity and the zinc finger and carboxyl-terminal domains to interact with substrate polypeptide. Mdj1 assists mtHsp70 to fold mitochondrial proteins after import but is not involved in regulating mtHsp70 activity in its role of driving protein translocation through the TIM23 complex (15–18). Instead, the J-protein Pam18/Tim14 is anchored in the inner membrane in association with the TIM23 complex to display a J-domain close by the site of polypeptide translocation. Pam18/Tim14 stimulates the ATPase activity of mtHsp70 during import (19–21). Interestingly, neither Pam18/Tim14 nor the functionally related J-like protein Pam16/Tim16 (22–26) has domains capable of interaction with substrate polypeptide and therefore could not couple ATP hydrolysis to substrate binding.

We hypothesized that Pam18/Tim14 and perhaps other J-proteins in yeast might represent “fractured” J-proteins where the J-domain and a functionally related zinc finger domain might be found in trans. Therefore we scanned the yeast genome for proteins homologous to the zinc finger domains of the archetypal J-protein DnaJ. In addition to the five J-protein homologues we found two proteins that might represent the zinc finger subunits of fractured J-proteins. One of these, Zim17, is found in the mitochondrial matrix, is essential for viability of yeast cells, and has homologues in a range of eukaryotic species. Decreased expression of Zim17 leads to specific defects in protein translocation into the matrix, and in vitro assays show that Zim17 binds to unfolded precursor proteins. We suggest that Zim17 is an essential component of the import motor acting together with Tim14/Pam18 to present substrate polypeptides to mtHsp70 for their translocation through the mitochondrial membranes.
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FIG. 1. Zinc finger domains in type III J-proteins, Hua1 and Zim17. A. BLAST analysis revealed sequence similarity between the zinc finger domains of five type I J-proteins and the novel proteins Hua1 and Zim17. The domain structures for each of the proteins are represented to scale. Both Hua1 and Zim17 align with the central region of the J-proteins. B. Alignment of Zim17 homologs from diverse species of fungi (Candida albicans, Gibberella zeae, N. crassa, S. cerevisiae, Schizosaccharomyces pombe), the coelenterate (Ciona intestinalis), the worms (Caenorhabditis elegans, Schistosoma japonicum), fly (Drosophila melanogaster), vertebrates (Silurana tropicalis, Homo sapiens), protozoan (Trypanosoma brucei), and plant (Arabidopsis thaliana). Acidic residues are colored to show chemically similar residues (pink, basic; blue, acidic; green, polar; red, non-polar), and the conserved cysteine residues are highlighted. Asterisks represent those residues conserved in all species. 6.

EXPERIMENTAL PROCEDURES

Plasmids, Yeast Strains, and Media—DNA fragments corresponding to Zim17 (YNL310c), Pam18/Tim14 (YJR088c), Tim44 (YIL022w), Tim23 (YNR017w), Mj16 (YHL014w), mtHsp70 (YJR045c), Mdj1 (YFL016c), and Mdj2 (YNL328c) were amplified by PCR using primers that generated in-frame restriction sites. PCR products were cloned into pYPGE2 under the control of the PGK promoter (27). Zim17 was further cloned into a centromeric plasmid (URA3 gene for selection) to encode Zim17-GFP called pSF23 or into a centromeric plasmid (with the LEU2 gene for selection) expressing Zim17 under control of the GAL promoter. Those two plasmids were used for transformation into the Δzim17 strain (MATα leu2 ura3 rme1 trp1 his3 leu2-3,112 met15Δ HMLα zim17Δ:kanMX5 pSF23 or pSF19). Yeast was grown at 30°C on YPAD (2% w/v glucose, 1% w/v yeast extract, 2% (w/v) peptone supplemented with adenine sulfate) or YPGal (2% w/v galactose, 1% (w/v) yeast extract, 2% (w/v) peptone supplemented with adenine sulfate).

Preparation of Mitochondria, in Vitro Imports, Na₃CO₃ Extraction, and Protease Sensitivity—Mitochondria were isolated according to procedures described in Ref. 28 and in vitro transcription; radiolabeling of precursor proteins and import into mitochondria were conducted according to procedures described in Ref. 29. To de-energize mitochondria, imports were performed in presence of carbonyl cyanide m-chlorophenyl hydrazone (Sigma). Osmotic shock treatment to produce “mito-plasts” from mitochondria and trypsin treatments was performed as described (30, 31). Membranes were extracted by resuspension in 100 mM Na₃CO₃ and incubation for 30 min on ice with intermitochondial vortex. Soluble and insoluble proteins were separated by centrifugation at 100,000 × g in a Beckman Airfuge. Samples of mitochondrial protein (100 μg) were separated by Tris-glycine SDS-PAGE and Western blots were carried out according to published methods (31, 32).

Fluorescence Microscopy—For fluorescence microscopy, cells were visualized directly or after staining with MitoTracker (MitoTracker red CM-H2X Ros) according to the standard protocol from Molecular Probes. All fluorescence images were captured using a Bio-Rad MRC1024 confocal scanning laser microscope mounted on a Zeiss Axioscope.

Serial Dilutions—Transformants of pYPGE2-s constructs were grown in selective minimal media with glucose as a carbon source. Each transformant was diluteln to an A₆₀₀ of 0.2, and 6-μl drops of serial 5-fold dilutions were spotted onto selective minimal media containing glucose (to repress Zim17 expression) or galactose (to induce Zim17 expression).

Protein Purification and in Vitro Binding Assay—The coding region of Zim17 was amplified by PCR and cloned into pﺴPE1 for expression in E. coli. After transformation into BL21 cells and growth to an A₆₀₀ of 0.8, protein expression was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested 2 h after induction and disrupted by two bursts (each of 2-min duration) in a mini-beadbeater-8 (BioSpec Products) using silica/zirconia beads. Cell debris was removed by centrifugation at 500 × g for 5 min at 4°C. 50 μl of glutathione-agarose was incubated with cleared extracts for 2 h at 4°C.

Unbound protein was removed by washing with washing buffer (25 mM Tris, pH 8.0, 400 mM KCl, 10% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride). Beads with bound protein were resuspended in 300 μl of lysis buffer (50 mM Hepes, pH 7.4, 0.1 mM KCl, 1% glycerol, 1% Triton X-100, and 1 mM EDTA) and incubated with in vitro-synthesized [35S]-labeled proteins for 1 h at 4°C. Beads were washed eight times with washing buffer (50 mM Hepes, pH 7.4, 0.3 mM KCl, 1% glycerol, 1% Triton X-100, and 1 mM EDTA) and eluted in 25 μl of SDS-PAGE sample buffer for separation on SDS-PAGE and analysis by autoradiography.

RESULTS

The zinc finger domain of DnaJ is 56 amino acids in length and centered around four pairs of cysteine residues (4, 6). Using the zinc finger domain of the bacterial J-protein DnaJ as a probe, BLAST analysis of the yeast genome revealed matches with seven proteins: Ydj1, Xdj1, Apj1, Sqj1, Mdj1, Hua1, and Zim17 (Fig. 1A). Five of the yeast proteins (Mdj1, Sqj1, Apj1, Xdj1, and Ydj1) are Type I J-proteins carrying a J-domain as well as the DnaJ-type zinc finger domain (25). Hua1 and Zim17 are novel proteins, and apart from the zinc finger domain neither Zim17 nor Hua1 sequences conforms to known domain signatures. There are a minimum of 14 different classes of zinc finger domains (33), and although yeast encode at least 50 proteins with zinc finger domains (34) none of these others are sufficiently well conserved to be detected by pairwise alignments with the zinc finger domain of the J-proteins.

Hua1 is a cytoplasmic protein with a possible role in assembly and disassembly of the actin cytoskeleton (35). Zim17 is a novel protein, one of about 800 proteins found in the mitochondrial proteome (36). The mature protein as analyzed from isolated mitochondria has an apparent molecular mass of 17 kDa, as determined from the mitochondrial proteome (36). The mature protein as analyzed from isolated mitochondria has an apparent molecular mass of 17 kDa, as determined from the mitochondrial proteome (36).

Reannotation of the YNL310c (ZIM17) Gene—In Saccharomyces cerevisiae, Zim17 is encoded by the nuclear gene YNL310c. However, we found that the open reading frame originally assigned to the YNL310c gene is likely in error because of a fortuitous upstream ATG sequence.

By comparisons of the total genome sequence from six related species of Saccharomyces, Clifton et al. (37) recently...
showed that discrete, conserved intergenic regions corresponding to promoter elements can be detected. In addition to discovering these important gene control elements, comparative genomics of this sort can be used to re-define open reading frames and intron-exon boundaries (37–39). Whereas sequences corresponding to open reading frames tend to be near identical between closely related species, non-coding regions are more prone to vary in sequence because of genetic drift. Inspection of the genomic sequence for *S. cerevisiae* and five related species shows a box of conserved sequence situated 100 bases upstream of a conserved ATG codon in all six ZIM17 genes (Fig. 2A). Only in *S. cerevisiae* is there an upstream ATG sequence that could have served as a start codon, though situated so close to the putative promoter element it would not be included in the transcript made from the ZIM17 gene.

The mitochondrial targeting sequences of hundreds of proteins from many species are known, often amino-terminal segments rich in positively charged residues that form two to three turns of a helix with amphipathic character (40). Predotar is a neural network predictor trained on hundreds of such sequences also validated in tests on simulated genomes (41). If the ATG designated at 93 in Fig. 2A were used as a start codon, the protein made predicts poorly (score 0.739) as a mitochondrial targeting sequence, whereas the amino-terminal segment of the shorter form of the protein (made from the ATG designated 1) predicts strongly (score 0.978) as a mitochondrial targeting sequence. In vitro transcription of a construct engineered to have both ATG sequences present drives translation of two protein products: one of 33 kDa apparent molecular mass corresponds to the ATG designated 93 (Fig. 2B), and the shorter protein corresponds to the product derived from ATG 1. Only the shorter, 23-kDa form binds to energized mitochondria in vitro (Fig. 2C). We re-cloned a construct to express only the 23-kDa precursor (starting at ATG 1, see Fig. 2B) for incubation with energized mitochondria, the 23 kDa precursor is imported rapidly and processed to a 17-kDa form (Fig. 2C) corresponding in size to the mature form.
of Zim17 found in mitochondria. When either the 33-kDa (starting at ATG −93) or the 23-kDa (starting at ATG +1) precursor forms of Zim17 were cloned to be under the control of a heterologous promoter and the plasmid transformed into a Δzim17 yeast strain, only the 23-kDa precursor could efficiently maintain viability (data not shown).

Zim17 Is a Mitochondrial Matrix Protein—If the ATG (−93) can be used in vivo, the long form of the protein might represent a non-mitochondrial population of protein. To determine whether any non-mitochondrial form of Zim23 exists in vivo, we generated two yeast strains coding for Zim17-GFP. In one, a DNA fragment encoding green fluorescent protein was integrated into chromosome XIV directly behind YNL310c. These cells are viable. In the other, a plasmid-coded form of Zim17-GFP was used to transform heterozygous diploid ZIM17/Δzim17 cells, and after sporulation the Zim17-GFP fusion complements haploid Δzim17 yeast. In each case, analysis by confocal microscopy revealed localization of Zim17-GFP exclusively to mitochondria, confirmed by co-staining with MitoTracker red (Fig. 3A). When mitochondria were isolated and incubated in iso-osmotic buffer in the presence of trypsin, outer membrane proteins like Tom70 were degraded, but Zim17 remained protected from proteolysis (Fig. 3B, lane 3). If trypsin is added to mitochondria in hyperosmotic buffer, where the outer membrane ruptures, proteins like cytochrome b2 in the intermembrane space are degraded by trypsin (lane 4). However only if the inner membrane is permeabilized with the detergent Triton X-100 is Zim17 degraded as is the matrix-located protein Mdj1 (lane 5). Zim17 is not integral in the inner membrane as treatment of mitochondria at pH 11.5 shows Zim17 in the soluble fraction, identical to the matrix protein mtHsp70. Integral membrane proteins, like porin, remain in the membrane fraction (Fig. 3C). Zim17 is exclusively a mitochondrial protein and is located in the matrix.

Zim17 Is an Essential Component of the Import Machinery—YNL310c, the gene encoding Zim17, is essential for viability (data not shown) (42). Mitochondrial proteins that are essential for viability play roles in mitochondrial biogenesis: either protein import (43), Fe-S cluster assembly (44), or protein folding (9). To distinguish in which of these processes Zim17 functions, we generated a Δzim17 yeast strain kept alive with a single-copy plasmid encoding Zim17 under the control of the glucose-repressible GAL promoter. This Zim17 ∆ strain grows on media containing galactose (Fig. 4A) or lactate (data not shown) as a carbon source, but is inviable on media containing glucose (Fig. 4A). A culture of Zim17 ∆ cells grown on media containing galactose was split in two with one half resuspended in fresh media containing galactose (Zim17 ∆) and the other resuspended in media containing glucose (Zim17 ∆). After 4 h of culture, mitochondria were purified and assayed for endogenous protein levels, which were similar under either growth condition (Fig. 4B). The Zim17 ∆ cells remain viable for at least
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16 h on glucose medium (data not shown).

To test if depletion of Zim17 has an effect on import of precursor proteins, mitochondria purified from cultures of Zim17 \( \uparrow \) or Zim17 \( \downarrow \) cells. To minimize indirect effects, cells were isolated from the cultures after only 4 h on either galactose or glucose and mitochondria were purified and assayed for protein import in vitro (Fig. 5A). Import that involves the TIM23 complex was decreased in Zim17-depleted mitochondria; the precursor proteins Su9-DHFR, a fusion protein consisting of the presequence of subunit 9 of F\(1\)F\(0\)-ATPase from *Neurospora crassa* and mouse dihydrofolate reductase and chaperonin 10 (Cpn10) are shown as examples for import into the matrix. Comparison of the amount imported at each time point shows the rate of import into mitochondria from the Zim17 \( \uparrow \) cells is ~25% of the rate into mitochondria deplete with Zim17. Approximately 2-fold decreased import rates were observed for cytochrome \( \text{c} \text{h} \), which is a processed for the intermembrane space only after translocation of its mitochondrial targeting sequence into the matrix (30). Import of inner membrane proteins like the phosphate carrier (PIC) and the ADP-ATP carrier (AAC1) that depend on the TIM22 translocase were not affected. Likewise, the rate of insertion of porin into the outer membrane remained unchanged in mitochondria from Zim17 \( \downarrow \) cells.

As an initial assessment of whether Zim17 can interact with unfolded precursor proteins, we expressed in *Escherichia coli* and purified both Zim17-GST\( ^2 \) and GST. Immobilized on glutathione-agarose beads (0.1 mg protein/ml resin). After incubation with 100% of the eluate are shown.

The abbreviation used is: GST, glutathione S-transferase.
mid overexpressing the matrix J-protein Mdj1 partially suppresses the growth defects for Zim17 cells shifted onto growth medium containing glucose (Fig. 6). It might be that the zinc finger domain of Mdj1 can overlap functionally for lost Zim17, albeit with reduced effectiveness.

**DISCUSSION**

Three classes of J-proteins have been identified, distinguished by their domain structures (25, 45). All three classes of J-proteins have the signature J-domain for interaction with a Hsp70 partner, but Type I J-proteins also have a zinc finger domain and carboxyl-terminal domain, whereas Type II J-proteins have only the additional carboxyl-terminal domain. The Type I and Type II J-proteins use these ancillary domains to bind non-native substrate polypeptide and assist it in eventually attaining a native structure and are thereby considered molecular chaperones (3). Type III J-proteins are not molecular chaperones in their own right, given their inability to bind substrate polypeptides. A recent survey of the yeast genome revealed that yeast express 22 J-proteins, of which 13 fall into the Type III category (25). Type III J-proteins can stimulate ATP hydrolysis in their partner Hsp70 yet alone would be incapable of delivering substrate or serving to couple ATP hydrolysis with substrate binding.

In isolation, the zinc finger domain from DnaJ forms an extended β-hairpin structure, exposing negatively charged patches and a single hydrophobic groove, and the isolated domain can bind hydrophobic peptides derived from substrate proteins (46). However, given the limited binding of peptides and larger substrates observed for both the zinc finger domain from DnaJ and from the yeast homolog Ydj1, it seems likely that the zinc finger domain normally co-operates with other domains of DnaJ to load non-native substrates onto a Hsp70 partner (5, 6, 46).

We propose that Zim17 is a partner of the Type III J-protein Pam18/Tim14 and that together they represent a frayed J-protein with the J-domain of Pam18/Tim14 being complemented in trans by a substrate-binding domain in Zim17. We do not exclude a previous suggestion that substrate binding surfaces in subunits of the TIM23 complex, in particular provided by Tim44, might also contribute to loading substrates onto the mitochondrial Hsp70 (19). The substrate binding domains of Type I J-proteins like Ydj1 and Mdj1 are around 35 kDa, including the zinc finger and additional carboxyl-terminal domain, and additional domains might collaborate with the zinc finger domain of Zim17 to assist mtHsp70 in binding substrate. In this context we note the small but reproducible increase in levels of Tim44 that occur in response to the decreased levels of Zim17 in the shut-down strain (Fig. 4).

Of the 800 or so proteins that are targeted to mitochondria, it is likely that approximately half are imported into the matrix via the TIM23 complex. The steps in this import pathway are becoming clear. Precuror proteins are made in the cytosol, are recognized by the receptors of the TOM complex for insertion into the channel formed by the Tom40 subunit, and are passed onto domains of the TIM23 complex exposed in the intermembrane space (7, 47–49). The amino terminus of the precursor is then inserted into the TIM23 complex, presumably crossing the inner membrane via a tightly gated channel of the complex (50–53). The peripheral subunit of the TIM23 complex, Tim44, serves as a docking point for mtHsp70 (54). The extended translocating polypeptide chain is taken up into the substrate-binding pocket of mtHsp70, and it is at this stage that we propose Zim17 acts by assisting the loading of substrate onto mtHsp70 in an analogous way to the loading of substrates by Type I J-proteins. The J-domain from Pam18/Tim14 interacts with the ATPase domain of mtHsp70, and multiple rounds of ATP hydrolysis by mtHsp70 effectively displace the substrate from the TIM23 complex segmentally.

Interactions made between Hsp70 chaperones and their J-protein partners are transient, and in the case of the proposed interaction between mtHsp70 and Zim17 we have been unable to stabilize any putative “complex” with either substrate polypeptide or chemical cross-linking agents. Nor have we been able to cross-link Zim17 to the Pam18/Tim14, Tim44, or Tim23 subunits of the translocation complex. Being unable to show a direct interaction of Zim17 with subunits of the import motor, we can not dismiss the possibility that Zim17 might also act at a later stage of peptide translocation, assisting in the Mdj1-driven loading of substrate onto mtHsp70 for protein folding. Like Pam18/Tim14 (19), Zim17 appears to be ubiquitous in eukaryotes suggesting that the modular nature of this frayed J-protein is a fundamental feature of the protein import motor. Although it remains speculation, the frayed arrangement of a Pam18/Tim14-Zim17 J-protein might assist in shedding mtHsp70 from the TIM23 complex, with Zim17 then being replaced by Mdj1 to initiate substrate-loading onto mtHsp70 and coupled ATP hydrolysis. For some substrates, the chaperone activity of mtHsp70 is sufficient to finalize folding of the substrate, whereas in many cases the partially folded substrate is then passed onto the chaperonin complex made up of Cpn10 and Cpn60 (55). For both translocation into the matrix and folding of imported substrates mtHsp70 also depends on the ADP-ATP exchange co-factor Mge1, a homolog of bacterial GrpE (9, 56–58).

Fractured J-proteins might be more widespread and found in other subcellular compartments. Another zinc finger domain with sequence similarity to that of Type I J-proteins was found in Hua1, a cytoplasmic protein that plays a role in actin patch assembly. The assembly and disassembly of actin and actin-related proteins in oligomeric patches might well be a job for Hsp70 in the cytosol, and one of the three type III J-proteins in the cytosol (Dj1, Dj2, or Dj3 (25)) might function in trans with Hua1 to modulate assembly and disassembly of parts of the actin cytoskeleton.

**Acknowledgments**—We thank Ana Traven for advice on promoter elements in yeast, Billie Guida for her contribution to the early work on this project, and Nikolaus Pfanner for unpublished data on the size of Zim17.

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