J-domain Protein, Jac1p, of Yeast Mitochondria Required for Iron Homeostasis and Activity of Fe-S Cluster Proteins*

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Roy Kimi‡, Sandeep Saxena§, Donna M. Gordon¶, Debkumar Pain†, and Andrew Dancis‡‡
From the ‡Department of Medicine, Division of Hematology-Oncology, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the ¶Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

J-proteins are molecular chaperones with a characteristic domain predicted to mediate interaction with Hsp70 proteins. We have previously isolated yeast mutants of the mitochondrial Hsp70, Ssq1p, in a genetic screen for mutants with altered iron homeostasis. Here we describe the isolation of mutants of the J-domain protein, Jac1p, using the same screen. Mutant jac1 alleles predicted to encode severely truncated proteins (lacking 70 or 152 amino acids) were associated with phenotypes strikingly similar to the phenotypes of ssq1 mutants. These phenotypes include activation of the high affinity cellular iron uptake system and iron accumulation in mitochondria. In contrast to iron accumulation, Fe-S proteins of mitochondria were specifically deficient. In jac1 mutants, like in ssq1 mutants, processing of the Yfh1p precursor protein from intermediate to mature forms was delayed. In the genetic backgrounds used in this study, jac1 null mutants were found to be viable, permitting analysis of genetic interactions. The Δjac1 Δssq1 double mutant was more severely compromised for growth than either single mutant, suggesting a synthetic or additive effect of these mutations. Overexpression of Jac1p partially suppressed ssq1 slow growth and vice versa. Similar mitochondrial localization and similar mutant phenotypes suggest that Ssq1p and Jac1p are functional partners in iron homeostasis.

Iron is required as a cofactor for critical proteins involved in diverse biological processes including oxidation-reduction, cellular respiration, oxygen interaction, and metabolic conversions (1). However, iron can also be extremely toxic. The toxicity of iron results from interaction with reactive oxygen intermediates, generating highly toxic hydroxyl radicals that damage nearby macromolecules, including lipids, DNA, and proteins (2). Cells cope with this dual nature of iron in part by homeostatic regulation of cellular iron uptake. In Saccharomyces cerevisiae and other organisms, iron uptake is induced by iron deprivation and repressed by iron sufficiency (3). The components of the iron uptake system of yeast include a surface reductase encoded by FRE1 that is regulated by iron levels through changes in its transcription (4). We used this property of the FRE1 gene to select mutants that failed to repress FRE1 expression in response to iron. The types of mutants selected included mutants with perturbed cellular iron uptake (5, 6), iron sensing (7), or intracellular iron distribution (8, 9). This last category of mutants, which included ssq1 mutants, exhibited misregulated activation of high affinity cellular iron uptake and accumulation of iron within mitochondria (8). The SSQ1 gene encodes a low abundance Hsp70 chaperone of the mitochondrial matrix (10). We now describe the isolation of jac1 mutants with similar phenotypes using the same selection scheme. Jac1p contains a J-domain with a signature HPD (histidine, proline, aspartate) tripeptide motif (11) predicted to mediate interaction with Hsp70s (12). Thus, Jac1p may be the functional partner of Ssq1p with activity in controlling iron trafficking into the cell and mitochondria.

Culotta and co-workers (11) have identified jac1 and ssq1 mutants by using a different genetic screen that selects for improved growth of a yeast strain deficient in cytosolic superoxide dismutase (Sod1p). Mutations of jac1 or ssq1 were associated with deficient activities of Fe-S proteins (11). Fe-S clusters are modular units in which iron and sulfur are coordinated in various combinations and linked to the peptide backbone of proteins via cysteine sulfurs. Because of their ability to donate or accept electrons with tremendously varied range of potentials, these clusters are involved in many fundamental biological processes, ranging from cellular respiration to metabolic conversions (13). Although the clusters can be synthesized non-enzymatically in vitro (14), recent genetic and biochemical evidence suggests that their in vivo formation is catalyzed by specific enzymes (15–18). In bacteria, several of these proteins are associated together on the isc operon (16), and their precise biological roles in Fe-S cluster synthesis are the subject of ongoing work (19–22). Homologs of Ssq1p (called HscA or Hsc66) and Jac1p (called HscB or Hsc20) have been found on the isc operon, suggesting a role in Fe-S cluster synthesis or maintenance (16, 23). Overexpression of these proteins is required for overexpression of Fe-S proteins in bacteria (24). In a recent study, Vickery and co-workers (21) demonstrated a three-way interaction of the Hsp70 (Hsc66), a J-protein co-chaperone (Hsc20), and another isc operon-encoded protein, IcuU. IcuU may provide a scaffold for assembling intermediates of the Fe-S cluster assembly process (20).

Here we characterize the phenotypes of jac1 mutants with altered iron homeostasis and their similarity to phenotypes of ssq1 mutants. We also demonstrate genetic interactions between JAC1 and SSQ1.

EXPERIMENTAL PROCEDURES

Growth Media

Rich medium consisted of 1% yeast extract, 2% peptone, 100 μg/ml adenine, and various carbon sources. In some experiments 2% raffinose...
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was used (YPAD), and in other experiments 2% glucose was used (YPAD). To induce the GAL10 promoter, the carbon source used was 2% raffinose and 0.5% galactose. Expression from the GAL10 promoter was turned off by growth in identical medium without galactose. For experiments with different concentrations of iron, standard defined medium with 0.6 M sorbitol was modified by omission of iron. Medium was autoclaved and iron was added back from a filter sterilized stock of 100 mM ferric ammonium sulfate. To achieve severe iron deprivation, defined medium without added iron was supplemented with 10 μM chelator bathophenanthrene disulfonate (Fluka). Methods for growing yeast, crossing strains, sporulation, tetrad dissection, and yeast transformation have been described (25).

Yeast Strains, Mutant Selection, and Crosses

Haploid strain 61q (MATa, trpl–3, leu2–3,112, gcn4–101, his3–609, ura3–52, FRE1-HIS3, URA3, SSQ1::LEU2) was derived from strain 61 by insertion of an additional copy of SSQ1 carried on plasmid pRS405 and integrated at the BatwlII site of the leu2–3,112 locus.

Mutant Selection—61q was subjected to mutagenesis by UV irradiation to produce a mortality of 40–70%. The mutagenized population was diluted and allowed to form colonies from single cells on YPAD agar plates. These colonies were replicated to agar plates containing defined medium supplemented with 10 μM copper sulfate and 20 μM ferric ammonium sulfate. After 4 days, some of the colonies developed papillae, which were streaked for single clones on YPAD plates and evaluated further. The mutants derived from 61q by this procedure included UV6.3 (jac1–1), UV6.2, and UV6.30 (rhoA–). The parent strain for UV6.30 was 609 (ura3–52, rhoA–) and UV6.30 (rho+) was the 2C (jac1–50, rhoA–) strain and inserted into the same vector. Several clones of each were subjected to DNA sequencing. The wild-type and mutant JAC1 genomic fragments with StelI and XhoI ends were subcloned into pRS406 for integration into the genome at the ura3–52 locus. The wild-type JAC1 genomic fragment was also subcloned into the same sites in pRS318 for use in plasmid shuffling. For obtaining high levels of regulated expression, the JAC1 ORF and truncated variants (with amino acids 1–114 or amino acids 1–32) were amplified from the F1 plasmid with 5′ BamHI and 3′ XhoI sites and cloned into pMELbyex4–i (9). The plasmids, called GAL10-Jac1, GAL10-jac1(1–114), and GAL10-jac1(1–32) could be linearized with a unique StelI restriction site for integration at the ura3–52 site. The SSQ1 ORF was inserted into the BamHI site of the same vector and was linearized as a BamHI–XhoI fragment. The unique StelI site of the JAC1 ORF, and His− strains were introduced by plasmid YCplac22. BamHI sites were introduced at the start and stop of the open reading frame, allowing for replacement of the entire ORF with a cassette containing the HIS3 gene. The knockout construct could be released by digestion with NotI and StelI. To delete JAC1, diploids CM3263, YPH501 (28), and W303 (29) were transformed with this fragment, and transformants were selected for growth in the absence of histidine. In each case, correct integration of the knockout was verified by PCR. For interruption/deletion of SSQ1, a HIS3 cassette with BamHI ends was inserted into BglII sites within a 2.2-kb BglII-EcoRI SSQ1 genomic fragment carried on the vector behind a ribosomal RNA promoter and disruption of the expressed product was achieved by EcoRI digestion. The plasmid was linearized with EcoRI, released a 3.5-kb fragment prior to transformation. The pRS318-SSQ1 plasmid carried a 3461-bp EcoRI-Sall genomic fragment containing SSQ1.

Bacterial Expression and Antibodies

For expression in bacteria, the JAC1 ORF was amplified with 5′ NdeI and 3′ XhoI sites and cloned into the corresponding sites of pET21b (Novagen), forming an in-frame fusion with 6 histidines (His6). Expression in BL21(DE3) was induced by 1 mM isopropyl–b-D-1-thiogalactopyranoside for 3 h at 37 °C. The overexpressed Jac1p-His6 fusion protein was isolated from inclusion bodies, purified on a nickel–nitrilotriacetic acid M-chelator column at 4 °C, and His6 was excised from the gel and used to inject rabbits for generation of polyclonal antibodies. The carboxyl-terminal 109 amino acids of the SSQ1 ORF was expressed in pET21b with His6 tag, purified from the soluble fraction of the bacterial lysate using the nickel-nitrilotriacetic acid column as above, and used to immunize rabbits. Antibodies directed against Aco1p (30), Mir1p, and Por1p (31) have been described previously. Commercially available antibodies used in some experiments included mouse monoclonal antibodies directed against Cox3p or Pkg1p (3-phosphoglycerate kinase) (Molecular Probes).

Mitochondria were isolated as described (32). The post mitochondrial fraction was centrifugated at 386,000 × g for 20 min, and the supernatant was used as a cytosolic fraction. Intact mitochondria were resuspended in 20 mM Tris-HCl, pH 7.5, 0.6 M sorbitol. For determining the iron

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1 The abbreviations used are: ORF, open reading frame; PCR, polymerase chain reaction; SDH, succinate dehydrogenase; MPP, matrix processing peptidase; bp, base pair(s); kb, kilobase pair(s); INT, p-iodonitrotetrazolium violet.

2 Plasmids and DNA Constructions: F1 was identified by screening a yeast genomic library (27) for correction of the slow growth and non-repressed reductase of 8A (jac1–3). F1 contained a 5.3-kb insert in the vector YCP50. The insert consisted of bp 458483–463769 of chromosome VII and included JAC1. The JAC1 ORF with 500 bp of flanking DNA was amplified from genomic DNA isolated from YPH501 by PCR using Pho polymerase and inserted into the vector pCR2.1-TOPO (Invitrogen). Similarly, the ORFs and flanking regions were amplified from the 8A (jac1–3) and 2C (jac1–30) strains and inserted into the same vector. Several clones of each were subjected to DNA sequencing. The wild-type and mutant JAC1 genomic fragments with StelI and XhoI ends were subcloned into pRS406 for integration into the genome at the Strl in the ura3–52 locus. The wild-type JAC1 genomic fragment was also subcloned into the same sites in pRS318 for use in plasmid shuffling. For obtaining high levels of regulated expression, the JAC1 ORF and truncated variants (with amino acids 1–114 or amino acids 1–32) were amplified from the F1 plasmid with 5′ BamHI and 3′ XhoI sites and cloned into pMELbyex4–i (9). The plasmids, called GAL10-Jac1, GAL10-jac1(1–114), and GAL10-jac1(1–32) could be linearized with a unique StelI restriction site for integration at the ura3–52 site. The SSQ1 ORF was inserted into the BamHI site of the same vector and was linearized as a BamHI–XhoI fragment. The unique StelI site of the JAC1 ORF, and His− strains were introduced by plasmid YCplac22. BamHI sites were introduced at the start and stop of the open reading frame, allowing for replacement of the entire ORF with a cassette containing the HIS3 gene. The knockout construct could be released by digestion with NotI and StelI. To delete JAC1, diploids CM3263, YPH501 (28), and W303 (29) were transformed with this fragment, and transformants were selected for growth in the absence of histidine. In each case, correct integration of the knockout was verified by PCR. For interruption/deletion of SSQ1, a HIS3 cassette with BamHI ends was inserted into BglII sites within a 2.2-kb BglII-EcoRI SSQ1 genomic fragment carried on the vector behind a ribosomal RNA promoter and disruption of the expressed product was achieved by EcoRI digestion. The plasmid was linearized with EcoRI, released a 3.5-kb fragment prior to transformation. The pRS318-SSQ1 plasmid carried a 3461-bp EcoRI-Sall genomic fragment containing SSQ1.

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Fractionation of Cells: Mitochondria were isolated as described (32). The post mitochondrial fraction was centrifugated at 386,000 × g for 20 min, and the supernatant was used as a cytosolic fraction. Intact mitochondria were resuspended in 20 mM Tris-HCl, pH 7.5, 0.6 M sorbitol. For determining the iron
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content of mitochondria, wild-type or mutant yeast cells were grown to steady state for 16 h in defined medium supplemented with varying concentrations of iron. A small amount (100 nm) of tracer $^{55}$Fe (Amer¬

## Mitochondrial Import

Radiolabeled precursors (Jac1p, Yfh1p, Put2p) were synthesized in a cell-free translation in the presence of a mixture of $^{[35]S}$methionine and $^{[35]S}$cysteine (31). Import reactions contained mitochondria (100 µg), radiolabeled preprotein, and 4 mM ATP and 1 mM GTP (31). Following import at 20 °C for 15 min, reaction mixtures were treated with trypsin (0.1 mg/ml) for 30 min at 0 °C. The protease was inactivated, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis and exposed to film. Use of purified matrix processing protease (MPP) to test processing of mitochondrial precursor proteins was as described (33).

## Assays for Iron Uptake, Aconitase, Succinate Dehydrogenase, and Heme Proteins

High affinity cellular iron uptake was measured as described (5). Aconitase was assayed by measuring the formation of cis-aconitate at 240 nm as described (34). Succinate dehydrogenase was assayed on mitochondria lysed in 0.5% Triton X-100 by following the reduction of p-iodonitrotetrazolium violet (INT) to INT-formazan as described (35). For detection of heme, mitochondrial proteins (100 µg) separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose were incubated with peroxide developer and chemiluminescent substrates (SuperSignal ECL; Pierce) for 5 min. The covalently bound heme group of cytochrome c (Cyt1p) and the heme group of the bc1 complex (Cyt1p) were visualized by exposing the blot to film (36).

## RESULTS

### Mutant Alleles of jac1 Isolated in a Screen for Mutants with Altered Iron Homeostasis—FRE1 encodes the major cell surface reductase and is required for iron acquisition from ferric iron chelates (4). The FRE1 promoter, which is repressed in response to iron, was fused to the HIS3 coding region. Mutants that failed to respond to iron-mediated repression were selected for their ability to grow in the presence of iron and in the absence of histidine (5). The most commonly selected mutants were defective in the SSQ1 gene. Therefore, in order to avoid selecting more of these mutants, the screen was modified by insertion of an additional copy of SSQ1 in the haploid genome.

Two new mutants, UV6.3 and UV6.30 (Fig. 1A), were selected and found to exhibit small colony size, non-repressing surface ferric reductase, and increased high affinity ferrous iron uptake. The mutant phenotypes were found to be recessive. The mutants were backcrossed with a wild-type parental strain, and the slow growth and iron related phenotypes segregated in a 2+2− pattern in the tetrad, consistent with involvement of a single nuclear locus. Crosses revealed that the two independent isolates contained allelic mutations.

The wild-type allele for the mutant gene in 8A was then cloned by complementation of the slow growth and reductase phenotypes. The complementing activity was further localized to a minimal construct containing only the JAC1 ORF and 500 bp of 5′- and 3′-flanking DNA. To rule out the possibility that complementation by the JAC1-containing genomic DNA fragment could be indirect, the JAC1 ORF with its native promoter and a URA3 marker gene were inserted into the unique MacI site of the chromosomal JAC1 locus in a wild-type strain. This strain JM1 (URA3::JAC1) was crossed with 8A (jac1−3, ura3−52) and sporulated. All Ura− tetrad clones exhibited slow growth and non-repressing reductase (mutant phenotype), indicating absence of recombination between the marked wild-type JAC1 and the 8A mutant. Hence, meiotic mapping also supported that the mutation in 8A responsible for the mutant phenotypes was in JAC1 or closely linked to this locus. The mutant alleles were rescued from strains 8A (jac1−3) and 2C (jac1−30) by PCR of the JAC1 ORF and flanking regions. DNA sequence analysis revealed single nucleotide changes in each case. For the jac1−3 allele, nucleotide 344 was changed from T to G, altering codon 115 from T to G, generating a stop codon at position 115. The mutation in jac1−30 was found to alter nucleotide 97 from C to T, generating a stop codon at position 33.

### Null Phenotype of jac1: Viability Depends on Genetic Background—The severity of the truncation of the predicted JAC1 ORF in allele jac1−30 suggested that it would be non-functional or null. The viability of this mutant was surprising in view of an earlier work indicating that JAC1 was an essential gene required for vegetative growth (11). To directly address the question of essentiality of JAC1 in the CM3283 genetic background in which the mutants were selected, the entire ORF was deleted in this diploid, and the correctness of the deletion was verified by genomic PCR. The heterozygous knockout was sporulated and spore clones were dissected on rich (YPAD) medium and incubated at 30 °C. All four spores formed colonies, although the histidine prototrophs carrying the knockout constructs formed much smaller colonies than the wild-type spore clones (Fig. 2A). The tetrad clones containing the jac1 deletions could be maintained in culture, although they were often overgrown by more rapidly growing suppressor mutants. Similarly, in another yeast genetic background, YPH501, jac1 knockouts germinated and formed small colonies (Fig. 2A). This slow growth phenotype of the jac1 deletion was more severe when incubated at elevated (37 °C) or lower (23 °C) temperatures (data not shown). Thus, in both of these yeast strains, jac1 deletion was deleterious but still allowed germination and vegetative growth. We also examined the deletion phenotype in W303, another commonly used parental strain. In this background, the jac1 deletion underwent germination and formed tiny macroscopic colonies consisting of several thousand cells (Fig. 2A, enlarged panel). However, these clones could not be propagated. Perhaps small amounts of Jac1p car-

![Fig. 1. Panel A, strain nomenclature. The original mutant isolates obtained from the selection scheme were named UV 6.3 and UV6.30. After backcrossing to the wild-type, tetrad clones 8A and 2C were identified containing mutant alleles, called jac1−3 and jac1−30, respectively. The alleles were sequenced and the mutation in jac1−3 was found to alter nucleotide 344 of the coding from T to G, generating a stop codon at position 115. The mutation in jac1−30 was found to alter nucleotide 97 from C to T, generating a stop codon at position 33. Panel B, domain structure of Jac1p. The predicted domain structure of Jac1p includes a mitochondrial signal sequence (S), J-domain (J), and carboxyl-terminal domain of unknown function. The J-domain includes the characteristic HDP (histidine, proline, aspartate) at residues 48–50. The locations of jac1−3 and jac1−30 mutations are depicted by upward arrows. The domain structure of the bacterial J-protein Hsc20 is shown. Identity refers to the percentage of amino acid identity between Hsc20 and Jac1p in the J domain or C-terminal domain.](http://www.jbc.org/)
was photographed at higher magnification and this is shown in the right-hand panel. Panel B, JAC1 alleles examined by plasmid shuffling. JAC1 shuffle strain, 729–4B (Δjac1::HIS3, pRS318-JAC1), was transformed with various constructs targeted to the ura3–52 locus. These constructs were: 1, pRS406, empty vector; 2, pRS406-JAC1, wild-type JAC1 allele including the native promoter; 3, GAL10-Jac1, JAC1 ORF under the control of the GAL10 promoter; 4, GAL10-jac1–(1–96); truncated Jac1p of 96 amino acids under the control of the GAL10 promoter. The transformants were analyzed by plating 5-fold dilutions of $1 \times 10^5$ cells in a 5-µl volume on agar plates containing YPAR supplemented with 0.5% galactose to induce the GAL10 promoter and 10 µg/ml cycloheximide to counterselect against the covering plasmid. Plates were incubated at 30 °C for 4 days before photography. For measurement of high affinity ferrous iron uptake, cells were taken from the photographed plates and grown under inducing conditions (YPAR with 0.5% galactose) prior to assay of high affinity ferrous iron uptake using $^{55}$Fe radionuclide.

**Fig. 2.** Panel A, null phenotype of jac1 depends on genetic background. Heterozygous deletions of JAC1 were constructed in diploid strains: YPH501, CM3263, and W303. These strains were sporulated; tetrads were dissected, arrayed in vertical columns on rich medium agar plates, and incubated at 30 °C for 4 days. In the YPH501 panel, column 5 was not a true tetrad. In the W303 panel, column 4 (tetrad 4) was photographed at higher magnification and this is shown in the right-hand panel. Panel B, JAC1 alleles examined by plasmid shuffling. JAC1 shuffle strain, 729–4B (Δjac1::HIS3, pRS318-JAC1), was transformed with various constructs targeted to the ura3–52 locus. These constructs were: 1, pRS406, empty vector; 2, pRS406-JAC1, wild-type JAC1 allele including the native promoter; 3, GAL10-Jac1, JAC1 ORF under the control of the GAL10 promoter; 4, GAL10-jac1–(1–96); truncated Jac1p of 96 amino acids under the control of the GAL10 promoter. The transformants were analyzed by plating 5-fold dilutions of $1 \times 10^5$ cells in a 5-µl volume on agar plates containing YPAR supplemented with 0.5% galactose to induce the GAL10 promoter and 10 µg/ml cycloheximide to counterselect against the covering plasmid. Plates were incubated at 30 °C for 4 days before photography. For measurement of high affinity ferrous iron uptake, cells were taken from the photographed plates and grown under inducing conditions (YPAR with 0.5% galactose) prior to assay of high affinity ferrous iron uptake using $^{55}$Fe radionuclide.
counting. At the lowest medium iron concentration of 0.1 pmol/mg, isolated and radiolabeled iron was determined by scintillation counting. After 16 h of growth, mitochondria were added as a tracer, and, after 16 h of growth, mitochondria were isolated and the iron content determined by scintillation counting of radiolucence in the samples. The iron content is displayed as picomoles of iron per microgram of mitochondrial protein.

In the absence of iron, Aft1p binds to these sequences and activates transcription. Aft1p thereby controls iron-dependent expression of the components of the high affinity ferrous transport system (37). We wondered if the aberrant induction of high affinity ferrous transport observed for the jac1 mutants was mediated via Aft1p. To address this question, the AFT1 gene was interrupted and high affinity ferrous uptake was measured in the jac1Δaft1 double mutants. The results showed equivalent low levels of uptake for all strains lacking AFT1, regardless of the presence or absence of jac1 mutations (Fig. 3C). Therefore, the increased cellular iron uptake due to jac1 mutations was completely dependent on an intact copy of AFT1.

Mitochondrial Iron Accumulation in jac1 Mutants Subjected to Iron Stress—Wild-type and jac1 mutant strains were grown in media containing different concentrations of iron with 55Fe added as a tracer. After 16 h of growth, mitochondria were isolated and radiolabeled iron was determined by scintillation counting. At the lowest medium iron concentration of 0.1 pmol/mg, mitochondrial iron in the wild-type was 0.36 pmol/µg of protein versus 1.0 and 1.0 for the jac1–3 and jac1–30 mutants. These mitochondrial iron values for the mutants are equivalent or below levels of mitochondrial iron in wild-type strains grown in standard defined medium (usually 1–3 pmol/µg). With increasing amounts of iron in the growth media, jac1–3 and jac1–30 mutants accumulated tremendously increased amounts of iron in mitochondria (Fig. 4). For the jac1–3 mutant grown in 1, 5, or 50 µM iron, mitochondrial iron was increased to 26, 127, or 395 pmol/µg protein. For the jac1–30 mutant the levels were 32, 186, and 348 pmol/µg protein, respectively. These results indicate that the jac1–3 and jac1–30 mutations lead to abnormal trafficking of iron to mitochondria.

Deficient Iron-Sulfur and Heme Proteins in jac1 Mutants—Iron-sulfur proteins were evaluated in the jac1–3 and jac1–30 mutants. Aconitase (Aco1p) is a mitochondrial protein (38), which contains a 4Fe-4S cluster required to catalyze the conversion of citrate to isocitrate (34). Aconitase protein level and activity in the wild-type strain were not affected by iron content of the growth medium (Fig. 5A). By contrast, in the mutants, aconitase protein was so severely decreased that it was not detected by immunoblotting. The activity assay was more sensitive, and some residual activity was noted in the jac1 mutants. Interestingly, this residual activity was dependent on the iron content of the mitochondria. In mitochondria isolated from the mutants grown in low iron media, aconitase activity was present at ~10% of wild-type levels, whereas in the mitochondria isolated from iron stressed cells exposed to 50 µM iron in the medium, activity was further decreased or undetectable.

In summary, aconitase protein and activity were markedly diminished in the jac1 mutants under all growth conditions. Iron accumulation within mitochondria could be correlated with abortion of the small amount of residual aconitase activity, suggesting a toxic effect. Succinate dehydrogenase (SDH), a protein complex of the inner membrane containing Fe-S and heme cofactors, was also evaluated (39). Like aconitase, SDH activity was markedly decreased in the mutants under all conditions, and the small amount of residual activity (6% or less) appeared to be further compromised by growth in high iron media (Fig. 5B).

Iron is inserted into heme in mitochondria (40), and many heme proteins are found in mitochondria. Therefore, the status of several heme proteins was evaluated in the jac1 mutants. Cytochrome c (Cyt1p) and cytochrome bc1 (Cyt1p) are nuclear-encoded mitochondrial proteins that contain covalently bound heme. The heme in these proteins can be visualized using enhanced chemiluminescence substrates by virtue of its intrinsic peroxidase activity (36). Cyt1p and Cyc1p heme levels in mitochondria were markedly decreased in the jac1–1 and jac1–30 mutants (Fig. 6), even at the lowest medium iron concentration tested. The levels were further reduced in mitochondria with increased iron content. Likewise, mitochondrial encoded cytochrome oxidase subunit 3 protein (Cox3p) was also reduced in the jac1–3 and jac1–30 mutants (Fig. 6). This decrease could reflect lack of heme or destruction of heme in the cytochrome oxidase complex, because the proteins of the
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Complex are destabilized in the absence of heme cofactor (data not shown). Alternatively, this decrease of Cox3p in jac1 mutants could result from mitochondrial DNA damage. Cellular Depletion of Jac1p—Jac1p was placed under the control of the inducible GAL10 promoter, and the wild-type allele was replaced with this regulated construct using the shuffle strategy. As expected, under inducing conditions for the GAL10 promoter, Jac1p was highly expressed (Fig. 7A). This strain grew well and was phenotypically indistinguishable from the wild-type. When the inducer was removed, Jac1p was no longer expressed and was depleted from the cells (Fig. 7A), and yet surprisingly, even after prolonged incubations or addition of glucose, which is known to repress this promoter, no slowing of growth was noted (data not shown). When we further evaluated this strain grown in the absence of inducer, phenotypes noted for the jac1 mutants were found to be present to variable degrees. High affinity iron uptake was increased 3-fold compared with wild-type (Fig. 7A). Aconitase and SDH activities were decreased to 56% and 49% of wild-type levels, respectively (Fig. 7B). In contrast with the jac1 mutants in which aconitase protein and activity were coordinately decreased, in these Jac1p depletion experiments, aconitase activity was decreased but Aco1p protein level was unaltered. The implication is that inactive Aco1p was being generated. The inactive protein might contain a 3Fe-4S cluster as has been described for oxidatively damaged aconitase (13). Alternatively, it may contain no cluster at all (apoprotein) due to loss of the cluster or failure to insert the cluster into newly synthesized protein. Furthermore, in the Jac1p depletion experiments, heme in Cyt1p and Cyc1p was not affected, as distinguished from the jac1 mutant phenotype (Fig. 7C). Thus, the phenotypes resulting from Jac1p depletion were more mild than the phenotypes of the jac1 mutants, perhaps because some minimal level of Jac1p was expressed from the GAL10 promoter even under non-inducing conditions.

Mutant (jac1–3) Is Not Generally Defective in Precursor Protein Translocation but Is Defective in Yfh1p Processing—The J-proteins of mitochondria, Mdj1p and Mdj2p, cooperate with the Hsp70, Ssc1p, in mediating import and folding of precursor proteins (41, 42). To determine if jac1 mutants were generally defective in precursor import, we compared translocation of Put2p precursor into mitochondria isolated from wild-type and jac1–3 strains. Put2p is a mitochondrial matrix protein involved in proline biosynthesis. The Put2p precursor was synthesized in reticulocyte lysate in the presence of [35S]methionine (Fig. 8A, lane 1). When incubated with mitochondria, a more rapidly migrating mature form was generated due to removal of the mitochondrial signal sequence (lane 2), and this mature form was protected from externally added protease (lane 3). The import, processing, and generation of the mature protected Put2p were unaffected in ssq1–4 mutant mitochondria (lanes 4 and 5) or in jac1–3 mutant mitochondria (lanes 6 and 7). In summary, a general import defect was not observed, and consistent with the import results, the steady state levels of Put2p were not diminished in jac1–3 mitochondria (see Fig. 5A).

An unusual defect in the processing of Yfh1p precursor was observed in ssq1 mutant mitochondria (8, 43). Yfh1p, the yeast frataxin homolog, is required for iron homeostasis (44). The precursor is nuclear encoded, imported into mitochondria, and proteolytically processed at the amino terminus in two steps by the matrix processing peptidase (45, 46). In ssq1 mutants, the second processing cleavage is impaired. Although the functional significance of this observation is not yet clear, we reasoned that if Ssq1p functions with Jac1p, the jac1 mutant might exhibit a similar defect in Yfh1p processing. Therefore, Yfh1p precursor was radiolabeled (Fig. 8B, lane 1) and imported into wild-type and mutant mitochondria. In the wild-type mitochondria, the precursor was rapidly processed to the mature form (m) and protected from external protease (Fig. 8B, lanes 2 and 3). The intermediate form (i) generated by the first processing cleavage was not observed. In ssq1 mitochondria, most of the precursor remained in this intermediate, partially processed form (i), and this was protected from external protease (Fig. 8B, lanes 4 and 5). In the jac1–3 mitochondria, the
Mitochondrial J-domain Protein, Jac1p, in Iron Homeostasis

The abundance of Jac1p was assayed using antibodies directed against mature Jac1p (Fig. 9 A). CM3260 revealed a specific signal migrating at 23 kDa representing mature Jac1p (lanes 4 and 5), or 8A (jac1–3, lanes 6 and 7). After 15 min, samples were exposed to 0.1 mg/ml trypsin (Trypsin, lanes 3, 5, and 7), and the protease was inactivated. The mitochondria were retrieved by pelleting at 12,000 × g and analyzed by SDS-polyacrylamide electrophoresis. The Trypsin (p) and mature forms (m) are indicated. Panel B, the Yfh1p precursor radiolabeled by synthesis in reticulocyte lysate (lane 1) consisted of the full-length product (p) and a truncated form arising from internal initiation (*). The mitochondrial import assay was performed as described in panel A. The Yfh1p precursor was processed in two steps, and in ssq1–4 and jac1–3 mutant mitochondria, both the intermediate form (i) and mature (m) forms were observed.

Jac1p Localized to Mitochondria in the Wild-type and Undetected in the Mutants—The initial description of Jac1p noted a potential mitochondrial leader sequence at the amino terminus of the predicted ORF (11). To experimentally address the question of whether the JAC1 ORF includes a mitochondrial signal sequence, the Jac1p precursor protein was synthesized and radiolabeled in reticulocyte lysate (Fig. 9A, lane 1). When the Jac1p precursor was incubated with purified MPP, the protein was processed to a more rapidly migrating form (lane 2). Incubation with mitochondria also generated a species with identical mobility (lane 3). The generation of the mature product was dependent on the presence of a membrane potential and was completely inhibited by the addition of valinomycin (lanes 4 and 6). The mature processed species formed after import was protected from externally added protease indicating that this is the translocated, processed form (lane 5). Thus, Jac1p preprotein is imported into mitochondria and proteolytically processed to a mature form by MPP. Comparison of the precursor and mature forms would be consistent with the removal of a 13-amino acid mitochondrial signal sequence at the amino terminus of Jac1p.

The localization of Jac1p in cells was examined. Immunoblotting of purified mitochondria from the wild-type strain CM3260 revealed a specific signal migrating at 23 kDa representing mature Jac1p (Fig. 9B). The abundance of Jac1p was unchanged in mitochondria isolated from wild-type yeast grown in widely different concentrations of iron, from 0.1 to 50 μM, indicating lack of iron dependent regulation of protein levels (Fig. 9B). In the mutants, jac1–3 and jac1–30, Jac1p was not detected (Fig. 9B), even with overexposure of the immunoblot (data not shown). Thus, the jac1–3 and jac1–30 mutants appeared to be null mutants. A cytoplasmic fraction was examined by immunoblotting. Jac1p and Yfh1p, a mitochondrial marker protein, were not detected in this fraction, whereas 3-phosphoglycerate kinase (Pgk1p), a cytoplasmic protein, was present (Fig. 9C). The data indicate that, within the detection limits of our antibody, Jac1p was found in mitochondria but not in cytoplasm.

Genetic Interaction of JAC1 and SSQ1—The viability of ssq1 and jac1 individual deletions provided an opportunity to perform epistatic analysis of these two genes. Shuffler strains carrying ssq1 and jac1 deletions were mated. After counterselection against the covering plasmids, the double heterozygote was sporulated and tetrads were analyzed. Many tetrads contained four viable spore colonies, and parental diploids, non-parental diploids, and tetratypes were identified (Fig. 10A, columns 1, 2, and 3–5, respectively). The Δssq1 spore clones formed small colonies (∆s), Jac1p spores formed smaller colonies (∆j), and the double mutants formed tiny, albeit viable colonies (∆sj). In many cases, the double mutant spore clones did not grow after inoculation of liquid or solid media cultures. Thus, the double mutants were clearly more compromised than either of the single mutants in terms of viability and growth. The data suggest that the two genes may serve some overlapping and some non-overlapping functions.
tions of 105 cells were plated on agar plates containing 2% raffinose, 5% galactose, and 10 mM 3-AT. Strains 61 (Jac1–3), 191-33C (ssq1–4), and 729–4B were similarly transformed with the integrating plasmids: pRS406-SSQ1, pRS406-JAC1, or GAL10-Jac1 (JAC1). The corresponding mutant genotypes are shown in the table below:

| Strain | Genotype |
|--------|----------|
| 61     | ssq1::HIS3 jac1::HIS3 |
| 191-33C | ssq1::HIS3 Jac1::HIS3 |
| 729–4B | jac1::HIS3 SSQ1::HIS3 |

Panel B, overexpression effects. The SSQ1 shuffle strain (MK4–6) was transformed with integrating plasmids linearized at SstI and targeted to the ura3–52 locus: pRS406 (1), pRS406-SSQ1 (2), GAL10-SSq1 (3), or GAL10-Jac1 (4). The JAC1 shuffle strain (729–4B) was similarly transformed with the integrating plasmids: pRS406 (5), pRS406-JAC1 (6), GAL10-SSq1 (7), and GAL10-Jac1 (8). The transformants were suspended in water, and 5-fold dilutions of 10^8 cells were plated on agar plates containing 2% raffinose, 0.5% galactose, and 10 μg/ml cycloheximide to counterselect against the covering plasmids. Panel C, strains YPH499 (1) or YPH499 transformed with GAL10-SSq1 (2) or GAL10-Jac1 (3) were grown in medium containing 2% raffinose and 0.5% galactose. Strains 61 (4), 191-33C (ssq1::HIS3) (5), and 8A (jac1–3) (6) were grown in medium containing 2% raffinose. Mitochondria were isolated and analyzed by immunoblotting with antibodies to Ssq1p, Jac1p, and Put2p.

Additional support for this assertion is provided by overexpression experiments. Overexpression of Jac1p in Δssq1 mutants clearly suppressed the slow growth phenotype (Fig. 10B, compare 1 versus 4). Overexpression of Ssq1p in Δjac1 mutants also enhanced growth, but the effect was not as marked (Fig. 10B, compare 7 to 3). Approximately 3-fold overexpression of Jac1p was achieved without overexpression of Ssq1p, and vice versa overexpression of Ssq1p was achieved without overexpression of Jac1p (Fig. 10C). In the mutant 33C (ssq1–4), Ssq1p was undetectable without alteration of the Jac1p level, and in strain 8A (jac1–3), Jac1p was undetectable without alteration of the Ssq1p level (Fig. 10C).

**DISCUSSION**

Mutant jac1 allele mutants were isolated by their ability to activate the FRE1-HIS3 gene fusion, an indicator of abnormal iron homeostasis. Mutant jac1 cells failed to repress cellular iron uptake appropriately in response to iron exposure, and the excess iron accumulated within mitochondria. The iron regulatory phenotype was tightly coupled with Fe-S protein deficiency. The most straightforward explanation for the findings is that Jac1p is involved in the biogenesis of one or more iron-sulfur protein regulators (47). Loss of Jac1p function, then, might result in failure to properly form these regulatory Fe-S cluster proteins, which in turn leads to failure to turn off iron uptake to the cell and iron accumulation in mitochondria. The identity of putative Fe-S cluster protein regulators of iron trafficking remains to be determined. Aft1p, an iron-sensor regulator that works by controlling transcription of target genes (7), is required for the induced cellular iron uptake of jac1 mutants. Aft1p might itself contain an Fe-S cluster or interact with another protein that contains a cluster. However, mitochondrial iron accumulation does not occur with activated AFT1 alleles, and so accumulation of mitochondrial iron may be due to effects of mutated jac1 on an unidentified regulatory protein. IRP1 in mammals is an Fe-S protein with regulatory effects on iron trafficking (48), although an orthologous protein in yeast has not been described.

Jac1p belongs to the class of J-domain chaperones predicted to interact with Hsp70 proteins. We have now provided evidence to suggest that Ssq1p is the Hsp70 partner for Jac1p. The mutant phenotypes resulting from loss-of-function mutations in either jac1 or ssq1 were extremely similar. Mutant alleles of both genes were selected in screens for abnormal iron homeostasis or Δssd1 suppression (11). The iron trafficking abnormalities were similar for both mutants, characterized by induction of high affinity cellular iron uptake and accumulation in mitochondria. Mutants of jac1 and ssq1 (11, 49) exhibited multiple Fe-S protein deficiencies. Protein translocation into jac1 or ssq1 (10) mutant mitochondria was not generally impaired. However, a delay in the second processing cleavage of translocated Yfh1p was observed for both mutant mitochondria in in vitro import assays. Finally, genetic evidence for the interaction exists. The double mutant (Δssq1 Δjac1) was even more slow growing than either single mutant, indicating an additive or synthetic effect. The overexpression of Jac1p was able to significantly compensate for the slow growth of the Δssq1 mutant, and reciprocally the overexpression of Ssq1p was able to slightly compensate for the slow growth of the Δjac1 mutant. Finally, both Ssq1p (10) and Jac1p have been localized to mitochondria and thus reside in the same cellular compartment, as would be required for them to interact.

Hsp70s are structurally similar proteins that contain an ATP-binding domain and a peptide-binding domain evolved to interact with unfolded proteins (50). Family members exist in various cellular compartments and function by binding unfolded proteins, thereby preventing aggregation or assisting translocation (50). A chief function of J-proteins is to enhance delivery of chaperone substrates to the partner Hsp70. The J-protein may also enhance the ATPase activity of the Hsp70 (51). Subsequently, after ATP hydrolysis, the peptide substrate binds more tightly to the chaperone, allowing it to fold (50). However, J-proteins and Hsp70s may interact with different unfolded protein substrates and may even exhibit independent chaperone activities (50). The data on the synthetic effects of the mutants and the cross suppression data are consistent with the existence of both independent and cooperating functions for Hsp70 and J-proteins.

A key question relates to the identity of the substrates for this specialized chaperone system. In view of the specific impairment in various Fe-S cluster proteins in the jac1 and ssq1 mutants, Fe-S cluster proteins might constitute chaperone substrates. Most mitochondrial proteins, including Fe-S proteins,
are synthesized on cytoplasmic ribosomes and translocated into mitochondria as unfolded intermediates (52). At this stage these apoproteins probably lack Fe-S cofactors and may be prone to aggregation. Specifically dedicated chaperones could protect the apoprotein while an Fe-S cluster is inserted and then assist folding or assembly of the active conformation. Alternatively, specialized proteins that mediate Fe-S cluster synthesis might be the chaperone substrates. Elegant work suggests that several proteins associated on the isc operon of E. coli mediate in vivo synthesis or maintenance of Fe-S clusters (16, 20), and these might require special chaperones for their assembly or activation. Recent data from Vickery and co-workers (21) show that the IscU protein, proposed to be a scaffold on which Fe-S cluster assembly occurs, interacts with high affinity with the HscA (homologous to Sqsp1) and HscB (homologous to Jac1p) chaperones. This strongly suggests that, in bacteria, IscU is a physiologic substrate, although perhaps not the only one. In yeast, the IscU homologs, Isu1p and Isu2p, are mitochondrial proteins that are important for iron homeostasis (49). Another protein that could be a substrate for the Sqsp1 and Jac1p chaperones is Yfh1p. Yfh1p, the yeast frataxin homolog, is clearly involved in cellular and mitochondrial iron homeostasis (44), although the prokaryotic homolog is not located on the isc operon (16).

Many questions remain, and an interesting unresolved question relates to the evolutionary conservation of these chaperones. IscU and IscU proteins involved in Fe-S cluster formation (20), are conserved from bacteria (isc operon) to yeast to humans (53, 54). Sqsp1 and Jac1p are related to orthologous genes on bacterial isc operons implicated in Fe-S cluster synthesis or maintenance (16, 23). Therefore, it is possible that Jac1p and Sqsp1 chaperones will also be found in higher eukaryotes. If human homologs of Jac1p and Sqsp1 exist, they might be important in human diseases of iron trafficking, such as some neurodegenerative diseases (55) and sideroblastic anemias (56–58).

REFERENCES

1. Brittenham, G. (2000) in Hematology: Basic Principles and Practice (Hoffman, R., Benz, E., Shattil, S., Furie, B., Cohen, H., Silberstein, L., and McGlave, P., eds) pp. 397–428, Churchill Livingstone, Philadelphia
2. Halliwell, B., and Gutteridge, J. M. C. (1991) Free Radicals in Biology and Medicine, Clarendon, Oxford, United Kingdom
3. Askwith, C. C., de Silva, D., and Kaplan, J. (1996) Mol. Microbiol. 20, 27–34
4. Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G., and Klausner, R. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5869–5873
5. Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J., and Klausner, R. D. (1995) J. Cell Biol. 129, 839–849
6. Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D., and Dancis, A. (1996) Science 271, 1552–1557
7. Yamaguchi-Iwai, Y., Dancis, A., and Klausner, R. D. (1996) EMBO J. 15, 3377–3384
8. Gangloff, S. P., Marguet, D., and Lauquin, G. J. (1990) Mol. Cell. Biol. 10, 3551–3561
9. Dibrov, E., Fu, S., and Lermine, B. D. (1993) J. Biol. Chem. 268, 23042–23048
10. Rowley, N., Prip-Buus, C., Westermann, B., Brown, C., Schwarz, E., Barrell, B., and Neupert, W. (1994) Cell 77, 249–259
11. Westermann, B., and Neupert, W. (1997) J. Biol. Chem. 272, 477–483
12. Voisine, C., Schüke, B., Ohlsen, M., Beinert, H., Marszalek, J., and Craige, E. A. (2000) Mol. Cell. Biol. 20, 3677–3684
13. Babcock, M., de Silva, D., Oakes, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfi, M., and Kaplan, J. (1997) Science 276, 1709–1712
14. Branda, S. S., Cavadini, P., Adamec, J., Kalousek, F., Taroni, F., and Isaya, G. (1999) J. Biol. Chem. 274, 22763–22769
15. Gordon, D. M., Kogan, M., Knight, S. A., Dancis, A., and Pain, D. (2001) Hum. Mol. Genet. 10, 259–269
16. Beinert, H., and Kiley, P. J. (1999) Curr. Opin. Chem. Biol. 3, 152–157
17. Klausner, R. D., Rouault, T. A., and Harford, J. B. (1993) Cell 72, 19–28
18. Schüke, B., Voisine, C., Beinert, H., and Craig, E. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10206–10211
19. Hartl, F. U. (1986) Nature 318, 571–580
20. Craig, E. A. (1998) Science 260, 1902–1903
21. Schatz, G., and Doberstein, B. (1996) Science 271, 1519–1526
22. Land, T., and Rouault, T. A. (1998) Mol. Cell 2, 807–815
23. Ting, W. H., and Rouault, T. A. (2000) EMBO J. 19, 5692–5700
24. Knight, S. A. B., Kim, R., Pain, D., and Dancis, A. (1999) Am. J. Hum. Genet. 64, 365–371
25. Wiley, J. S., and Moore, M. R. (2000) in Hematology: Basic Principles and Practice (Hoffman, R., Benz, E., Shattil, S., Furie, B., Cohen, H., Silberstein, L., and McGlave, P., eds) pp. 11428–11445, Churchill Livingstone, Philadelphia
26. Allikmets, R., Raskind, W. H., Hutchinson, A., Schueck, N. D., Dean, M., and Koebler, D. M. (1999) Hum. Mol. Genet. 8, 743–749
27. Bekri, S., Kispal, G., Lange, H., Fitzmissoe, E., Tolmje, J., Lill, R., and Bishop, D. F. (2000) Blood 95, 3256–3264
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Roy Kim, Sandeep Saxena, Donna M. Gordon, Debkumar Pain and Andrew Dancis

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