Mt-Hsp70 Homolog, Ssc2p, Required for Maturation of Yeast Frataxin and Mitochondrial Iron Homeostasis*

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Here we show that the yeast mitochondrial chaperone Ssc2p, a homolog of mt-Hsp70, plays a critical role in mitochondrial iron homeostasis. Yeast with ssc2-1 mutations were identified by a screen for altered iron-dependent gene regulation and mitochondrial dysfunction. These mutants exhibit increased cellular iron uptake, and the iron accumulates exclusively within mitochondria. Yfh1p is homologous to frataxin, the human protein implicated in the neurodegenerative disease, Friedreich's ataxia. Like mutants of yfh1, ssc2-1 mutants accumulate vast quantities of iron in mitochondria. Furthermore, using import studies with isolated mitochondria, we demonstrate a specific role for Ssc2p in the maturation of Yfh1p within this organelle. This function for a mitochondrial Hsp70 chaperone is likely to be conserved, implying that a human homolog of Ssc2p may be involved in iron homeostasis and in neurodegenerative disease.

Iron is required as a cofactor for critical proteins within mitochondria of eukaryotic cells. These proteins include heme and iron-sulfur proteins involved in diverse processes such as cellular respiration and the synthesis of metabolic intermediates. However iron is also extremely toxic, capable of generating damaging free radicals (3). Therefore, homeostatic mechanisms exist that regulate iron levels and iron protein levels within mitochondria. Yfh1p is a mitochondrial protein of Saccharomyces cerevisiae that is involved in this homeostasis (4–8). Yeast with mutations in yfh1 accumulate iron within mitochondria (4, 5) and yet are deficient in some mitochondrial iron proteins (5, 6). Yfh1p is homologous to the human protein frataxin (7, 8), and mutations in frataxin are associated with the neurodegenerative disease Friedreich's ataxia (9). At the cellular level, iron accumulation occurs in affected tissues in these patients, and iron proteins such as aconitase and cytochrome oxidase are deficient (6).

The manner in which Yfh1p in the yeast (or frataxin in humans) affects iron homeostasis of mitochondria has not been defined. This work implicates a member of the class of Hsp70 proteins in this process. Two distinct Hsp70 proteins are found in mitochondria of S. cerevisiae (1). One of these, Ssc1p, is essential for viability and is involved in the import and subsequent folding of nuclear-encoded proteins in mitochondria (10–13). The second, Ssc2p,1 is one thousand-fold less abundant, and its physiological role has not been previously determined (1). We show here that Ssc2p plays a role in mitochondrial iron usage and in the maturation of Yfh1p.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—Methods for yeast manipulations and growth media have been described (14). In YE the carbon source was replaced with 3% ethanol. For some experiments with rho− strains, the carbon source was 2% raffinose. The yeast strains used were: CM3260, CM3262, 61, and 81 (15). Strains 81 or 61 were exposed to ethidium bromide, creating strains 81rho6 and 61rho6 respectively. Strain 35-5B (MATα trp1-63 leu2-3, 112 gen4-101 his3-609 FRE4-HIS3::URA3 ssc2-1) carried the ssc2-1 allele. The following strains were derived from backcrosses of this mutant: 191-33C (MATα ssc2-2); a complete tetrad 191-36A; 191-36B(ssc2-1), 191-36B/ssc2-1, 191-36D/ssc2-2, Ura− derivatives 341-2B and 341-2A (MATα ura3-52 ssc2-1); 341-4A and 341-8A (MATα ura3-52 ssc2-1). The yfh1 deletion strain, Δyfh1, was generously provided by Dr. Jerry Kaplan (4) and was backcrossed to strain 61, creating 5DΔyfh1 (MATα Δyfh1::HIS3).

Assays—The assay for ferric reductase was a filter lift assay (16), modified by the addition of 50 μM copper sulfate and 10 μM ferric ammonium sulfate to YPD agar plates for growth of the colonies to be assayed. Measurement of high affinity radioactive iron uptake rate has been described (15). To assess mitochondrial iron, the cells were grown for 16 h (6–8 doublings) in SD raffinose with different concentrations of radioactive 55Fe, and mitochondria were purified (17). The cells for the microscopy were grown in SD raffinose with 5 μM ferric ammonium sulfate as above. The preparation of yeast for electron microscopy has been described (18). The electron microscope was a Jeol 100CX model and was fitted with an Energy Dispersive Spectrophotometer (19). Mitochondrial import studies were described previously (20). Briefly, import reactions containing 100 μg of mitochondrial were initiated by adding urea-denatured preprotein (30–40 ng). Import reaction mixtures contained 4 mM ATP and 1 mM GTP. Following import at 20 °C for 5 or 15 min, reaction mixtures were treated with trypton (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 fluorography.

Plasmids and DNA Manipulations—Plasmid pSc30, isolated from a yeast genomic library (21), contained yeast genomic sequences from Chromosome XII, coordinates 858900-870300, and included open reading frames SUR4, ROM2, ARC18, and SSC2 (YLR369W). Plasmid pSc30-3, containing SSC2, was created by subcloning the EcoRI-KpnI genomic fragment. The plasmid pSc30-3Δnot contained a frameneshift mutation in the open reading frame at the unique NotI site. For meiotic mapping, the EcoRI fragment from within ROM2 inserted into Yip5 (prom2-YIp5) was integrated into CM3260 at its unique SacI site, and this strain was crossed with 35-5B (ssc2-1). The YFH1 open reading frame was inserted into the NdeI-Khol sites of vector pET21b (Novo-

1 Note on nomenclature: the gene, SSR1, corresponding to the open reading frame YLR369W was initially renamed SSIQ1 while this paper was in press. The gene can be found in the S. cerevisiae Genome Database under the name SSIQ1. The name SSI1 was used in a previous publication (1), but that name also designates YBR283C (2).
RESULTS

The present investigation was an outgrowth of our general interest in iron trafficking in *S. cerevisiae*. Yeast cells were subjected to a selection procedure designed to detect mutants with abnormal iron metabolism. The iron-repressible promoter of the *FRE1* gene was fused to the HIS3 coding region and integrated into the genome of a haploid yeast strain. Cells harboring this gene fusion were then cultured in a medium supplemented with iron but without histidine. Mutants were selected that were unable to repress *FRE1*-driven gene transcription, indicating a defect in iron uptake (15), iron sensing (22), or iron distribution. A subset of these mutants was identified that was unable to grow on medium containing ethanol as a carbon source, an indicator of mitochondrial dysfunction.

One such mutant, 35-5B, was chosen for further study. The mutant retained ferric reductase activity under conditions (available iron and copper) that led to repressed activity in the wild-type. This assay, used to track the mutation in genetic analyses, indicated that the mutant phenotype was recessive. Sporulation of this diploid strain which yielded 30 tetrads showing 2:2 segregation of the mutant phenotype indicated that the mutation was at a single locus. The mutant was then transformed with a genomic library, and a complementing plasmid pSC30 was isolated (21). The complementing activity was retained by pSC30-3 which contained the single complete open reading frame from *SSC2*, and this activity was abrogated by the frameshift mutation introduced into pSC30-3amot. Rescue of the *ssc2-1* allele and sequence analysis identified a single T to G point mutation at nucleotide 658 within the open reading frame, thereby generating a stop codon within the amino-terminal portion of the predicted protein. The correctness of the identification of *SSC2* as the wild-type allele of the mutation in the 35-5B strain was further verified by meiotic mapping. A *URA3* marked allele of the genomic fragment carried on pSC30 was integrated into the parental strain and crossed with the *ura3* mutant strain 35-5B. Recombination between the mutant phenotype and the *URA3* marker was not observed in 12 tetrads analyzed.

To evaluate mitochondrial function in the *ssc2-1* strain, we investigated the growth of this strain on media with nonfermentable carbon sources. Heterogeneity arose because of loss in strain 341-5B (*ssc2-1*). Strains 81rho0 (1. rho<sup>0</sup>) and 341-5B (2. *ssc2-1*) were crossed and zygotes were manipulated. Other haploid controls were 191-SCC (3. *ssc2-1*), 61 (4. WT), 61rho0 (5. rho<sup>0</sup>). Haploids and diploid clones arising from the cross were transferred to YPD (Ethanol) or YPD (Glucose). Failure of the diploid clones to grow on ethanol plates is diagnostic of mtDNA inactivation in the parental strain, 341-5B (*ssc2-1*). B. growth characteristics of the mutants: temperature and iron sensitivity. Diploid x191-36 was sporulated, and spore clones carrying the mutant allele *ssc2-1* (A, B) or the wild-type allele *SSC2* (C, D) were grown on YE agar at different temperatures (30 °C, 23 °C, 37 °C) or for growth on SD medium containing 1 mM ferrozine (Chelator, no added iron; *Iron*, 250 µM ferric ammonium added). The relative concentrations of the inocula spotted onto the plates are indicated by 1 (10<sup>3</sup> cells/10 µl) and 1:10. The wild-type clone C appeared pigment because of a genetic trait unlinked to *SSC2*. C. high affinity cellular iron uptake increased in the *ssc1-2* mutants. The spore clones were grown to logarithmic phase in YPD, and iron uptake was assayed using 1 µM <sup>55</sup>Fe radionuclide in 50 mM sodium citrate buffer, pH 6.5, as described. Data are the mean ± S.D. of triplicate measurements. D. mitochondrial iron content increased in the *ssc1-2* mutants. Strains 61 (WT), 61rho<sup>0</sup> (rho<sup>0</sup>), and 191-SCC (*ssc2-1*) were grown in media with 0.9, 1.8, or 5 µM iron, and the mitochondrial iron content was assayed as described.

To directly assess the iron content of the mitochondria, cells from the wild-type, a congenic rho<sup>0</sup> strain, and the *ssc2-1* mutant were cultured in media containing different concentrations of iron-55 radionuclide. Mitochondria were purified (17), and the total radioactive iron content was evaluated. In the wild-type, mitochondrial iron content varied little with the different iron concentrations of the growth medium (Fig. 1D, wild-type values 1.8 to 2.6 pmol/µg of protein). By contrast, the *ssc2-1* mutant strain accumulated iron within mitochondria in proportion to the iron content of the growth medium. When grown in 0.9, 1.8, or 5 µM <sup>55</sup>Fe-containing medium, the mutant accumulated 9.5, 29.4, or 107.2 pmol/µg of mitochondrial protein, respectively (Fig. 1D). The rho<sup>0</sup> strain did not exhibit comparable mitochondrial iron accumulation, and so the effect could not be ascribed to the absence of mtDNA. We wondered if the increased mitochondrial iron content in the mutant represented a primary problem or a consequence of the increased cellular iron uptake “spilling over” into the mitochondria. When the iron content of cellular fractions was analyzed, iron accumulation in the mutant was observed exclusively within the mitochondrial fraction. The post-mitochondrial supernatant, in fact, appeared moderately depleted of iron in the mutant compared with the wild-type strain (5.5 compared with 9.2 pmol/µg of protein). These results suggest that the increase in mitochondrial iron in the mutant was not a secondary effect resulting from increased cytosolic iron but rather a primary defect.
The accumulation of iron in the ssc2-1 mutant mitochondria was so great that it was visible by electron microscopy. The mitochondria were packed with electron-dense material in over 50% of the cells (Fig. 2A). The fact that the deposits indeed contained iron was confirmed by Energy Dispersive X-ray Spectroscopy. The wild-type yeast strain contained no such iron deposits, and a congeneric rho0 strain showed only rare deposits in less than 5% of cells, indicating that this appearance was specific for the ssc2-1 mutant. Under higher magnifications, the mitochondrial double membrane could be seen (arrow m, Fig. 2B), and the iron deposits were evident within the mitochondrial matrix. The deposits were granular and discontinuous in appearance, as if separated by intramitochondrial cristae (Fig. 2, B and D). In some cells, the deposit-laden mitochondria were arrayed around the nucleus (Fig. 2D). We conclude that a loss of homeostatic control in the ssc2-1 mutant leads to accumulation of vast quantities of iron as electron-dense bodies within the mitochondria.

Some of the features described here for the ssc2-1 mutant have been reported for yeast with mutation in yfh1. Therefore, we compared the two mutant strains directly. Both were slow growing and exhibited frequent destabilization or inactivation of the mitochondrial genome (4, 7). Both retained ferric reductase activity under conditions that repress activity in the wild-type. Both exhibited elevated levels of high affinity iron uptake (354 pmol/10^6 cells/h for the ssc2-1 mutant and 464 for the yfh1 mutant, compared with 17 for the wild-type). Most striking was the similar phenotypes of

**FIG. 2.** Electron microscopy showing iron-laden mitochondria. A, ssc2-1 cells from strain 191-33C with accumulated iron visible as dense bodies. B, higher magnification (× 11,000) view of a single ssc2-1 cell showing accumulated iron within mitochondria. Mitochondria (m) and nucleus (n) are indicated. C, wild-type cell from strain 61. D, single ssc2-1 cell with iron-laden mitochondria ringing the nucleus. The calibration bar in each panel represents 1 μm.

The increased iron within mitochondria in both strains occurred without an increase in cytosolic iron (2.2 pmol of iron/μg of protein for the ssc2-1 mutant and 1.2 for the yfh1 mutant compared with 3.0 for the wild-type). Thus, the ssc2-1 and yfh1 mutants strongly resemble each other with respect to their mutant phenotypes.

The similar phenotypes of ssc2-1 and yfh1 mutants suggested that the corresponding proteins might function together. We therefore considered that Ssc2p might function specifically in the import or folding of Yfh1p, analogous to the known effects of Sec1p on import and folding of other mitochondrial preproteins. To test this hypothesis, mitochondria were isolated from the wild-type (WT) and ssc2-1 mutant (M) strains, and the import of Yfh1 preprotein was allowed to proceed for 5 min (5') or 15 min (15') at 20 °C. Where indicated, unimported precursor was digested by trypsin. p, i, and m signify the precursor, the intermediate, and the mature form, respectively. Lane 1 in each panel (Std) indicates 35% of the precursor used per import assay.

**FIG. 3.** Yfh1 preprotein processing impaired in ssc2-1 mitochondria. Import of urea-denatured precursors of Yfh1 (A), Yfh1-Protein A (B), or Put2 (C) were evaluated in mitochondria purified from wild-type (WT) or ssc2-1 (M) strains. Import reactions were allowed to proceed for 5 min (5') or 15 min (15') at 20 °C. Where indicated, unimported precursor was digested by trypsin. Two new fragments (i and m) acquired trypsin resistance, suggesting that the import of Yfh1 preprotein was followed by two processing cleavages (Fig. 3A). The Yfh1 preprotein (p) migrated at a molecular mass ~29 kDa, although the predicted size was only 19.5 kDa, perhaps because of the acidic nature of the protein. The initial processing cleavage removed ~2 kDa from the amino terminus of the preprotein and generated an intermediate size polypeptide (i) migrating at ~27 kDa. A subsequent cleavage removed ~4 kDa from the amino terminus of the intermediate form, generating a mature product (m) of ~23 kDa that was also trypsin-resistant (Fig. 3A). In the ssc2-1 mutant, by contrast, import of Yfh1 preprotein was efficient as judged by the appearance of the protease-resistant intermediate polypeptide (i), but the conversion to the mature form was impaired. After 5 min of incuba-
tion, the level of the mature form was decreased compared with the wild-type (Fig. 3A, m in lanes 2, 3, 4, and 5), whereas the level of the intermediate form of Yfh1 was increased compared with the wild-type (Fig. 3A, i in lanes 2, 3, 4, and 5).

Import studies of the preYfh1-Protein A fusion similarly generated two protease-resistant polypeptide forms, differing from the precursor by ~2 and ~6 kDa (Fig. 3B). This experiment also demonstrated that the proteolytic processing steps must be occurring at the amino terminus of the Yfh1 preprotein, because Yfh1 and Yfh1-Protein A precursors were processed identically. The level of the mature Yfh1-Protein A fusion protein (m) was again decreased in the ssc2-1 strain (M) compared with the wild-type (WT) (Fig. 3B, m in lanes 2, 3, 4, and 5). A reciprocal increase in the intermediate form was noted in the early (5 min) time point in the mutant, consistent with an inefficient second processing step (Fig. 3B, i in lanes 2, 3, 4, and 5). We also studied the import of prePut2 (20), the precursor of a mitochondrial matrix protein involved in proline biosynthesis, and in this case, no difference in the appearance of protease-protected forms was observed in the ssc2-1 mutant compared with the wild-type (Fig. 3C). Consistent with our prePut2 control, earlier studies failed to demonstrate alterations in the import or processing of several other preproteins by mitochondria isolated from ssc2 mutant strains (1). These data suggest that the defect in preprotein processing that exists in the ssc2-1 strain is specific for the Yfh1 preprotein.

**DISCUSSION**

We present the following model to explain these findings (Fig. 4). i) The primary defect in the ssc2-1 mutant leads to impaired maturation of Yfh1p (yellow in Fig. 4). ii) In the ssc2-1 mutant, iron uptake into the mitochondria is greatly increased, reducing cytoplasmic iron concentrations. The iron sensor-regulator, Aft1p, which ordinarily does not affect mitochondrial iron levels, responds to the decreased cytoplasmic iron by activation of the cellular iron uptake system (blue in Fig. 4) (22). Thus, iron is continually fed from the medium to the cytoplasm to the mitochondria (red in Fig. 4). The iron accumulates as dense bodies in the mitochondria that are visible by electron microscopy. iii) Despite the excess iron, the activities of a number of mitochondrial iron proteins are decreased (e.g. in yfh1 mutants, respiratory chain complexes I, II, III, IV, and the iron-sulfur protein, aconitase (5, 6)). In this model, Ssc2p is required for the generation of mature Yfh1p, thereby regulating iron usage and assembly of iron proteins within the mitochondria. A direct role for Ssc2p in these processes is also possible.

We have shown that Ssc2p participates in the second processing cleavage of Yfh1 following an initial cleavage of the extreme amino-terminal signal sequence. To do this, Ssc2p might itself be acting as the processing protease. The association of proteolytic and chaperone activities in a single complex has been described for mitochondrial proteins such as Lon (23) and Afg3p and Rca1p (24). Alternatively, Ssc2p could mediate maturation of Yfh1 preprotein indirectly via effects on folding or complex formation. The iron-sulfur protein of the cytochrome bc1 complex provides an example of an association between preprotein assembly and two-step proteolytic processing. The iron-sulfur preprotein is imported into the matrix, and the signal sequence is cleaved by the matrix processing peptidase. A second processing cleavage by the mitochondrial intermediate peptidase then occurs upon assembly of the mature protein into complex II of the mitochondrial inner membrane (25). In analogous fashion, Ssc2p might mediate processing and insertion of Yfh1p into a complex. However, physical interaction between Yfh1p and Ssc2p has not yet been demonstrated, and assembly partners for Yfh1p are not known.

Ssc2p function is necessary for normal iron homeostasis, and defects of Ssc2p are correlated with iron accumulation within the mitochondria. This may result from increased activity of mitochondrial iron importers or decreased activity of exporters. Another possibility is that diversion of iron into an inactive or inaccessible form induces increased iron import into mitochondria, causing the massive accumulations that we have observed. The iron, like intermediates in some storage diseases (26), may accumulate in a metabolic dead end, causing deficiencies of iron proteins and iron-protein complexes (5, 6). Ssc2p, through its effects on the maturation and assembly of Yfh1 and other proteins, might regulate this iron accumulation process.

Yfh1p is homologous to the human protein frataxin, which is defective in most cases of the neurodegenerative disease Friedreich’s ataxia (4–9). Specialized Hsp70 proteins within different cellular compartments are also conserved between yeast and humans (e.g. BiP in the endoplasmic reticulum and mt-Hsp70 in the mitochondria) (10). Therefore, in humans, a specialized mitochondrial form of Hsp70, analogous to Ssc2p, is likely to be involved in the maturation of human frataxin. Our inability to identify such a homolog in the human sequence data bases at this time may relate to the incomplete nature of these data bases and the low abundance of the transcript. The human homolog of Ssc2p might be defective in forms of Friedreich’s ataxia that are not explained by frataxin mutations (27) or in other neurodegenerative diseases with a mitochondrial basis.

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**FIG. 4. Model for the involvement of Ssc2p in Yfh1 preprotein processing and iron homeostasis.** Ovalis represent yeast cells, the wild-type on the left and the mutant (ssc2-1) on the right. The box enclosed by the double line represents the mitochondria. Yellow indicates protein trafficking and processing pathways (Yfh1p translocation and maturation). Red indicates iron trafficking pathways, with boxes showing steady state iron pools. Blue indicates regulatory loops, with Aft1p controlling uptake at the plasma membrane and Yfh1p/Ssc2p controlling iron accumulation within the mitochondria.
