CCC1 Suppresses Mitochondrial Damage in the Yeast Model of Friedreich’s Ataxia by Limiting Mitochondrial Iron Accumulation*

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Deletion of YFH1 in Saccharomyces cerevisiae leads to a loss of respiratory competence due to excessive mitochondrial iron accumulation. A suppressor screen identified a gene, CCC1, that maintained respiratory function in a Δyfh1 yeast strain regardless of extracellular iron concentration. CCC1 expression prevented excessive mitochondrial iron accumulation by limiting mitochondrial iron uptake rather than by increasing mitochondrial iron egress. Expression of CCC1 did not result in sequestration of iron in membranous compartments or cellular iron export. CCC1 expression in wild type cells resulted in increased expression of the high affinity iron transport system composed of Fet3p and Ftr1p, suggesting that intracellular iron is not sensed by the iron-dependent transcription factor Aft1p. Introduction of AFT1
to, a constitutive allele of the iron transcription factor, AFT1, that also leads to increased high affinity iron transport did not prevent Δyfh1 cells from becoming respiratory-incompetent. Although the mechanism by which CCC1 expression affects cytosolic iron is not known, the data suggest that excessive mitochondrial iron accumulation only occurs when cytosolic free iron levels are high.

Friedreich’s ataxia is a lethal disorder affecting the nervous system and heart. The disorder is due to a triplet expansion in the first intron of the Frataxin gene that results in decreased levels of Frataxin mRNA (1), which encodes a mitochondrial protein (2). Insight into the function of Frataxin resulted from studies on the Saccharomyces cerevisiae gene, yeast Frataxin homologue (YFH1), which is an orthologue of mammalian Frataxin. In the absence of Yfh1p, iron accumulates within mitochondria, leading to a loss of respiratory activity through the generation of mitochondrial DNA mutations. It is thought that decreased respiratory activity is a consequence of iron-induced oxygen radicals (3, 4). The observation of increased iron deposits in Friedreich’s ataxia heart biopsies (5) and the finding of increased iron in mitochondria of Friedreich’s ataxia fibroblasts (6, 7) indicate that the yeast model may be an accurate reflection of the pathophysiology of Friedreich’s ataxia.

We initiated a search for genes that could suppress the respiratory deficit in yeast lacking Yfh1p. Here we report that CCC1, previously characterized as a putative Golgi Ca\(^{2+}/Mn^{2+}\) transporter (8, 9), allowed Δyfh1 cells to maintain respiratory activity by preventing the toxic accumulation of mitochondrial iron. The restriction on mitochondrial iron accumulation resulted from reduced mitochondrial iron uptake rather than increased mitochondrial iron efflux. The observation that respiratory activity as well as leucine biosynthesis was unaffected in Δyfh1 cells overexpressing CCC1 implies that Yfh1p is not significantly involved in the biosynthesis, assembly or export of iron-sulfur clusters. Overexpression of CCC1 in wild type cells activated the iron-dependent transcription factor Aft1p, resulting in an increase in iron uptake and cytosolic iron accumulation. Activation of Aft1p suggests that cells sense that cytosolic iron is low, indicating that much of the cytosolic iron was not bioavailable. This reduced concentration of “free” iron did not affect the growth of Δyfh1 cells but did prevent excessive mitochondrial iron accumulation. These studies demonstrate that toxic mitochondrial iron levels resulting from the loss of Yfh1p may only occur when cytosolic free iron levels are high.

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CTC TTA AGC GGC CGC ACC TTC TTG GCT TTT AGA AAT GGC CT-3'. Cells showing a polymerase chain reaction product at 2.2 kilobases, but not 0.55 kilobases, the size of an intact YFH1 gene, were chosen for further study. Plasmids were rescued from candidate clones by electroporation into DH5α. DNA sequence analysis of the rescued plasmids revealed the presence of the two primers specific to the region upstream and downstream of the ccc1 gene (CT-3'). Sequences were not identical to the ccc1 gene, and in whole (YLR218c) and part of the sequences (YLR219w) that encompassed the sequence of 2243 base pairs were cloned into pRS314, bearing the LEU2 selectable marker. Methionine-dependent expression of Ccc1p-FLAG was confirmed by Western blot using an antibody against FLAG epitope. The FLAG epitope did not influence the ability of Ccc1p-FLAG to rescue MET3-deficient cells, and cells containing Ccc1p-FLAG were growth-competent in glucose-containing medium. The Ccc1p-FLAG protein was synthesized in yeast cells and was detected by Western blot using an antibody against FLAG epitope. The FLAG epitope did not influence the ability of Ccc1p to maintain respiratory activity in Δyfh1 cells. The METCC1 strain was generated by transforming METCC1 strain with pMetCC1. The transformed cells were "cured" of the pMetYFH1 plasmid by growth on uracil-containing medium. Identification of CCC1 as a High Copy Suppressor of Δyfh1—Since Δyfh1 cells are respiratory-deficient, we used a plasmid shuttle approach to identify high copy suppressors that could rescue Δyfh1 cells from respiratory deficiency (Fig. 1). A diploid heterozygous for chromosomal deletion of YFH1 was transformed with a URA3 plasmid in which YFH1 was placed under the control of the MET3 promoter (pMetYFH1). Diploids were sporulated in the absence of methionine, which permitted YFH1 gene expression and maintained respiratory competence as shown by growth on glycerol-ethanol. These haploids, designated METYFH1 (Δyfh1, pMetYFH1), were transformed with a high copy (YEp) genomic library that contained the LEU2 gene as a selectable marker. Transformants were selected on plates lacking leucine, uracil, and methionine. The cells were then plated under conditions that promoted the loss of the YFH1-containing URA3 plasmid: growth on medium lacking leucine but containing methionine and uracil followed by replica-plating to medium lacking leucine but containing 5-fluoroorotic acid. Cells capable of growth on glycerol-ethanol medium were examined for the presence or absence of an intact YFH1 gene and were capable of growth on glycerol-ethanol were selected for further study. Screening of 3,000 transformants resulted in four colonies that could grow on glycerol-ethanol plates but did not contain an intact YFH1. To demonstrate that growth on glycerol-ethanol required the LEU2 library plasmid, cells were grown in leucine-containing medium to permit plasmid loss. All cells that showed a leucine auxotroph were also found to be respiratory-incompetent. Plasmids were able to be rescued from transformed cells and, when re-transformed into the METYFH1 strain, permitted growth on respiratory substrates. Four of the five library clones contained overlapping regions of yeast chromosome XII. The only open reading frame present in common was a previously identified gene CCC1 (8) (9). This gene was cloned from one of the library clones and, when placed in a high copy vector, was able to support the growth of a Δyfh1 strain in glycerol-ethanol medium (Fig. 2A). Cells lacking YFH1 are viable but grow poorly in glucose medium, particularly in the presence of high levels of iron (10). Overexpression of CCC1 markedly enhanced growth in glucose-containing medium to the presence of high levels of iron (Fig. 2B). It is possible that overexpression of CCC1 is not sufficient for respiratory competence, as the selection system may have resulted in the generation of obligate endogenous suppressors. Additional mutations may have occurred during the process of selecting for cells that lost the pMetYFH1 plasmid. To test this possibility we took advantage of the ability to regulate YFH1 expression through the MET3 promoter. The addition of me-
thionine repressed YFH1 expression, resulting in an iron concentration-dependent increase in rho^-cells. Cells overexpressing CCC1 maintained respiratory activity, even in the face of high iron levels (Fig. 3A). If cells resistant to 500 μM iron were allowed to lose the CCC1 plasmid through growth on leucine-containing medium, they also lost the ability to grow on glycerol-ethanol medium, becoming petite in an iron concentration-dependent manner (Fig. 3B). These results suggest that growth of Δyfh1 cells on glycerol-ethanol is solely dependent on the CCC1 plasmid. The suppression of iron-dependent petite formation by overexpression of CCC1 demonstrates that CCC1 is both necessary and sufficient for maintaining respiratory activity of Δyfh1 cells.

CCC1 Prevents Mitochondrial Iron Accumulation—Our previous studies indicated that mitochondrial iron accumulation was the proximal cause of respiratory incompetence in Δyfh1 cells (10). If CCC1 maintains respiratory competence in Δyfh1 cells, it could do so by either increasing antioxidant defenses or by limiting mitochondrial iron accumulation. Growth of cells in medium containing H₂O₂ showed that CCC1 overexpression had at best a marginal protective effect. This effect was seen with the plasmid that contained just CCC1 (pSCCC1) but not with the original library clone (pS222). Yet both plasmids could maintain the respiratory activity of Δyfh1 cells. These results suggest that it is unlikely that the respiratory sparing ability of CCC1 is due to increased antioxidant defenses (Fig. 2C). We then examined the effect of CCC1 overexpression on mitochondrial iron accumulation. When METYFH1 cells were grown in the presence of methionine (Yfh1p “off”), there was a 10-fold increase in the total amount of cell-associated ^{59}Fe, and more than 50% of that was localized in mitochondria (Fig. 4). The accumulation of mitochondrial iron was not seen if cells were grown in the absence of methionine (Yfh1p “on”). In CCC1-overexpressing METYFH1 cells, the amount of cell-associated ^{59}Fe was similar in the presence or absence of methionine (see below). Yet regardless of whether Yfh1p was expressed or not, CCC1-overexpressing cells did not accumulate mitochondrial iron.

The inability of CCC1-overexpressing cells to accumulate mitochondrial iron could reflect a restriction on mitochondrial iron import or an acceleration of mitochondrial iron export. We took advantage of the ability to regulate CCC1 expression to distinguish between these possibilities. A strain, METCCC1 (Δyfh1, pMetCCC1), was constructed that had a chromosomal deletion of YFH1 and contained a high copy plasmid of CCC1 under the control of the MET3 promoter. Cells grown in the presence of methionine overnight did not show evidence of CCC1 mRNA by Northern blot analysis. Maximal levels of CCC1 mRNA, however, were reached within 2 h of methionine
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FIG. 3. Effect of CCC1 on petite formation. METYFH1 transformed with vector only or pSCCC1 were grown in glucose medium containing methionine and iron (ferrous ammonium sulfate) as indicated. A, iron-dependent petite formation was determined by replicating to glycerol-ethanol-containing media. METYFH1 transformed with pSCCC1, grown in 500 μM iron, was allowed to lose the pSCCC1 plasmid. B, iron-dependent petite formation was measured.

FIG. 4. The effect of CCC1 expression on mitochondrial iron accumulation. METYFH1 transformed with vector only or pSCCC1 were grown in the presence or absence of methionine for 8 h. Cells were washed, incubated with 59Fe for 10 min, and then resuspended in growth medium containing nonradioactive iron for 2 h. Cells were harvested, spheroplasted, and homogenized, and the membrane fraction was applied to an iodixanol gradient. A, the amount of radioactivity in each fraction of the gradient was determined. B, distribution of organelle markers on iodixanol gradients was determined by Western blot analysis using antibodies directed against mitochondrial porin, prevacuolar Pep12p, and vacuolar alkaline phosphatase (ALP).

Ccc1p was originally identified as a Mn2+/Ca2+ Golgi transporter, and it is possible that it may transport iron as well. One explanation for the decrease in mitochondrial iron may be that Ccc1p transports iron out of the cytosol. Decreased cytosolic iron may limit iron entry into mitochondria. Analysis of the distribution of 59Fe within cells, however, did not provide evidence that CCC1 overexpression led to accumulation of iron in any membranous compartment (Figs. 4 and 5). A representative gradient of subcellular organelles is shown in Fig. 4B. We did not detect any accumulation of iron within vacuoles, prevacuoles, or Golgi vesicles. Our fractionation procedure resulted in less than 5.0% release of vacuolar carboxypeptidase Y to the cytosolic fraction (data not shown). Greater than 75% of accumulated iron was found to be cytosolic in both wild type and yfh1Δ cells overexpressing Ccc1p (data not shown). We also considered the possibility that CCC1 overexpression resulted in the appearance of Ccc1p on the cell surface, where it might act as an efflux channel. Pulse-chase experiments, however, provided no evidence that Ccc1p overexpression resulted in efflux of cellular iron (data not shown).

Overexpression of CCC1 Affects the Iron Regulon—Cells with a YFH1 deletion show increased expression of genes regulated by the iron-dependent transcription factor Aft1p (3). This increased expression results from sequestration of iron within mitochondria, resulting in a decreased cytosolic iron concentration. Overexpression of CCC1 did not prevent the increased rate of iron uptake in Δyfh1Δ cells and resulted in increased iron transport in wild type cells (Fig. 6A). Increased iron uptake was mediated by the high affinity iron transport system, as deletion of FET3 resulted in no iron transport in Ccc1p-overexpressing cells. No increased uptake activity was seen in a Ccc1p-overexpressing Δyfh1Δ strain, indicating that the effect of CCC1 required the iron transcription factor Aft1p. Ccc1p-overexpressing cells had a higher iron content than wild type cells, as measured by atomic absorption spectroscopy (control cells, 60.8 fmoles of iron/106 cells; pSCCC1-transformed cells, 99.0 fmoles of iron/106 cells). The increase in iron content was similar to that
seen in Δyfh1 cells grown in the same medium but was less than expected, based on the measurement of iron transporter activity. The high affinity iron transport system is regulated primarily by iron through Aft1p. Ferrireductase activity, which is required to convert ferric iron to ferrous iron (the substrate for the high affinity transport system) is regulated by several factors including iron (Aft1p), copper (Mac1p), and cyclic AMP (14). That ferrireductase activity was rate-limiting was shown by measuring iron transport in the presence and absence of ascorbate (Fig. 6B). In the absence of ascorbate, which bypasses the need for a reductase, CCC1-overexpressing cells took up more iron than control cells (vector only) but much less iron than in the presence of ascorbate. A similar pattern of iron transport was observed for cells that had increased expression of the high affinity iron transport system through the action of a constitutive allele of AFT1 (AFT1up).

The finding that overexpression of CCC1 results in induction of at least some iron-regulated genes leads to the question of whether activation of the iron regulon will suppress the respiratory phenotype of Δyfh1. Introduction of the AFT1up allele into wild type cells resulted in an increase in high affinity iron transport to the same extent as CCC1 overexpression (Fig. 6B), yet AFT1up did not prevent iron-dependent petite formation in Δyfh1 cells (Fig. 7).

**DISCUSSION**

A genetic screen revealed that CCC1 is a high copy suppressor of the respiratory deficit of Δyfh1 cells. High copy plasmid expression of CCC1 prevented Δyfh1 from becoming petite, even in the presence of high iron concentrations. CCC1 overexpression does not prevent cellular iron uptake but does prevent the increased mitochondrial iron accumulation expected of a Δyfh1 strain. This result confirms previous studies demonstrating that the respiratory defect in Δyfh1 cells is due to excessive accumulation of iron within mitochondria (10). Heart biopsies from patients with Friedreich’s ataxia have shown a selective defect in the activity of mitochondrial iron-sulfur proteins (15). A recent report indicated that in the absence of Yfh1p, even in low iron conditions, there was a decrease in aconitase activity, suggesting that Yfh1p was involved in regulating formation of iron-sulfur clusters (16). A caveat to those experiments is that aconitase protein levels were not measured. In our strain of yeast, however, deletion of YFH1 does not result in significant loss of iron-sulfur-containing enzyme ac-
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A

B

overexpression.

A. wild type (DY150), Δccc1, and Δaft1 were transformed with vector or CCC1 subcloned into the vector pTF63, and high affinity iron uptake activity was determined. B. iron uptake activity was determined in the presence and absence of ascorbate in wild type cells transformed with vector only, AFT1Δ, or pGCCCC1.

iron export from cells or sequestration of iron in membranous compartments. Our results indicate that iron accumulates in cytosol and is not recognized by the iron-sensing transcription factor Aft1p. We do not know the form or species (Fe^{2+} or Fe^{3+}) of this stored iron. Cells overexpressing Ccc1p perceive an apparent “low” iron content and increase the transcription of the iron regulon, resulting in increased activity of the high affinity iron transport system.

Increased activity of the high affinity iron transport in CCC1-overexpressing cells is an indication that cells “sense” low iron levels. Expression of the high affinity iron transport system, however, does not necessarily result in iron-limited growth; the activities of a number of essential iron-containing proteins, such as methyl sterol oxidase, Δ9-fatty acid desaturase, are unaffected as shown by robust rates of cell growth. The mechanism by which mitochondria can adjust iron accumulation with respect to cytosolic iron levels is unknown. Restriction of cytosolic “free iron” either by environmental iron restriction (10, 16), genetic ablation of high affinity iron transport systems (10), or genetic manipulation of intracellular iron content (CCC1 overexpression) prevents excessive mitochondrial iron accumulation in cells lacking YFH1.

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