The budding yeast *Saccharomyces cerevisiae* can grow for generations in the absence of exogenous iron, indicating a capacity to store intracellular iron. As cells can accumulate iron by endocytosis we studied iron metabolism in yeast that were defective in endocytosis. We demonstrated that endocytosis-defective yeast (Δend4) can store iron in the vacuole, indicating a transfer of iron from the cytosol to the vacuole. Using several different criteria we demonstrated that *CCC1* encodes a transporter that effects the accumulation of iron and Mn2+ in vacuoles. Overexpression of *CCC1*, which is localized to the vacuole, lowers cytosolic iron and increases vacuolar iron content. Conversely, deletion of *CCC1* results in decreased vacuolar iron content and decreased iron stores, which affect cytosolic iron levels and cell growth. Furthermore Δccc1 cells show increased sensitivity to external iron. The sensitivity to iron is exacerbated by ectopic expression of the iron transporter *FET4*. These results indicate that yeast can store iron in the vacuole and that *CCC1* is involved in the transfer of iron from the cytosol to the vacuole.

While iron is a required element for all eucaryotes, it is also potentially toxic. Organisms tightly regulate the concentration of cytosolic iron through regulation of iron uptake and storage. In the past few years the mechanisms that mediate plasma membrane iron transport in the budding yeast *Saccharomyces cerevisiae* have been described in molecular detail (1). Many of the genes required for plasma membrane transport have been cloned. Much less is known, however, about iron storage. Yeast is distinguished from most eucaryotes in not having ferritin as an iron storage molecule. In this regard yeast are more analogous to plants than vertebrates. In plants, ferritin is restricted to the vacuole. Overexpression of *CCC1* is analogous to plants than vertebrates. In plants, ferritin is restricted to the vacuole. Overexpression of *CCC1* is analogous to plants than vertebrates. In plants, ferritin is restricted to the vacuole.

### EXPERIMENTAL PROCEDURES

**Yeast Strains, Plasmids and Media**—The yeast strains employed in this study were derived from DY150, whose parental strain was W303 (8). The Δ fet3 Δ fet4 strain has been described previously (9). The Δ end4 strain has also been described (10). The Δ ccc1 strain was generated by double fusion polymerase chain reaction (11). The primers were: 5′-TGT CCG TCT GGA CCA ATC GC-3′, 5′-GTC GTG ACT AGT GCT AAA ACC CTG GCG ACT GCG TTC TTT AGT GCT AC-3′, 5′-TCC TGT GGT AAA TTG TTA GCC TTG GCC ATG AGT GGC AGT TC-3′, 5′-GCC ACC AAA TGA CGA ATT AG-3′. The HIS3 gene was used as the selectable marker. The polymerase chain reaction fusion products were transformed into DY150 wild type cells, and the deletion of the Δ ccc1 gene was confirmed by polymerase chain reaction. Overexpression of *CCC1* was accomplished using a multicopy plasmid that contained *CCC1* under its own native promoter as described in (12). Overexpression of *FET4* was accomplished using a plasmid (pCB1) in which *FET4* was placed under the control of the GAL1 promoter (13). DNA transformations of *S. cerevisiae* and *Escherichia coli* were performed using standard procedures (14, 15). Cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose), CM (a synthetic medium of yeast nitrogen base, amino acids, and glucose), CM deficient in specific amino acids, CM made iron-limited by the addition of BPS (1 mM), and CM supplemented with iron or Mn2+ as detailed in specific experiments.

**Labeling with Fluorescent Dyes**—FM4–64 and DCFDA were purchased from Molecular Probes. For FM4–64 labeling, cells were grown in YPD medium followed by incubation with 30 μM final concentration of FM4–64 for 1 h. For DCFDA labeling, cells were grown in CM medium and then incubated with 100 μM DCFDA for 1 h. Cells were washed once with YPD or CM, then fixed on concanavalin A (Sigma)-coated slides, and examined by fluorescent microscopy.

**Isolation of Vacuoles and Measurement of Metals by ICP-AAS**—Vacuoles were prepared and measured by ICP-AAS as detailed in specific experiments.

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* This work was supported by National Institutes of Health Grants NIDDK-5238 and NIDDK-DE-30534. Support for use of Core facilities was provided by a grant from the NCIC, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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peptatin A) were added to the Ficoll buffer. 50 μg/ml DEAE-Dextran (Amersham Pharmacia Biotech) was added to the spheroplasts, and the sample was incubated for 3 min on ice and for 5 min at 30 °C. MgCl₂ was added to the lysate to give a final concentration of 1.5 mM. The lysate (3.5 ml) was transferred to two SW41 tubes (Beckman Instruments) and overlaid with 3 ml of 8% Ficoll, 4 ml of 4% Ficoll, and 1 ml of 0% Ficoll. The tubes were centrifuged at 110,000 × g for 90 min. The vacuolar fraction was collected from the 0%/4% interphase.

To determine metal content the vacuolar fraction or spheroplasts were pelleted and resuspended in 1 ml of MIB buffer (0.6M mannitol, 20 mM HEPES-KOH, pH 7.4). Samples were incubated with DCFDA (Sigma) and used at a dilution of 1:500. Alexa-594-conjugated goat anti-mouse antibody purchased from Molecular Probes and used at a dilution of 1:200.

**RESULTS**

**Isolation of Vacuoles**—Vacuoles were isolated by a modification of the procedure of Conradt et al. (16). To demonstrate that the preparation contained intact vacuoles, cells were exposed to DCFDA, a fluorescent dye that accumulates in vacuoles. To assess endocytic activity, cells were exposed to FM4–64, a dye that intercalates into the plasma membrane and accumulates in vacuoles through membrane internalization (20). Both wild type cells and cells with a deletion in the EN4 gene were examined by fluorescent microscopy and photographed. The vacuolar fraction obtained from wild type cells labeled as in C was examined for DCFDA (E).

**TABLE I**

|          | end4(vector) | end4/CCC1 |
|----------|--------------|-----------|
| iron     |              |           |
| µg iron/mg protein |
| A Whole cells | 0.158 ± 0.057 | 0.498 ± 0.041 |
|          | 0.095 ± 0.012 | 0.177 ± 0.15 |
|          | 0.177 ± 0.002 | 0.710 ± 0.006 |
| B Whole cells | 0.006 ± 0.006 | 0.028 ± 0.0037 |
|          | 0.006 ± 0.0004 | 0.015 ± 0.0022 |
|          | 0.006 ± 0.0000 | 0.060 ± 0.0001 |

**Vacular Iron Storage**

**Isolation of Vacuoles**—Vacuoles were isolated by a modification of the procedure of Conradt et al. (16). To demonstrate that the preparation contained intact vacuoles, cells were exposed to DCFDA, a fluorescent dye that accumulates in vacuoles. To assess endocytic activity, cells were exposed to FM4–64, a dye that intercalates into the plasma membrane and accumulates in vacuoles through membrane internalization (20). Both wild type cells and cells with a deletion in the EN4 gene showed DCFDA laden vacuoles (Fig. 1). Wild type cells showed FM4–64 in vacuoles, but Δend4 cells showed dye only in the plasma membrane. These results are consistent with published free medium. At specified times, lysates were prepared, and β-galactosidase activity were assayed as described previously (19).

**RESULTS**

**Isolation of Vacuoles**—Vacuoles were isolated by a modification of the procedure of Conradt et al. (16). To demonstrate that the preparation contained intact vacuoles, cells were exposed to DCFDA, a fluorescent dye that accumulates in vacuoles. To assess endocytic activity, cells were exposed to FM4–64, a dye that intercalates into the plasma membrane and accumulates in vacuoles through membrane internalization (20). Both wild type cells and cells with a deletion in the EN4 gene showed DCFDA laden vacuoles (Fig. 1). Wild type cells showed FM4–64 in vacuoles, but Δend4 cells showed dye only in the plasma membrane. These results are consistent with published
studies showing that Δend4 cells are unable to endocytose but contain a normal vacuolar compartment. Examination of a vacuolar-enriched fraction from a homogenate of either wild type cells or Δend4 cells showed DCFDA in large intact vacuoles (Fig. 1E). The vacuolar fraction showed a great enrichment over spheroplasts for the soluble vacuolar enzyme carboxypeptidase Y (Fig. 2A). Vacuoles isolated from Δend4 cells showed a similar enrichment in enzyme activity. As these step gradients are bottom-loaded, the presence of the CPY and DCFDA in the low-density fraction confirms that vacuoles are intact. The yield of vacuoles, based on enzyme activity, indicated a recovery of 10–15% of the vacuole population. Although the yield was low, the population was highly enriched, as shown by the low level of marker proteins for other organelles (Fig. 2B). The only organelle that showed some enrichment in the vacuolar fraction was the prevacuole, and the degree of enrichment was much less than that of vacuolar markers.

Yeast Can Live on Stored Iron—Previous studies have indicated that yeast can store iron, as they can grow for generations in iron-free media (4). If iron can accumulate in cells through endocytosis, it might be expected that cells unable to endocytose would show less growth in iron-free media. Incubation of Δend4 cells in iron-deficient media results in a decreased rate of cell growth relative to wild type cells. The decreased growth is not due to an inability to transport iron across the plasma membrane as the growth rate of Δend4 cells is greater than that of Δfet3 Δfet4 cells, which show a profound decrease in plasma membrane iron transport (Fig. 3A). The fact that wild type cells can grow longer in low iron media than Δend4 cells suggests that iron accumulated by endocytosis can be used for growth. Upon prolonged incubation in iron-free media both wild type cells and Δend4 cells will show a growth arrest. That the growth arrest is due to iron limitation is shown by the observation that addition of iron for as little as 15 min to iron-starved cultures results in the restoration of cell growth for both wild type and Δend4 cells (Fig. 3B). This result suggests that Δend4 cells can accumulate iron through the high affinity iron transport system, demonstrating that the defect in growth in iron-poor media may reflect the absence of iron accumulated through endocytosis. That the Δend4 cells can grow for generations after being exposed to iron for just 15 min indicates that cells can store iron. Where do cells store that iron? Wild type cells exposed to high iron accumulate iron in a membrane fraction enriched in vacuoles. Δend4 cells exposed to high iron also accumulate iron in the vacuole, but only about half as much as wild type cells (Fig. 4). We surmise from this result that vacuoles in wild type cells can obtain iron both from endocytic activity and via transport from the cytosol. Transfer of iron from cytosol to vacuole would constitute proof of iron
storage. Evidence in support of that view comes from examination of the kinetics of accumulation of vacuolar iron in Δend4 cells that cannot accumulate iron by endocytosis. In these cells there is a delay between the exposure of cells to iron and the appearance of iron in vacuoles. Vacuoles isolated immediately after a 15-min pulse shows low levels of iron but there is a dramatic increase in vacuolar iron content over the ensuing three hours (Fig. 3C). This result again indicates that the vacuole can store iron independently of endocytosis indicating a transfer of iron from cytosol to vacuole.

Identification of CCC1 as a Transporter That Can Effect Vacular Iron Storage—Cells with a deletion in YFH1 accumulate high concentrations of iron in their mitochondria leading to a loss of respiratory function (21). The loss of respiratory function is iron-dependent, and reduction in cytosolic iron maintains the ability of Δyfh1 cells to grow on respiratory substrates. We identified CCC1 as a high copy suppressor of the Δyfh1 phenotype (12). Further study indicated that overexpression of CCC1 resulted in a decrease in the cytosolic iron pool. In our initial study we did not find evidence that iron accumulated in vacuoles. We have since recognized that the membrane isolation procedure employed, which preserves mitochondrial iron, resulted in release of vacuolar contents. Using the vacuolar isolation procedure described above we determined that CCC1 expression resulted in increased vacuolar iron content. Expression of CCC1 in wild type cells resulted in an increase in vacuolar iron (Fig. 4). It is formally possible, however, that some of this iron can enter cells through endocytic activity. To test that possibility we measured vacuolar iron in Δend4 cells overexpressing CCC1. Because Δend4 cells have a relatively low vacuolar iron content, we used cells grown in 50 µM iron. Overexpression of CCC1 in Δend4 cells resulted in an enormous increase in vacuolar iron content over Δend4 cells with a control vector (Table IA). CCC1 was previously identified as a transporter that can lower cytosolic Mn2+ (22). Overexpression of CCC1 also resulted in an increase in vacuole-associated Mn2+ (Table IB). A FLAG-tagged Ccc1p was localized to the vacular membrane, providing further evidence that the site of iron and Mn2+ storage is the vacuole (Fig. 5).

How does expression of Ccc1p alter vacuolar/cytosolic iron? First, vacuolar iron is lower in Δccc1 cells than in control cells (Fig. 4). Second, Δccc1 cells show a decreased growth rate in iron-free media (data not shown) compared with wild type cells or CCC1 overexpressing cells. If Δccc1 cells are unable to store iron in the vacuole, then when placed in iron-free media they may be more iron-starved than control cells. Increased iron starvation would be manifested by a more rapid increase in transcripts for the high affinity iron transport system. A FET3-β-galactosidase reporter construct was used to examine this possibility. When placed in iron-free media Δccc1 cells show a more rapid increase in β-galactosidase activity than wild type cells (Fig. 6).

It is thought that one function of iron storage is to protect cells from the deleterious effects of iron. Thus, a defect in
Vacuolar iron accumulation may result in increased iron toxicity. Incubation of Δccc1 cells in high iron media resulted in decreased viability. A decrease in viability was also seen when cells were exposed to high levels of Mn²⁺ but was not seen following exposure to Zn²⁺, Cu²⁺, or Co²⁺ (data not shown). If the toxicity is due to increased cytosolic iron, then increasing iron uptake should exacerbate the effect. This possibility was examined by expressing the low affinity iron transporter FET4 under the control of the GAL1 promoter. In glucose media, Δccc1 cells were more sensitive to iron than control cells and did not grow at concentrations higher than 3 mM (Fig. 7A). In the galactose media, Δccc1 cells transformed with the FET4-containing plasmid were more sensitive to iron at a lower concentration while Δccc1 cells transformed with a control plasmid did not show the increased iron sensitivity (Fig. 7B).

**DISCUSSION**

In S. cerevisiae, high affinity iron transport is regulated at the level of transcription by Aft1p, which activates transcription of the iron-regulon in low iron conditions (23). Once transcribed, the activity of the high affinity iron transport system is not inhibited by high iron (24). In high iron media the expression of Fet3p/Ftr1p slowly declines, and cells can continue to accumulate iron even though they are iron replete. The inability to reduce high affinity iron transport when exposed to high concentrations of metal distinguishes the iron transport system from transport systems for Cu²⁺, Mn²⁺, and Zn²⁺ (1). The activity of these transport systems is regulated by the metal, which accelerates the degradation of the transporters. Because of continued iron uptake even in the face of high iron stores, yeast must find ways of accommodating iron. That yeast can store iron is demonstrated by the observation that cells can grow for generations in the absence of exogenous iron.

In this communication we presented direct biochemical data that show that iron can be stored in the vacuole. A previous concern with other studies that demonstrated vacuolar iron was the possibility that iron entered the vacuole through endocytic activity (3, 4). Endocytosis is a constitutive process, and exposure of cells to iron-rich media would lead to the accumulation of iron in vacuoles. To determine whether iron could enter the vacuole independently of endocytosis, we took advantage of yeast strains with a deletion in the CCC1 gene. While unable to internalize, has normal vacuoles. In the absence of endocytosis, however, Δend4 cells can accumulate iron in the vacuole, which can then be mobilized for cellular growth. These observations provide compelling evidence that the vacuole can function as an iron storage organelle.

Further evidence that supports the hypothesis that vacuoles can store iron is the observation that CCC1 can effect vacuolar iron accumulation. CCC1 was first identified as a suppressor of a mutant that was unable to grow in high Ca²⁺ (25). The mutant had a defect in the synthesis of a mannosyl-lipid. A subsequent study identified CCC1 as a suppressor of Mn²⁺ hypersensitivity resulting from a deletion in pnr1. Analysis of the effect of CCC1 suggested that it was a transporter that mediated the accumulation of Mn²⁺ into a membranous compartment. Subcellular fractionation and immunofluorescence suggested that an overexpressed hemagglutinin-tagged CCC1 was localized to the Golgi, and it was concluded that CCC1 was a Golgi Mn²⁺ transporter (22). Overexpressed CCC1 could not, however, suppress a glycosylation defect of Δpnr1 and provide Mn²⁺ to Golgi enzymes.

We identified CCC1 as a suppressor of a Δyfh1 strain (12). Δyfh1 strains can accumulate toxic levels of mitochondrial iron resulting in the selection of respiratory incompetent strains. Reduction in cytosolic iron can maintain the respiratory competence of Δyfh1 strains and permit them to grow on glycerol-ethanol even in high iron (26). We have discovered that iron accumulates within vacuoles in cells overexpressing CCC1. Iron sequestration within vacuoles would result in a low cytosolic iron level that precludes mitochondrial iron accumulation. Overexpression of CCC1 results in increased vacuolar iron accumulation in Δend4 cells, indicating that iron can enter the vacuole independently of endocytic activity. We have localized a FLAG-tagged CCC1 to the vacuolar membrane. Thus, our data are consistent in identifying the vacuole as a site of iron storage and CCC1 as a vacuolar iron transporter. Our studies also indicate that CCC1 can lead to Mn²⁺ accumulation in vacuoles. This result suggests that CCC1 may be a Fe²⁺/Mn²⁺ transporter consistent with the finding that most transporters that recognize Mn²⁺ also recognize Fe²⁺.

While there is one high affinity cell surface iron transporter there are also a number of low affinity iron transporters (1). There appear to be at least two transporters that can extract iron from the vacuole (FET5/FTH1, SMM3) (5, 6). Thus, each membrane system has redundant iron transporters. We do not know if there are other transporters capable of mediating Fe²⁺ or Mn²⁺ accumulation in vacuoles. An attempt to examine this possibility by measuring vacuolar iron content in a Δend4Δccc1 deletion strain has been unfruitful as the strain grows poorly. We have also not been able to identify mammalian homologues of CCC1. There are, however, homologues in both Arabidopsis thaliana and Orzya sativa, indicating that plants may also store iron within vacuoles.

**Acknowledgments**—We thank our colleagues in the Utah metal group for their help in editing this manuscript.

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