Identification of a Vacuole-associated Metalloreductase and Its Role in Ctr2-mediated Intracellular Copper Mobilization*

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Copper is an essential trace metal whose biological utility is derived from its ability to cycle between oxidized Cu(II) and reduced Cu(I). Ctr1 is a high affinity plasma membrane copper permease, conserved from yeast to humans, that mediates the physiological uptake of Cu(I) from the extracellular environment. In the baker’s yeast Saccharomyces cerevisiae, extracellular Cu(II) is reduced to Cu(I) via the action of the cell surface metalloreductase Fre1, similar to the human gp91phox subunit of the NADPH oxidase complex, which utilizes heme and flavins to catalyze electron transfer. The S. cerevisiae Ctr2 protein is structurally similar to Ctr1, localizes to the vacuolar membrane, and mobilizes vacuolar copper stores to the cytosol via a mechanism that is not well understood. Here we show that Ctr2-1, a mutant form of Ctr2 that mislocalizes to the plasma membrane, requires the Fre1 plasma membrane metalloreductase for Cu(I) import. The conserved methionine residues that are essential for Ctr1 function at the plasma membrane are also essential for Ctr2-1-mediated Cu(I) uptake. We demonstrate that Fre6, a member of the yeast Fre1 metalloreductase protein family, resides on the vacuolar membrane and functions in Ctr2-mediated vacuolar copper export, and cells lacking Fre6 phenocopy the Cu-deficient growth defect of ctr2Δ cells. Furthermore, both CTR2 and FRE6 mRNA levels are regulated by iron availability. Taken together these studies suggest that copper movement across intracellular membranes is mechanistically similar to that at the plasma membrane. This work provides a model for communication between the extracellular Cu(I) uptake and the intracellular Cu(I) mobilization machinery.

The trace element copper plays important roles in a range of physiological functions that include transcripational regulation, energy generation, angiogenesis, neuuropeptide maturation, oxidative stress protection, pigmentation, connective tissue biogenesis, and iron uptake and distribution (1–3). Although the ability of copper to alternate between its oxidized (Cu(II)) and reduced (Cu(I)) forms is critical for its function as a redox cofactor, this same property has the propensity to generate damaging intracellular free radicals (4). Consequently, orga-
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Second, the essential Ctr1 methionine ligands that are conserved in all Ctr1 family members exhibit a stronger preference for coordination to Cu(I) than Cu(II) (22). Third, the reducing agent ascorbate stimulates Ctr1-mediated copper uptake (26). Moreover, yeast Ctr1 requires the action of plasma membrane metallochaperones, encoded by the FRE1 and FRE2 genes, to efficiently transport extracellular copper across the plasma membrane (26, 27).

Previous studies suggest that, similar to Ctr1, the S. cerevisiae Ctr2 protein has three transmembrane domains, exists as a homomultimer, and resides in the vacuolar membrane where it serves to mobilize vacuolar copper stores to cytosolic copper chaperones (28, 29). Although Ctr2 requires the conserved methionine residues in the amino terminus and second transmembrane domain for copper mobilization, it has not been established whether Ctr2 transports Cu(I) and, if so, whether vacuolar copper mobilization involves a metallochaperone. Furthermore, given that Ctr1 and Ctr2 function to import copper and mobilize copper stores, respectively, the mechanisms by which these two copper acquisition pathways communicate are not understood. Here we report that a mutant of Ctr2, Ctr2-1, that is mislocalized to the plasma membrane and drives copper uptake, requires a cell surface metallochaperone to functionally replace Ctr1. In addition, the conserved methionine residues critical for copper import by Ctr1 are essential for Ctr2-1 function at the plasma membrane. We identify Fre6 as a vacuolar membrane-localized metallochaperone that functions in the Ctr2-mediated vacuolar copper mobilization pathway. Furthermore, we demonstrate that steady state CTR2 and FRE6 mRNA levels are increased in response to iron deficiency. These studies support strong mechanistic similarities for Cu(I) transport across intracellular membranes and the plasma membrane and provide a model for mechanisms through which cells signal a need for extracellular Cu(I) uptake versus Cu(I) mobilization from intracellular stores.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—S. cerevisiae deletion strains were constructed by integration of the His3MX6, TRP1, or KanMX6 cassette (30) into the desired locus of MPY17 (MATa ura3-52 his3Δ200 trp1-901 ctp1::ura3::Knr ctp3::TRP1), BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), or SEY6210 (MATα ura3-52 leu2-3,112 his3Δ200 trp1Δ901 lys2-801 suc2Δ9) yeast strains. Wild-type and mutant alleles were amplified from genomic DNA by PCR. Site-directed mutagenesis was performed by the overlap extension method (31). The FRE6 open reading frame was subcloned into the p416ADH and p416GPD yeast expression vectors, and the FRE6 open reading frame was subcloned into the p416GPD vector. For the FRE6-3HA, FRE6-GFP, and FRE7-GFP fusions, a Ndel restriction enzyme site was introduced in-frame immediately upstream of the FRE6 stop codon in the p416ADH vector or the FRE7 stop codon, and the triple hemagglutinin (3HA)3 or green fluorescent protein (GFP) open reading frame was inserted. The integrity of the fusion between FRE6 or FRE7 and the epitope tags was verified by DNA sequencing. Yeast strains were transformed with plasmids using standard techniques and grown in synthetic complete (SC) selective media at 30 °C.

Functional Complementation, Growth, and Enzyme Assays—S. cerevisiae strains transformed with specific plasmids were grown in selective media to exponential phase at 30 °C with agitation. 10-Fold serial dilutions were spotted onto selective glucose media, ethanol (2%) and glycerol (3%) media (YPG), and yeast supplemented with copper sulfate (CuSO4) or ferrous ammonium sulfate containing 1.5% agar and incubated at 30 °C for 3–6 days. SC media was supplemented with 200 or 500 μM EDTA or 200 μM EDTA in addition to CuSO4 (10 μM), ZnCl2 (100 μM), ascorbate (10 μM), or the Cu(I) chelator bathocuproine disulfonic acid (100 μM) for spot assays.

For reductase assays, the fre1Δfre2Δ strain was transformed with an empty vector, p416GPD-FRE6, or p416GPD-FRE7. Cells were grown in selective media at 30 °C to exponential phase, and reductase assays were performed (32). Cells were pelleted by centrifugation and resuspended in reductase assay buffer (50 mM citrate, pH 6.6, and 5% (w/v) glucose). Optical density (A600) of the cells resuspended in reductase assay buffer was measured for cell number normalization. Assays were initiated by the addition of 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide (MTT) to a final concentration of 0.5 μg/ml, incubating for 1 h at 30 °C in the dark. The formazan crystals formed by the reduction of MTT were dissolved in acidified (0.04 M HCl) isopropanol, and the absorbance of the supernatant at 595 nm was measured.

Fluorescence and Indirect Immunofluorescence Microscopy—The fre6Δ strain was transformed with p416ADH-FRE6-GFP and grown in selective media at 30 °C to exponential phase. FM4–64 (Molecular Probes) was added to a final concentration of 20 μM, and cells were incubated at 30 °C for 15 min. Cells were then collected, resuspended in fresh media, and incubated for an additional 45 min before visualization. For localization of FRE7-GFP, the rsp5-1 temperature-sensitive mutant strain and corresponding isogenic wild-type strain were transformed with plasmids expressing FRE6-GFP and FRE7-GFP and grown in selective media at 30 °C to exponential phase. Two hours before visualizing cells, each culture was split, with half incubating at 30 °C and the other half incubating at the nonpermissive temperature of 37 °C. All cells were visualized with a Zeiss Axioskop upright wide field fluorescence microscope equipped with a filter wheel. Indirect immunofluorescence was performed as previously described (33). Polyclonal antibody against the amino terminus of Ctr2 was generated by Bethyl Laboratories, Inc. from the peptide sequence EGNAGHDHSDMHMGDGD-DTC (amino acids 38–57) and affinity-purified. Affinity-purified antibody against the intracellular loop region of Ctr2 (peptide sequence CVHKRQLSQRVLLPNRSLTK) was also generated and purified by Bethyl Laboratories, Inc.

Vacuole Purification and Immunoblotting—Vacuoles were isolated by Ficoll gradient fractionation as previously described (29, 34). Whole cell extracts made by the Triton X-100/glass bead mechanical extraction method (9), or samples from the vacuole isolation were separated by 10% SDS-PAGE, trans-

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3 The abbreviations used are: 3HA, triple hemagglutinin; GFP, green fluorescent protein; SC, synthetic complete; GPD, glyceraldehyde-3-phosphate dehydrogenase; MTT, 2-(4,5-dimethyl-2-thiazoly)l-3,5-diphenyl-2H-tetrazolium bromide; YPEG, ethanol-glycerol media.
ferred to nitrocellulose membranes, and probed with α-Ctr2, α-HA (Berkeley Antibody Co., Inc.), α-Pma1 (Santa Cruz Biotechnology), α-3-phosphoglycerate kinase, α-alkaline phosphatase, or α-carboxypeptidase Y antibodies (all from Molecular Probes).

**RNA Blotting**—Cells from isogenic wild-type (CM3260), aft1Δ, aft2Δ, and aft1aft2Δ strains (35, 36) were grown in SC containing 100 μM ferrous ammonium sulfate or 100 μM iron(II) chelator bathophenanthroline disulfonic acid to exponential phase. Total yeast RNA was isolated with a modified hot phenol method (37). PCR-amplified DNA fragments were gel purified and radiolabeled with [32P]dCTP and used as hybridization probes. A probe to detect actin (ACT1) mRNA was used as a gel loading control.

**RESULTS**

**Conserved Methionine Residues Are Essential for Ctr2-1 Function**—Although *S. cerevisiae* Ctr1 and Ctr2 are integral membrane proteins involved in copper transport, they share less than 25% amino acid sequence identity overall, with highest similarity occurring in the three transmembrane regions. However, both proteins possess a methionine residue ~20 amino acids upstream of the first transmembrane domain and a MX₃M motif in the second transmembrane domain that are essential for normal copper transport function (22, 29). In previous work we described a mutant allele of *CTR2*, *CTR2-1*, that expresses a protein which partially mislocalizes to the plasma membrane and stimulates extracellular copper uptake and accumulation (29). The Ctr2-1 protein contains a substitution of arginine for tryptophan within the amino terminus and a 16-amino acid truncation at the carboxyl terminus. However, the integrity of the methionine residues, conserved in all Ctr family members identified to date, is maintained in Ctr2-1. We used the Ctr2-1 allele to further test whether Ctr2 may transport copper in a manner mechanistically similar to Ctr1. A *ctr1Δctr3Δ* yeast strain cannot grow on a non-fermentable carbon source such as ethanol and glycerol (YPEG) due to a defective mitochondrial respiratory chain that results from the inability of cytochrome c oxidase to obtain its copper cofactor (22). The results shown in Fig. 1A demonstrate that whereas expression of the Ctr2-1 protein can suppress the growth defect of *ctr1Δctr3Δ* cells on YPEG, growth on YPEG is abolished by mutation of the amino-terminal methionine residues (M59A,M61A) or mutation of both methionine residues in the second transmembrane MX₃M (M148L,M152L) motif. The addition of copper to the media restored growth of all strains, indicating that the growth defect on YPEG is due to insufficient intracellular copper levels and not a secondary mutation that inactivates mitochondrial oxidative phosphorylation. Indirect immunofluorescence microscopy of cells expressing the methionine mutants of Ctr2-1 (M59A,M61A and M148L,M152L) with a polyclonal antibody directed against a region of Ctr2 that is conserved between the wild-type and mutant proteins shows that mutation of the methionine residues does not alter localization of the Ctr2-1 derivatives to the plasma membrane (Fig. 1B). Furthermore, immunoblot analysis of whole cell extracts demonstrates that Ctr2-1 and the Ctr2-1 methionine mutant proteins accumulate to similar steady state levels (Fig. 1C).

**CTR1 and FRE1 gene expression are coordinately activated in response copper deficiency by the Mac1 transcription factor** (38–41). For Ctr1 to import Cu(I) from the oxidizing extracellular environment that favors the existence of Cu(II), a cell surface flavocytochrome metalloreductase, encoded by the FRE1 gene, has been demonstrated to be required in vivo (26). Furthermore, both *CTR1* and *FRE1* gene expression are coordinately activated in response copper deficiency by the Mac1 transcription factor (38–41).

As a secretory pathway compartment derived from the endoplasmic reticulum, the interior of the yeast vacuole is likely an oxidizing environment, which would be predicted to favor the accumulation of Cu(II). Our previous studies demonstrated that Ctr2-1 stimulates extracellular ⁶⁴Cu uptake and intracellular copper accumulation (29). Given that the integrity of the conserved methionine residues is critical for Ctr2-1 to suppress the *ctr1Δctr3Δ* growth phenotype on YPEG, suggesting that...
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Like Ctr1 Ctr2-1 may transport Cu(I), we tested whether Ctr2-1 may also require cell surface metalloreductase activity to substitute for Ctr1 in copper import. As shown in Fig. 2, left panel, deletion of the FRE1 gene precludes Ctr2-1 from suppressing the loss of the Ctr1 plasma membrane copper importer for growth on YPEG. Interestingly, fre2Δ has no effect on Ctr2-1 suppression under these conditions, and loss of both the Fre1 and Fre2 plasma membrane metalloreductases phenocopies the fre1Δ phenotype. Supplementation of YPEG medium with copper or the inoculation of all strains on glucose medium (Fig. 2, center and right panels, respectively) allowed growth for all strains tested. Because Ctr2-1 expression results in only partial mislocalization of the protein to the plasma membrane, with some protein remaining on the vacuole membrane, one possible explanation for suppression of the ctr1Δctr3Δ growth defect on ethanol-glycerol medium is increased mobilization of copper from the vacuole by the Ctr2-1 protein. This is unlikely, given that overexpression of a wild-type Ctr2 protein from a plasmid under the control of the strong glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter shows complete vacuolar localization and does not permit growth on YPEG (29). Taken together, these data suggest that Ctr2-1 suppression of the Cu(I) uptake defect associated with ctr1Δctr3Δ strains requires a cell surface metalloreductase, and this is consistent with the plasma membrane pool of Ctr2-1 protein rather than that localized to the vacuole, being responsible for suppression of the ctr1Δctr3Δ phenotype.

Fre6 Metalloreductase Is Localized to the Vacuole—Our studies reported here support the hypothesis that Ctr2-1 transports Cu(I) across the plasma membrane and suggests the possibility that Ctr2 may mobilize vacuolar luminal copper stores as Cu(I). To date, the valence state and coordinating ligands of vacuolar copper stores have not been elucidated. However, if vacuolar copper is stored as Cu(II) and is mobilized to the cytosol as Cu(I), in a manner similar to Cu(I) import by Ctr1 and Ctr2-1, this would suggest the involvement of a metalloreductase to function in concert with Ctr2. In S. cerevisiae, at least six additional putative metalloreductases have been identified based on their sequence homology to Fre1 and Fre2 (42, 43). We identified two Fre family members as candidate metalloreductases that could function in Ctr2-mediated copper mobilization from the lumen of the vacuole. One, designated Fre6, has been preliminarily localized to the vacuole in a genome-wide localization study of GFP fusion proteins (44). The FRE6 gene has been shown to be transcriptionally activated by iron deficiency and the Aft1/2 iron-sensing transcription factors (42, 43). The other candidate vacuolar metalloreductase, designated Fre7, lacks published subcellular localization information but is encoded by the FRE7 gene that is transcriptionally activated by copper limitation via the Mac1 copper-responsive transcription factor (39, 42, 43). Fre7 is the only gene product of the Fre family identified to date that is up-regulated exclusively by copper limitation and not by iron deficiency. Given the Mac1-dependent regulation of the CTR1 and CTR3 copper importer genes and the FRE1 metalloreductase gene, all of which are involved in copper movement across membranes, the regulation of FRE7 by Mac1 strongly implicates a role for FRE7 in copper homeostasis.

To identify whether either or both of these putative metalloreductases are involved in vacuolar copper homeostasis, we first carried out experiments to localize the Fre6 and Fre7 proteins via a combination of fluorescence microscopy and measurement of cell surface reductase activity. In-frame translational fusions were constructed between the carboxyl termini of the FRE6 or FRE7 coding sequences and the GFP open reading frame. Interestingly, the Fre7-GFP fusion protein localized to both the plasma membrane and a perinuclear compartment consistent with the endoplasmic reticulum (Fig. 3A) as determined by 4,6-diamidino-2-phenylindole staining (data not shown). Because the secretion of some plasma membrane protein–GFP fusions that are partially trapped in the endoplasmic reticulum can be facilitated in mutants defective in the Rsp5 ubiquitin ligase (45), we also localized Fre7-GFP in an rsp5Δ temperature-sensitive mutant both at the permissive (30 °C) and restrictive (37 °C) temperatures. Although Fre7-GFP localized to both the endoplasmic reticulum and plasma membrane at 30 °C, it was found almost exclusively at the plasma membrane at 37 °C (Fig. 3A). These observations as well as additional data described herein suggest that the Fre7 protein is likely not a vacuolar membrane protein and may instead be localized to the plasma membrane. In contrast to Fre7, the Fre6-GFP fusion protein co-localized to the vacuolar membrane with the lipophilic dye FM4–64, a known vacuolar marker, with no detectable localization to the plasma membrane (Fig. 3B). Moreover, consistent with vacuolar residency, Fre6-GFP localization was not altered in the rsp5Δ mutant at the permissive or restrictive temperature (data not shown).

The localization of Fre6-GFP to the vacuole suggests that Fre6 could function together with Ctr2 in the mobilization of vacuolar copper stores. To begin to understand Fre6 function, isogenic ctr1Δctr3Δctr2Δ and ctr1Δctr3Δfre6Δ strains were constructed and compared for their growth on YPEG medium supplemented with copper. As shown in Fig. 3C, whereas ctr1Δctr3Δctr2Δ cells are unable to grow on YPEG without added copper or with 15 μM copper, transformation of this strain with a plasmid carrying wild-type CTR2 allowed growth on medium containing 15 μM added copper. Furthermore, the ctr1Δctr3Δfre6Δ strain phenocopied the ctr1Δctr3Δctr2Δ strain in this assay (Fig. 3C), and growth on YPEG containing 15 μM copper was restored via transformation with a plasmid expressing either wild-type FRE6, the FRE6–GFP fusion gene used for fluorescence microscopy (Fig. 3A), or a fusion gene encoding Fre6 fused to three
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FIGURE 4. Fre6 co-purifies with yeast vacuoles. A, a ctr2Δfre6Δ strain transformed with pRS416 and pRS414, or p416ADH-FRE6-3HA and pRS414, or p416ADH-FRE6-3HA and p414GPD-CTR2 was spheroplasted, permeabilized, and fractionated on a discontinuous Ficoll density gradient. Samples from each interface of the gradients (0/4, 4/8, and 8/15% Ficoll) were collected, separated by SDS-PAGE, and transferred to nitrocellulose membrane. Fre6-3HA was detected by immunoblotting with α-HA antibody, and Ctr2 was detected by α-Ctr2 antibody. Antibodies against alkaline phosphatase (ALP) and carboxypeptidase Y (CPY) were used as vacuole markers, and 3-phosphoglycerate kinase (Pgk1) was used as a cytosolic control. The black circles correspond to the expected size of the monomer (●), dimer (●●), and trimer (●●●) of Ctr2. B, vacuoles were purified as described in A from a fre6Δ strain expressing empty vector or p416ADH-FRE6-3HA. Pma1 is a plasma membrane marker.

For metal transporters that function as heteromultimer protein complexes, such as the S. cerevisiae Ftr1/Fet3 iron permease-ferroxidase complex (46, 47) and the Schizosaccharomyces pombe high-affinity plasma membrane copper transporter complex Ctr4/Ctr5 (13), expression of each gene product is critical for function and trafficking of both components of the complex (13, 47–49). However, as shown in Fig. 4A, Fre6-3HA co-fractionates with vacuolar markers in the absence of Ctr2, and in a fre6Δ strain Ctr2 remained in the vacuole fraction (Fig. 4B), suggesting that whereas both Ctr2 and Fre6 reside on the vacuole membrane, their localization is not interdependent. Note that the three different sizes of proteins detected by the anti-Ctr2 antibody in Fig. 4A correspond to the expected sizes of the monomeric, dimeric, and trimeric multimerization states of Ctr2. In Fig. 4B, where we are detecting endogenous Ctr2 and not expressing Ctr2 constitutively from a plasmid, the predom-

copies of the hemagglutinin epitope (FRE6-3HA). Given that both the Fre6-GFP and Fre6-3HA proteins complement the growth phenotype of fre6Δ cells (Fig. 3C), these alleles encode functional Fre6 fusion proteins.

To independently assess the vacuolar localization of Fre6 observed by fluorescence microscopy of cells expressing Fre6-GFP, yeast vacuoles were isolated from ctr2Δfre6Δ cells harboring an empty vector or a vector expressing the Fre6-3HA protein alone or co-expressing the Ctr2 and Fre6-3HA proteins. Immunoblotting analysis of fractions enriched for vacuoles (0/4% Ficoll), plasma membrane (4/8% Ficoll), and cytosol (8/15% Ficoll) shows that Fre6-3HA co-fractionates with Ctr2 and resident vacuolar proteins (ALP and CPY) (Fig. 4A), supporting the vacuolar localization observed by fluorescence microscopy in Fig. 3B.
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FIGURE 5. **FRE7, but not FRE6, expression stimulates extracellular reductase activity.** A, fre1Δfre2Δ cells expressing vector, GPD-FRE6, or GPD-FRE7 were grown to exponential phase, harvested, and resuspended in reductase assay buffer, and cell surface reductase activity was measured using the tetrazolium salt MTT as substrate. Data represent the mean and S.D. for three experiments. B, serial dilutions of the same cells as in A were spotted onto selective glucose media (Sc-ura) with or without EDTA. C, serial dilutions of wild-type (FRE1FRE2) cells and isogenic fre1Δ (fre1ΔFRE2), fre2Δ (FRE1fre2Δ), and fre1Δfre2Δ (fre1Δfre2Δ) cells were spotted onto Sc media, Sc media containing 200 μM EDTA, and Sc media containing 200 μM EDTA with 10 μM copper, 100 μM zinc, 10 μM ascorbate (Asc), or 100 μM bathocuproine disulfonic acid (BCS).
Because copper is required for high affinity iron uptake into *S. cerevisiae* through the plasma membrane Fet3 copper-dependent ferroxidase working in concert with the Ftr1 iron permease, a secondary consequence of copper deficiency is an intracellular iron deprivation (46, 47). To distinguish whether the respiratory growth defects observed in both fre6Δ and ctr2Δ mutants is due primarily to a cellular copper deficiency or iron deficiency, ctr2Δ cells harboring an empty vector or plasmid-borne *CTR2* and fre6Δ cells harboring an empty vector or plasmid-borne *FRE6* were plated on YPEG media supplemented with increasing iron concentrations up to 100 μM iron (added as ferrous ammonium sulfate (Fig. 6B) or ferric chloride (data not shown)). Whereas the addition of 100 μM copper significantly enhanced the growth of all strains on YPEG, iron supplementation did not (Fig. 6B). Taken together, these data are consistent with a role for Fre6 in the Ctr2-mediated copper mobilization required for growth on respiratory carbon sources under conditions of copper limitation.

**FRE6 FAD Binding Site Is Essential for Function**—The Fre proteins utilize NADPH and flavin adenine dinucleotide (FAD) to facilitate an electron transfer process. All of the Fre proteins identified to date possess in their carboxyl termini the HPFTXXS sequence or a closely related sequence that is predicted to be an FAD binding motif as well as at least one glycine-rich or glycine-cysteine motif involved in NADPH binding (43). Binding of these cofactors to the protein is important for metalloreductase function; mutations in these motifs should affect the ability of Fre proteins to reduce Fe(III) and Cu(II) (54).

To determine whether the FAD binding motif present in the Fre6 amino acid sequence is essential for the ability of Fre6 to function in Ctr2-mediated vacuolar copper mobilization, we tested whether a Fre6 FAD binding mutant is able to complement the fre6Δ respiratory defect on copper-supplemented YPEG (Fig. 7A). Mutation of the HPFT sequence (amino acids 493–496) in Fre6 to alanine residues disrupts the putative FAD binding motif and results in an inability for Fre6 to complement the phenotype (Fre6(FADmut)) like wild-type plasmid-borne Fre6 (Fig. 7A). Epitope-tagging of this mutant with GFP (Fre6-GFP(FADmut)) showed a similar loss of function for Fre6 (Fig. 7A) and enabled visualization of the subcellular localization of this mutant protein by fluorescence microscopy (Fig. 7B). Both the mutant and wild-type GFP-tagged forms of Fre6 are readily detectable by immunoblotting with an anti-GFP antibody (data not shown). These data support the importance of FAD binding to Fre6 for its ability to function in Ctr2-mediated vacuolar copper mobilization and suggest that Fre6 functions as a vacuolar metalloreductase.

**CTR mRNA Levels Are Regulated by Iron Availability**—Under conditions of copper deficiency, *S. cerevisiae* cells increase the expression of genes involved in Cu(I) import, such as *FRE1*, *CTR1*, and *CTR3*, through the Mac1 copper-responsive transcription factor and the cis-acting copper responsive element, 5′-TTTGC(T/G)C(A/G)-3′ (38–41). The promoter region of *CTR2* does not contain a Mac1 binding site, and no evidence exists for increased *CTR2* steady state mRNA levels in response to copper deficiency. Interestingly, expression of *CCC2*, the P-type ATPase responsible for transporting copper from the cytosol to the secretory pathway (55, 56), and *ATX1*,
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The gene for the chaperone that delivers copper to Ccc2 (57), is controlled by the iron-responsive transcription factors Aft1 and Aft2 (57, 58). Because Ccc2 and Atx1 deliver copper to the copper-dependent iron uptake machinery in the secretory compartment, their expression is governed by iron availability.

The link between copper and iron availability and the approximate 2.5-fold increase in CTR2 mRNA levels upon deletion of the yeast frataxin homolog YFH1 as ascertained by DNA microarray analysis (59, 60) led us to investigate whether CTR2 mRNA levels are regulated by iron availability. Isogenic wild-type, aft1Δ, aft2Δ, and aft1Δaft2Δ cells (35, 36) were grown in the presence of adequate iron (ferrous ammonium sulfate) or the iron chelator bathophenanthroline disulfonic acid (BPA) for 6 h, total RNA was extracted, and the steady state levels of CTR2, ATX1, FET3, and ACT1 mRNAs were assayed by RNA blotting (Fig. 8). In wild-type cells, CTR2 steady state mRNA levels increase in cells grown in bathophenanthroline disulfonic acid compared with cells grown in the presence of iron. This elevation is evident in both the aft1Δ and aft2Δ mutants. Furthermore, in the aft1Δaft2Δ double mutant, CTR2 mRNA levels are increased compared with wild-type cells, even in the presence of added iron (Fig. 8). Although it is clear that CTR2 mRNA levels increase upon iron deprivation in wild-type cells, the AFT1/2-independent mechanism by which this occurs is unclear. However, given that both CTR2 and FRE6 mRNA levels are elevated in response to iron deficiency, this would provide a useful mechanism for increasing the abundance of these functionally related proteins.

**DISCUSSION**

In an environment where essential trace elements are limiting and, specifically in the case of copper and iron, not readily bioavailable, organisms must rely on transport mechanisms with high affinity and specificity to mobilize these elements across membranes into the intracellular environment where they are incorporated as enzymatic cofactors, enter a regulatory pool, or are sequestered in storage molecules or compartments. One mechanism whereby cells increase the bioavailability of copper or iron is through reduction of the insoluble, oxidized forms of these metals to their reduced valence states of Fe(II) and Cu(I). In *S. cerevisiae* this extracellular metal reduction occurs through the action of at least two plasma membrane metalloreducers, Fre1 and Fre2 (26, 27, 50–52). Fre1 and Fre2 possess preserved FAD and NADPH binding motifs and are homologous to the gp91phox subunit of the human phagocyte NADPH oxidase (43, 54) as well as a family of recently identified mammalian metalloreducers (61).

Although both Fre1 and Fre2 have been shown to have a role in Cu(I) uptake mediated by the high affinity plasma membrane Cu(I) transport proteins Ctr1 and Ctr3 and Fe(II) uptake by the yeast Ftr1/Fet3 complex, Fre1 is the major cell surface reductase involved in Cu(I) acquisition (26, 27). This may be due to the elevated expression of FRE1, but not FRE2, in response to copper limitation, which would render Fre1 the dominant cell surface reductase activity under these conditions (39, 40, 42, 43, 52). Consistent with these observations, deletion of FRE1, but not FRE2 or FRE6 (data not shown), abrogated the ability of the cell surface-localized Ctr1-2 protein to suppress the crr1Δctr3Δ growth phenotype under conditions of copper limitation on YPEG. Given these results with Ctr2-1 and other similarities between the Ctr1 and Ctr2 proteins including similar membrane topology, multimerization, and the conservation of methionine residues essential for function, these results suggest that both Ctr1 and Ctr2 may transport Cu(I) across distinct membranes via the action of a metalloreductase.

We have localized Fre6-GFP and Fre6-3HA proteins to the vacuolar membrane by fluorescence microscopy and via copurification with vacuoles. Data presented here support a physiological role for Fre6 in Ctr2-mediated Cu(I) mobilization from the vacuole. Although Fre6 and Fre7 were identified based on their sequence similarity to FRE1 and FRE2, Fre6- or Fre7-associated metalloreductase activity has not been directly demonstrated or correlated with their expression. Here we show that overexpression of FRE7, but not FRE6, in the absence of the Fre1/2 cell surface reductases results in an ~10-fold increase in cell surface metalloreductase activity and suppression of the EDTA-mediated growth phenotype. Furthermore, consistent with their distinct subcellular localizations, inactivation of FRE6, but not FRE7, phenocopies crr2Δ growth defects on YPEG. This growth phenotype on YPEG is attributable to a decreased ability to mobilize copper for its incorporation into cytochrome c oxidase but may also partly result from a loss of metallated Cu,Zn-superoxide dismutase needed to disproportionate the superoxide produced during mitochondrial respiration. Previous studies in *S. cerevisiae* and *Schizosaccharomyces pombe* have demonstrated that disrupting the function of vacuolar copper mobilization results in a decrease in Sod1 function (28, 62). In support of a role for Fre6 in vacuolar Cu(I) mobilization in a pathway that also involves Ctr2, we demonstrated that fre6Δ mutants exhibit a copper, but not an iron, remedial phenotype on YPEG. Whereas we have not been able to demonstrate Fre6-dependent metalloreductase activity with purified vacuoles or with vacuolar and cytosolic fractions mixed *in vitro*, mutagenesis experiments demonstrated that the Fre6 protein requires the structural integrity of the FAD binding site for functional complementation of the fre6Δ YPEG phenotype. Co-factors such as FAD and NADPH have been shown to be important for the function of this family of metalloreducers.
(54), and these results suggest that Fre6 functions as a metalloreductase.

Although this work provides new information on the identification of Fre6 as a vacuolar metalloreductase and its role in copper homeostasis, many questions remain. The importance of the Fre6 vacuolar metalloreductase suggests that copper is stored in the vacuolar lumen as Cu(II). However, this is not currently well understood nor is the chemical nature of protein or small molecule ligands that may coordinate to vacuolar luminal copper known. Recently, a low molecular weight copper chelate complex has been identified in the cytosol and mitochondria of yeast and mammalian cells (63), and it would be interesting if this same complex is present in the vacuolar lumen. It is also not clear how copper may be imported into the vacuole nor whether a known Cu(I) chaperone functions in the vacuolar copper delivery pathway. However, dedicated yeast vacuole membrane transporters exist and have been identified for both iron and zinc (64–70).

The studies reported here describe a functional interaction between Ctr2 and Fre6 in vacuolar Cu(I) mobilization. Currently, it is not clear whether a physical interaction exists between Ctr2 and Fre6 or even between Ctr1 and Fre1 on the plasma membrane. Spatial proximity could facilitate the transfer of Cu(I) to the site of transport without it being reoxidized. If there is a direct physical interaction between Fre6 and Ctr2, they are not interdependent for proper localization given that deletion of one still results in a vacuolar localization of the other. This is in contrast to what has been observed for the interdependence for proper localization and function of the Fet3/Ftr1 Fe(II) uptake complex at the plasma membrane in S. cerevisiae or the Ctr4/5 cell surface Cu(I) transport complex in S. pombe (13, 47–49).

Interestingly, although Ctr2 mediates copper mobilization from the vacuolar lumen and functions to facilitate growth in response to an external copper deficiency, CTR2 mRNA levels are elevated in response to iron deprivation rather than copper deficiency. Furthermore, consistent with its proposed role with Ctr2, FRE6 expression has been shown to be elevated in response to iron deficiency in a manner dependent on the Aft1/2 iron-responsive transcription factors (42, 43). Why would Ctr2, a copper transporter, function with an Aft1/2-regulated metalloreductase? In S. cerevisiae the expression of the copper chaperone ATX1 and the copper-transporting P-type ATPase CCC2, which are needed to load newly synthesized Fet3 with copper, is up-regulated by Aft1/2 when intracellular iron becomes limiting (57, 58). Microarray results of mRNA expression in a frataxin (yfh1Δ) mutant, a condition that simulates cytosolic iron deficiency due to increased sequestration of iron in the mitochondria, indicate that CTR2 mRNA is elevated under these conditions (59). Here we present evidence that suggests CTR2 mRNA levels, like ATX1 and CCC2, are increased upon iron deprivation. This regulation may serve as a way for the plasma membrane and vacuole to coordinate copper uptake with copper mobilization. For example, copper deficiency leads to the activation of FRE1 and CTR1 expression via Mac1 to increase copper uptake (40). However, if an extracellular copper deprivation persists, a secondary iron deficiency results. In response to this iron deficiency, yeast cells may elevate expression of the vacuolar Cu(I) export machinery to mobilize copper stores into the cytosol. Because CTR2 expression does not appear to be controlled directly by Aft1/2, future studies will investigate the mechanism of this iron regulation. Recent evidence from Guisbert et al. (71) suggests the potential for post-transcriptional regulation of CTR2 by the RNA-binding protein Nab4, which is involved in 3′ pre-mRNA processing. Whether Nab4-dependent post-transcriptional regulation or another mechanism of post-transcriptional or post-translational regulation is responsible for CTR2 induction under iron deficiency remains to be investigated.

Importantly, Ohgami et al. (61) have recently identified at least four putative metalloreductases in mammals. The family of proteins responsible for this reductase activity is the six-transmembrane epithelial antigen of the prostate (Steap) proteins (72). Like the characterized Fre proteins in yeast, three of these mammalian proteins, Steap2, Steap3, and Steap4, possess both ferric and cupric reductase activity (61). Steap3 is expressed highly in erythroid cells where it is involved in the reduction of transferrin-bound iron in endosomal compartments. Transfection of epitope-tagged Steap2 and Steap4 in HEK-293T cells shows some plasma membrane localization but also partial co-localization with transferrin and the transferrin receptor 1 in endosomes (61). It is possible that at least one Steap protein may be an intracellular metalloreductase functioning similarly to Fre6 in reducing Cu(II) before transport across intracellular membranes. Although mammalian cells express a protein homologous to S. cerevisiae Ctr2, it has not yet been well characterized. Co-localization studies of Ctr1, Ctr2, and the Steap proteins may provide evidence for a functional interaction in copper trafficking. Our work in yeast provides a working model for further investigations into mammalian Ctr2 and intracellular metal sequestration, reduction, and mobilization in metazoans.

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REFERENCES


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