Characterization of the *Saccharomyces cerevisiae* High Affinity Copper Transporter Ctr3*

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Copper is an essential nutrient required for the activity of a number of enzymes with diverse biological roles. In the bakers’ yeast *Saccharomyces cerevisiae*, copper is transported into cells by two high affinity copper transport proteins, Ctr1 and Ctr3. Although Ctr1 and Ctr3 are functionally redundant, they bear little homology at the amino acid sequence level. In this report, we characterize Ctr3 with respect to its localization, assembly, and post-transcriptional regulation. Ctr3 is an integral membrane protein that assembles as a trimer to form a competent copper uptake permease at the plasma membrane. Whereas the *CTR1* and *CTR3* genes are similarly regulated at the transcriptional level in response to copper, post-transcriptional regulation of these proteins is distinct. Unlike Ctr1, the Ctr3 transporter is neither regulated at the level of protein degradation nor endocytosis as a function of elevated copper levels. Our studies suggest that Ctr3 constitutes a fundamental module found in all eukaryotic high affinity copper transporters to date, which is sufficient for copper uptake but lacks elements for post-transcriptional regulation by copper.

Copper is an essential redox active metal that serves as a cofactor in a variety of enzymes such as cytochrome oxidase, Cu,Zn superoxide dismutase, ceruloplasmin, and l-lysyl oxidase (1). When allowed to accumulate in excess, copper is toxic due to its proclivity to participate in Fenton-like reactions that lead to the generation of highly reactive hydroxyl radicals (2). Consequently, organisms have developed sophisticated mechanisms for maintaining the balance between essential and toxic copper levels. Studies of copper uptake in *S. cerevisiae* have shown that copper is reduced from Cu(II) to Cu(I) by cell surface metallothioneins (3, 4) and transported by two high affinity copper transport proteins, Ctr1 and Ctr3 (5, 6). Within cells copper is distributed to specific subcellular compartments or proteins by copper chaperones that include Atx1 which delivers copper to the Fet3 high affinity iron transport subunit in the secretory compartment (7, 8), Cox17, which is required for delivery to mitochondrial cytochrome oxidase (9), and CCS, which inserts copper into Cu,Zn superoxide dismutase (10). Although inactivation of the yeast copper chaperone genes generates phenotypes specific for each copper delivery pathway, mutations in the both the *CTR1* and *CTR3* genes result in yeast cells that exhibit phenotypic defects associated with mutations in all three of the copper chaperone genes (5, 6).

In addition, these cells are defective in high affinity copper uptake resulting in poor growth on low copper media and defective in their ability to activate transcription of the *CUP1*-encoded metallothionein, except at copper concentrations beyond the high affinity range, due to an inability to provide copper to the metal regulatory transcription factor Ace1 (5, 6).

Because copper uptake at the cell membrane is a crucial step in copper acquisition, yeast genes encoding the high affinity copper transport proteins are tightly regulated in response to copper levels to ensure that sufficient copper is present for cellular needs. In *S. cerevisiae* transcription of the *CTR1* and *CTR3* genes is activated during copper starvation and repressed under conditions of copper adequacy (11–13). At the post-transcriptional level Ctr1 protein has been shown to be regulated by two distinct processes (14). At low copper concentrations (0.1–1 μM) Ctr1 undergoes copper-induced endocytosis, although the role of Ctr1 internalization in copper uptake is currently unclear. Furthermore, exposure of yeast cells to high copper concentrations (≥10 μM) triggers the degradation of Ctr1 at the plasma membrane in a mechanism that is independent of endocytosis and vacuolar proteases (14).

The Ctr1 and Ctr3 high affinity copper transport proteins are functionally redundant. However, there is little homology between their amino acid sequences, and they are structurally distinct (15). Although both proteins possess three potential membrane spanning domains, Ctr1 is a 406-amino acid protein that is highly glycosylated and harbors eight repeats of the potential metal-binding motif MX6XM (Mets domain) in the predicted amino-terminal extracellular domain (5). These motifs are repeated twice in the putative human and mouse copper transport proteins, hCtr1 and mCtr1, and five times in the fission yeast *Schizosaccharomyces pombe* copper transport protein Ctr4 (15). Ctr3 lacks this motif but has an abundance of cysteine residues throughout the protein (11 Cys of 241 total residues) and within its putative transmembrane domains. Since both methionine and cysteine are potential copper-binding ligands (16), these residues may be important for copper uptake. Alignment of the sequences of Ctr1 and Ctr3 with the *S. pombe* Ctr4, human and mouse Ctr1, and putative copper transport proteins in existing data bases from *Caenorhabditis elegans* and *Drosophila melanogaster* reveal the following observations. First, the copper transport family of proteins is defined by the presence of three transmembrane domains. Second, with the exception of *S. cerevisiae* Ctr1, the transmembrane spanning regions exhibit high similarity to the Ctr3 transmembrane domains with several highly conserved residues present in all sequences. Third, the predicted ectodomains of the known copper transporters, and some of the putative copper transporters, are similar to Ctr1 with respect to the
presence of the MX/MXM motif that is predicted to bind copper. The conservation of these domains between yeast and mammalian systems suggests an important functional role in copper binding and transport. Taken together, it appears possible that the fusion of *S. cerevisiae* Ctr1 and Ctr3-like proteins may be an early event that led to the evolution of high-affinity copper transporter proteins of other eukaryotes (15, 17).

Since most eukaryotic high-affinity copper transporters identified to date have strong homology to Ctr3, it is important to understand the structure, function, and regulation of this protein. Here we show that Ctr3 exists as a trimer at the plasma membrane to form the competent copper uptake permease. Of the 11 cysteine residues distributed throughout Ctr3, only mutations in four of these amino acid residues affect Ctr3 function and localization. These mutants can assemble as a trimer, as assessed by *in vitro* cross-linking experiments; however, the mutant protein complexes fail to localize at the plasma membrane suggesting that assembly of the competent permease takes place in the secretory pathway. At the post-transcriptional level, Ctr3 is distinctly regulated compared with Ctr1 in response to copper.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—The yeast strains used in this study are listed in Table I. Strains were maintained in YPD medium (1% yeast extract, 2% BactoPeptone, 2% dextrose) or in the corresponding drop-out media for the maintenance of yeast strains transformed with plasmids.

**Plasmids**—The plasmids pRS316-CTR3 and pRS316-CTR3-NotI carry the CTR3 open reading frame on a centromeric plasmid, with or without a NotI restriction site before the stop codon, and the CTR3 promoter region up to ~349 from the start codon for translation and the 3’ region up to +328 from the stop codon, cloned into the Xhol and SalI sites. The plasmids pRS426-CTR3 and pRS426-CTR3-NotI have the same CTR3 fragments on a 2 μm plasmid, pRS416-CTR3-GFP and pRS426-CTR3-GFP were constructed by inserting a NotI fragment containing the green fluorescent protein (GFP)1 (18) derived from pSF1-GP1 at the NotI sites of pRS415-CTR3-NOTI and pRS426-CTR3-NOTI. Construction of pRS426-CTR3-FLAG(2) was carried out as follows: two oligonucleotides, FLAG(2)UP (5’-AAGCTTATTTCTGAAGAAGACTTAGAC-3’) and FLAG(2)LOW (5’-GCCGAGAACAAAAGCTTATTTCTGAAGAAGACTTAGGCGAACAA-3’), were ligated into the SstI and XhoI sites of pRS416-CTR3-FLAG(2) using restriction enzymes, and CTR3-FLAG(2) was cloned into the pRS413-CTR3-FlagI and pRS423-GAL1-CTR3-FlagI respectively with the primers CTR3-START (5’-CGGCTTTTTAAGTGAGTATTGCCGCCGGCGATTGACGTCTTC-CTCT-3’) and CTR3-STOP (5’-CGGCGTTTTAAGTGAGTATTGCCGCCGGCGATTGACGTCTTCAGAACCC-3’). The polymerase chain reaction fragment was cloned into the SmaI and HindIII sites on p426-Gal1 (21). Plasmid p426-GAL1-CTR3-GFP was constructed by amplifying the CTR3 open reading frame from genomic DNA using *Pfu* Turbo polymerase and the primers CTR1tagS (5’-GGAGCGGCCGATGAAAGACTTAGGATATTGAGATTTGAGTGG-3’) and CTR1NPH (5’-CGGCGTTTTAAGTGAGTATTGCCGCCGGCGATTGACGTCTTCAGAACCC-3’). The tagged construct was confirmed by sequencing.

**Biochemical Methods**—For immunoprecipitation experiments, MPY17 cells were co-transformed with pRS416-CTR3-FLAG(2) and pRS413-CTR3-myc(2). Cells were grown on YPD medium (1% yeast extract, 2% BactoPeptone, 2% dextrose) and suspended in liquid YPD and immobilized on slides with 1% low melting agarose. Ctr3-GFP was visualized by fluorescence microscopy using a Zeiss Axioskop photomicroscope with filters for observing green fluorescence. Cells were photographed with Kodak TMAX 400 print film. The negatives were digitized to CD-ROM and optimized for contrast and sharpness using Adobe Photoshop 3.0. For copper-dependent endocytosis of Ctr1 and Ctr3, fluorescence microscopy was carried out using a Nikon Eclipse E800 fluorescent microscope equipped with a Hamamatsu ORCA-2 cooled CCD camera. Images were obtained using ESee and ISee software packages from Innovision, Corp. (Raleigh-Durham, NC) and processed using Adobe Photoshop 3.0.

**TABLE I  
Yeast strains used in this study**

| Strain    | Genotype         | Ref.  |
|-----------|------------------|-------|
| MPY17     | MATa ctr1;ura3;Kan-ctr3;TRP1 his3 lys2-802 CUP1   | 44    |
| SKY72     | MATa gal1 trp1-1 his3 ade8 CUP1 ctr1;TRP1       | 6     |
| RH1800    | MATa leu2 his4 ura3 bar1 (1wt)                  | 25    |
| RH3777    | MATa ura3 leu2 his4 bar1 ts end3-1              | 25    |
| W303      | MATa SUC2 ade2-1 can-100 his3-11,15 leu3-3,112 trp1-1 ura3-1 | 17    |
| KRY54-4   | MATa sec12-4 ura3-1 his3-11,-15 leu2-3,-112      | R. Fuller |

1 The abbreviations used are: GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; EGS, ethylene glycol bis(succinimidylsuccinate); BCS, bathocuproine disulfonic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
with PBS prior to cross-linking. After cross-linking, the membranes were incubated with 1% Triton X-100 for 30 min on ice to solubilize the membranes followed by centrifugation at 15,000 rpm for 15 min at 4 °C. The cross-linked complexes were immunoprecipitated from the clarified extracts using anti-FLAG M2 Affi-Gel beads for 1 h at 4 °C in the presence of fresh protease inhibitors. The beads were washed three times with lysis buffer containing 1% Triton X-100, and the complexes were eluted from the beads with 0.1 M glycine, pH 3.0 and neutralized with 1.0 M Tris-HCl, pH 8.0. The immunoprecipitated complexes were analyzed by SDS-PAGE under denaturing and non-denaturing conditions and immunoblotting.

**Degradation and Endocytosis Experiments**—For the analysis of copper-induced degradation of Ctr3 and Ctr1, W303 cells were transformed with p426-Gal1-CTR3-FLAG(2) and p426-Gal1-CTR1-myc. For copper-dependent endocytosis wild type (RH1800) cells and the corresponding endocytosis-deficient mutant, RH3777 (end3–1) (25), were transformed with p426-Gal1-CTR3-GFP and p426-Gal1-CTR1-GFP. Cells were grown and treated with copper or BCS as described previously (14). For endocytosis experiments, cells were incubated at 15 °C for 15 min while shaking at 300 rpm, followed by the addition of 10 µM copper sulfate or BCS. After 1 h incubation, cells were treated with one-tenth volume of KILL buffer (1.0 M Tris-HCl, pH 7.5, 100 mM sodium azide, 100 mM sodium fluoride) to stop endocytosis (26). Endocytosis of Ctr3-GFP and Ctr1-GFP was observed using a Nikon microscope as described above.

**Results**

**Localization of the Ctr3 Protein**—Our previous localization studies of Ctr3, using a Ctr3-3xHA-tagged allele, found that it predominantly localized to intracellular vesicles consistent with the secretory compartment (6). However, subsequent phenotypic analysis of the Ctr3-3xHA epitope-tagged protein demonstrated that it is only partially functional in high affinity copper transport. To facilitate a re-analysis of the localization and biochemical characterization of Ctr3, a plasmid-borne copy of CTR3 was engineered to add a NotI site at the carboxyl terminus just prior to the STOP codon for the insertion of different epitopes. Two copies of each of the FLAG epitope, c-myc epitope, and a single copy of the GFP were inserted into the NotI site. Plasmids expressing the tagged Ctr3 proteins were transformed into a ctr3Δ ctn3Δ strain, and a serial dilution series of each culture was analyzed for respiratory competency as compared with the wild type CTR3 gene. As shown in Fig. 1A, all of the epitope-tagged alleles used in this study functionally complement the respiratory deficiency of a ctn3Δ ctr3Δ strain in a manner indistinguishable from the CTR3 wild type allele.

The Ctr3-GFP fusion protein was localized to the plasma membrane by fluorescence microscopy (Fig. 1B). As a control, a functional fusion between the yeast heat shock transcription factor and GFP was localized to the nucleus (27), and the unfused GFP protein was distributed throughout cells. To ascertain if Ctr3 traverses the normal route for plasma membrane proteins through the secretory pathway for localization to the plasma membrane, Ctr3-GFP was expressed and localized in temperature-sensitive sec12-4 mutant cells. Although at the permissive temperature, protein turnover is normal in the sec12-4 background, at the non-permissive temperature (37 °C) transport from the ER to the Golgi is blocked in this mutant (28, 29). The sec12-4 mutant cells expressing Ctr3-GFP were grown at the permissive (25 °C) and non-permissive temperatures, and Ctr3-GFP protein was localized by fluorescence microscopy. At the permissive temperature in both wild type and sec12-4 cells Ctr3-GFP was localized to the plasma membrane.
EGS concentration is increased, Ctr3-FLAG(2) forms a ~54-kDa complex consistent with the size expected for a Ctr3 homodimer (Fig. 3A, lane 2), and at 0.5 mM EGS an ~82-kDa complex, equivalent to the size expected for a Ctr3 homotrimer, is detected (Fig. 3A, lane 3). As the EGS concentration is increased to 1.0 mM, the dimeric and trimeric forms are detected with concomitant disappearance of the 27-kDa monomeric Ctr3-FLAG(2) (lane 4). In the presence of 2 and 3 mM EGS, the monomeric and dimeric forms are completely chased into a trimer with no observed formation of additional higher molecular weight species at this or higher EGS concentrations (Fig. 3A, lanes 5 and 6).

To test whether Ctr3 exists as a trimer at the plasma membrane, cross-linking reactions were performed on intact plasma membranes obtained from lysed spheroplasts made from cells expressing Ctr3-FLAG(2), as well as untransformed MPY17 cells as a negative control. The EGS-cross-linked complexes were extracted from the plasma membrane as described under “Experimental Procedures.” The immunoprecipitates were suspended in non-denaturing buffer (no β-mercaptoethanol or SDS and 0.01 mM iodoacetamide to prevent the rearrangement of disulfide bonds) or denaturing sample buffer (5% β-mercaptoethanol and 2% SDS) (22) and analyzed by immunoblotting using anti-FLAG M2 antibody. As shown in Fig. 3B, under native conditions, in the absence of EGS, high molecular weight species migrating at ~75-kDa and higher are detected (lane 1), which dissociate into the 27-kDa monomeric and ~54-kDa dimeric forms under denaturing conditions (lane 5). As the EGS concentration increases, high molecular weight species that may correspond to homotrimers are detected under native conditions (lanes 2–4). Under denaturing conditions, in the presence of 1 and 2 mM EGS, the high molecular weight species are resistant to denaturation and migrate as a mixture of dimers and trimers (lanes 6 and 7). In the presence of 3 mM EGS, these species exist primarily as a trimer under both native and denaturing conditions (lanes 6 and 8). Under native conditions, the mobility of the high molecular weight complexes do not correspond to their predicted molecular weights (lanes
The presence of the potential metal-binding motif, MDx, in the aminoterminal ectodomain of Ctr1 from yeast and mammals suggests that these motifs may be involved in binding copper ions from the extracellular milieu for transport into the cell as predicted by TopPredII analysis. The biochemistry mechanisms by which copper transport proteins mediate the uptake of copper into cells is poorly understood. The chemical mechanisms by which copper transport proteins mediate the uptake of copper into cells is poorly understood. The transmembrane domain, resulted in a partially functional protein. The results of complementation analysis, summarized in Fig. 4B, show that mutation of Cys-16, within the putative extracellular domain, results in a Ctr3 protein that could not complement the respiratory defect or sensitivity to menadione of the double mutant strain (Fig. 4B, lanes 2 and 5). Mutation of the two cysteine residues Cys-48 and Cys-51, which are arranged in a CXXC or CC potential metal binding configurations (16, 35). These motifs are found in the copper metalloregulatory transcription factors Ace1, Amt1, and Mac1, in the copper chaperone Cox17, and the metallothionein Cup1. It seems that Ctr3 does not possess these motifs, several cysteine residues are present throughout the protein, some of which are arranged in a CXXC or CC potential metal binding configurations (16, 35). These motifs are found in the copper metalloregulatory transcription factors Ace1, Amt1, and Mac1, in the copper chaperone Cox17, and the metallothionein Cup1. To determine whether the cysteine residues are important for Ctr3 function, site-directed mutagenesis was used to convert each individual cysteine codon to that encoding serine. In addition, several Ctr3 tyrosine residues, which have the potential to bind Cu, were changed to phenylalanine in the same fashion. To assess the effects of these mutations on Ctr3 function, plasmids expressing the mutant proteins were transformed into a ctr1Δ ctr3Δ yeast strain. The ability of the Ctr3 derivatives to complement the respiratory deficiency and oxidative stress sensitivity of this strain was tested by growth on ethanol as sole carbon source and in the presence of glucose and the superoxide anion generator menadione, respectively. Fig. 4A shows a working model for the topology of Ctr3 at the plasma membrane as predicted by TopPredII analysis (36) and indicates the location of the cysteine and tyrosine residues based on this model. The results of complementation analysis, summarized in Fig. 4B, show that mutation of Cys-16, within the putative extracellular domain, results in a Ctr3 protein that could not complement the respiratory defect or sensitivity to menadione of the double mutant strain (Fig. 4B, lanes 2 and 5). Mutation of the two cysteine residues Cys-48 and Cys-51, which are arranged in a CXXC configuration within the first transmembrane domain, resulted in a partially functional protein that allowed very slow growth in ethanol after 5 days of incubation but failed to reverse the hypersensitivity to this concentration of menadione (Fig. 4B, lane 7). When the C48S,C51S mutations were combined with C199S, a cysteine residue within the third predicted transmembrane domain, the mutant protein lost residual function and failed to complement the respiratory deficiency and oxidative stress sensitivity of the double mutant (Fig. 4B, lane 8). In contrast, mutation of Cys-

**Fig. 2. Multimerization of Ctr3.** Strain MPY17 (ctr1Δ ctr3Δ) was transformed with FLAG-tagged (pRS316-CTR3-FLAG2) or Myc-tagged (pRS413-CTR3-Myc2) Ctr3 individually or with both plasmids (lane 4). As a control, these cells were also transformed with pRS316 and pRS413 vectors (lane 1). The transformants were grown to log phase and treated with 10 μM BCS for 3 h to induce expression of Ctr3. Triton X-100-solubilized extracts were used for immunoprecipitation and the immunoprecipitates (IP) (lanes 5–8) as well as total extracts (Total) (lanes 1–4) were separated on 12% SDS-PAGE gels and visualized by immunoblotting (WB). Extracts were immunoprecipitated with anti-FLAG M2 Affi-Gel and visualized with anti-FLAG M2 antibody. Ctr3-FLAG(2) and Ctr3-Myc(2) are indicated by labeled arrows. As a specificity control, blots were stripped and probed with anti-Gas1 antibody and anti-Pma1 antibody which are indicated by labeled arrows. The asterisk represents the position of the light chain from the immunoprecipitating antibody.

**Fig. 3.** Ctr3 assembles as a trimer. A, MPY17 cells (ctr1Δ ctr3Δ) were transformed with FLAG-tagged Ctr3 (pRS316-CTR3-FLAG2), grown to log phase, and treated with BCS for 3 h to induce Ctr3-FLAG(2) expression. In vitro cross-linking of Ctr3-FLAG(2) was performed using 25 μg of Triton X-100-solubilized total cell extracts using 0 (dimethyl sulfoxide solvent alone), 0.25, 0.5, 1.0, 2.0, and 3.0 mM EGS (indicated by ramp from left to right) and resolved by denaturing SDS-PAGE. Ctr3-FLAG(2) proteins were detected by immunoblotting with anti-FLAG M2 antibody. B, intact membranes from lysed spheroplasts prepared from MPY17 cells expressing Ctr3-FLAG(2) were grown as described above. Membranes were incubated with 0 (dimethyl sulfoxide solvent only) or 1, 2, and 3 mM EGS (lanes 2–4 and 6–8) in PBS for 30 min at room temperature. The cross-linked complexes were extracted as described under “Experimental Procedures.” Samples were re-suspended in nondenaturing sample buffer (50% glycerol, 25 mM Tris, 0.1% bromphenol blue, 0.01 M iodoacetamide) (Native) or denaturing sample buffer (Denaturing) (same as Native buffer but including 5% β-mercaptoethanol and 2% SDS and without iodoacetamide). For both A and B, the positions of the molecular weight standards are indicated to the left, and on the right, the monomeric, dimeric, and trimeric complexes are indicated by one, two, or three ovals, respectively.
a genomic copy of were transformed with pRS316. with wild type CTR3 domain was mutated to MSS, top indicated on menadione plates and 5 days on YPE plates. The specific photographed after incubation at 30 °C for 3 days on SC-ura plates and of the cysteine and tyrosine residues are indicated by a the plasma membrane as predicted by TopPredII analysis. The location of Ctr3 proteins were grown to log phase and spotted onto SC-ura agar, respectively. open circle, respectively. assays (Fig. 4 199 to serine alone had no effect on Ctr3 function by these notypes of the cysteine to alanine mutants were indistinguishable from the cysteine to serine mutants (data not shown). The results shown in Fig. 5 demonstrate that functional Ctr3 mutants can trimerize but are trapped in a secretory compartment suggests that Ctr3 assembles into a trimeric complex in this compartment. Failure of the mutant proteins to exit this compartment further suggests that Ctr3 multimer that is consistent with a trimer is observed in the absence of the cross-linker under denaturing conditions, and no monomer is detected suggesting that this mutant may be aggregated even though it retains residual function as assessed by its ability to confer slow growth in media containing ethanol over 5 days. The observation that these mutants can trimerize but are trapped in a secretory compartment suggests that Ctr3 assembles into a trimeric complex in this compartment. Failure of the mutant proteins to exit this compartment further suggests that at least Cys-16, Cys-48, and Cys-51 are essential for proper assembly of the complex or for interaction with other proteins that are required for exit from the ER and passage through the secretory pathway for localization at the plasma membrane.

Post-transcriptional Regulation of Ctr3—The CTR1 and CTR3 genes are transcriptionally regulated in response to copper or copper starvation through the interaction of the transcription factor Mac1 with copper-responsive elements in their promoters (12, 13, 37–40). In addition to transcriptional regulation, Ctr1 protein is further regulated by copper-stimulated endocytosis at copper ion concentrations from 0.1 to 10 μM, and copper stimulated degradation at the plasma membrane in the presence of copper concentrations equal to or in excess of 10 μM (14). To ascertain if Ctr3 undergoes similar forms of post-transcriptional regulation, changes in the steady state levels of Ctr3 and in Ctr3 endocytosis were examined in the absence or presence of copper and compared with Ctr1. The expression of both Ctr3-FLAG(2) and Ctr1-myc, as well as Ctr3-GFP and Ctr1-GFP, were placed under the control of the galactose-inducible glucose-repressible GAL1-10 promoter. To assess Ctr3 steady state levels as a function of added copper, cells were grown overnight in the presence of 2% raffinose and transferred to media containing 2% raffinose and 0.5% galactose to induce the expression of Ctr3-FLAG(2) and Ctr1-myc. Transcription of the copper transporter gene was extinguished by transferring the cultures to medium containing 2% glucose, and the effects of copper on steady state protein levels were assessed after treatment with either 10 μM BCS or 10 μM copper. The results shown in Fig. 6 demonstrate that although using EGS with 1% Triton X-100-solubilized extracts from cells expressing the mutant Ctr3 proteins fused to two copies of the FLAG epitope demonstrated that these proteins can assemble to form trimers (Fig. 5). In the case of the C48S,C51S and C48S,C51S,C199S mutants, however, some high molecular weight aggregates are observed in the wells of the SDS-PAGE gels (Fig. 5). For the C48S,C51S mutant, an apparent high molecular weight multimer that is consistent with a trimer is observed in the absence of the cross-linker under denaturing conditions, and no monomer is detected suggesting that this mutant may be aggregated even though it retains residual function as assessed by its ability to confer slow growth in media containing ethanol over 5 days. The observation that these mutants can trimerize but are trapped in a secretory compartment suggests that Ctr3 assembles into a trimeric complex in this compartment. Failure of the mutant proteins to exit this compartment further suggests that at least Cys-16, Cys-48, and Cys-51 are essential for proper assembly of the complex or for interaction with other proteins that are required for exit from the ER and passage through the secretory pathway for localization at the plasma membrane.

We assessed the expression and localization of the mutant Ctr3 proteins by tagging with GFP and localization by fluorescence microscopy. The phenotypes of MPY17 cells expressing the GFP-tagged mutants with respect to growth on respiratory complex or for interaction with other proteins that are required for exit from the ER and passage through the secretory pathway for localization at the plasma membrane.
Ctr3 degradation is stimulated in a time-dependent manner in the presence of 10 μM copper, the same copper concentration has little if any effect on the stability the Ctr3 as compared with the control culture. In several experiments, we observed the same rate of decrease in the steady state levels of Ctr3 in the presence of 10 μM BCS or 10 μM copper sulfate. Samples were taken at the indicated time points, and 50 μg of Triton X-100-solubilized total cell extracts were analyzed by immunoblotting. Ctr3-FLAG(2) was detected using anti-FLAG M2 antibody, and Ctr1-myc was detected with anti-c-myc 9E10 antibody. The arrow on the right indicates the molecular mass of Ctr3 (27 kDa) and Ctr1-myc (105 and 46 kDa).

To ascertain whether, like Ctr1 (14), Ctr3 endocytosis is stimulated by elevated copper levels, cells transformed with plasmids expressing the Ctr3-GFP or Ctr1-GFP fusion proteins from the GAL1-10 promoter were examined by fluorescence microscopy. The results shown in Fig. 7 demonstrate that, as previously shown using a Ctr1-myc protein (14), Ctr1-GFP undergoes enhanced endocytosis in the presence of copper as indicated by the presence of presumptive endocytic vesicles that appear in a time-dependent manner after copper addition but not in BCS-treated control cultures. Under these same conditions, endocytosis of Ctr3-GFP from the plasma membrane is not stimulated. As a control, both plasmids were also transformed into S. cerevisiae end3-1 mutants (25) that are blocked for endocytosis. In this strain, neither Ctr1-GFP nor Ctr3-GFP undergoes endocytosis in the presence or absence of copper (data not shown). Taken together these results demonstrate that whereas the genes encoding Ctr1 and Ctr3 are similarly regulated in a copper-dependent manner at the transcriptional level, at the post-transcriptional level the Ctr1 and Ctr3 proteins are regulated in a distinct manner.

**FIG. 6.** Ctr1 and Ctr3 steady state levels in response to elevated copper concentrations. S. cerevisiae (W303) cells transformed with p423-Gal1-CTR3-FLAG(2) or p426-Gal1-CTR1-myc were grown as described under “Experimental Procedures.” After glucose shut-off of Ctr3-FLAG(2) and Ctr1-myc expression, cells were treated with 10 μM BCS or 10 μM copper sulfate. Samples were taken at the indicated time points, and 50 μg of Triton X-100-solubilized total cell extracts were analyzed by immunoblotting. Ctr3-FLAG(2) was detected using anti-FLAG M2 antibody, and Ctr1-myc was detected with anti-c-myc 9E10 antibody. The arrow on the right indicates the molecular mass of Ctr3 (27 kDa) and Ctr1-myc (105 and 46 kDa).

**FIG. 7.** Ctr1 but not Ctr3 undergoes copper-stimulated endocytosis. S. cerevisiae wild type (RH1800) cells were transformed with p426-Gal1-CTR3-GFP and p426-Gal1-CTR1-GFP, grown, and harvested as described under “Experimental Procedures.” Cells were incubated at 15 °C for 15 min and then treated with 10 μM BCS (A and C) or 10 μM copper (B and D) for 60 min at 15 °C. Ctr1-GFP and Ctr3-GFP were visualized by fluorescence microscopy and photographed as described under “Experimental Procedures.”

firmly establish that, consistent with its role in copper uptake, Ctr3 is localized to the yeast plasma membrane. Like most plasma membrane proteins, Ctr3 traverses the secretory pathway for proper localization. Whereas most S. cerevisiae permeases and transport proteins possess 6–12 transmembrane domains (30), the copper transport family of proteins identified thus far possess only 3 such domains. To begin to understand the mechanism by which these proteins deliver copper into the cell, we carried out experiments to determine how the Ctr3 protein assembles as a functional copper transporting permease. By using co-immunoprecipitation experiments, we show that Ctr3 self-assembles to form a specific homo-multimeric complex. Through in vitro cross-linking with EGS using Triton X-100-solubilized total cell extracts and cross-linking of intact membranes, we show that assembly of the Ctr3 complex is consistent with a trimer. Nine transmembrane domains from three Ctr3 molecules could form a pore through which copper can be translocated into the cell. Although we have not assessed if Ctr1 and Ctr3 can form mixed multimers, this is clearly not obligatory for their function in high affinity copper transport since both proteins can function efficiently independent of the other.

How does Ctr3 interact with copper to deliver copper into the cell? The established or putative copper transporters in yeast, Ctr1 and Ctr4, hCtr1, and mCtr1 possess a conserved sequence, MX_4XM, in the predicted extracellular domain that is proposed to interact with copper or copper bound to another ligand in the environment (15). This motif, however, is lacking in Ctr3. Instead, Ctr3 has 11 cysteine residues throughout the protein that may be involved in copper binding. We assessed the potential role of these residues on Ctr3 function and biogenesis by mutating the cysteines to serines or alamines. Of these mutations, only Cys-16, Cys-48, and Cys-51 resulted in a non-functional or partially functional Ctr3 protein as assessed by the ability of these proteins to complement the respiratory deficiency and oxidative stress sensitivity of a ctr1Δ ctr3Δ mutant strain. By in vitro cross-linking, we show that these mutants are capable of trimerization, with the C48S,C51S and C48S,C51S,C199S mutants forming higher molecular weight aggregates. Through fluorescence microscopy on the GFP-tagged mutants, we found that these mutants fail to exit an intracellular compartment likely to be the ER. Although our results preclude us from addressing the role of these residues in copper uptake at the plasma membrane, they suggest that these cysteine residues may be important for the assembly of the Ctr3 complex in the secretory pathway. Since proper as-
Furthermore, whereas Ctr1 undergoes enhanced endocytosis in not affected by increased copper concentration in the media. It provides an important tool for dissecting the pathways copper transporters will provide insights into the mechanism in Ctr1 that are required for this process and their conservation in other eukaryotic copper transporters will provide insights into the mechanism by which these proteins can translocate copper into the cell. Since Ctr3 is a module that is present in all copper transporters, it provides an important tool for dissection of the pathways that govern this regulatory mechanism and the assembly of these proteins.

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