Copper is an essential nutrient that serves as a cofactor for enzymes involved in critical cellular processes including energy generation, peptide hormone maturation, oxidative stress protection, and iron homeostasis. Although genes have been identified from yeast and mammals encoding a homologous subunit of a plasma membrane high affinity copper transporter, the presence of additional subunits that function as part of a copper transport complex has not been reported. We observed that *ctr4*Δ, a previously identified copper transport protein from the fission yeast *Schizosaccharomyces pombe*, fails to complement bakers' yeast cells defective in high affinity copper transport and fails to be targeted to the plasma membrane. However, selection for *S. pombe* genes, which, when co-expressed with Ctr4, confer high affinity copper transport to *S. cerevisiae* cells resulted in the identification of *ctr5*Δ. Both Ctr4 and Ctr5 are integral membrane proteins, are co-regulated by copper levels and the copper-sensing transcription factor Cuf1, physically associate in vivo, are interdependent for secretion to the plasma membrane, and are each essential for high affinity copper transport. These studies in *S. pombe* identify Ctr4 and Ctr5 as components of a novel eukaryotic heteromeric plasma membrane complex that is essential for high affinity copper transport.

Copper is as an essential metal ion for life. Due to its ability to adopt both oxidized (Cu(II)) and reduced (Cu(I)) states, copper is an important redox co-factor for many copper-dependent enzymes, including cytochrome oxidase, copper/zinc superoxide dismutase, ceruloplasmin, and lysyl oxidase (1–3). Many human genetic diseases have been linked to aberrant copper homeostasis, including Menkes syndrome, Wilson disease, neurodegenerative diseases such as amyotrophic lateral sclerosis, and iron deficiency anemia (4, 5). Consequently, it is important to identify the components, and understand the mechanisms for how organisms acquire, distribute, and utilize copper.

The bakers' yeast *Saccharomyces cerevisiae* has been a valuable model system to study eukaryotic copper homeostasis due to its powerful genetics (2, 6). Cu(II) in yeast growth medium is thought to be reduced to Cu(I) by cell surface Fe3+/Cu2+ reductases encoded by several *FRE* genes (7–11), before being transported into the cell by two high affinity plasma membrane copper transporters, Ctr1 (12, 13) and Ctr3 (14, 15). Although Ctr1 and Ctr3 are functionally redundant, and both possess three potential transmembrane domains, they are otherwise dissimilar structurally. Ctr1 harbors eight copies of the “Mets” motif MX₃MX₅MX₃ (where M represents methionine and X represents any amino acid) in its putative amino-terminal extracellular domain, whereas Ctr3 is rich in cysteine residues but lacks the Mets motif. Recently, the isolation of genes encoding proteins involved in high affinity copper transport in the fission yeast *S. pombe*, mice, and humans indicates the presence of variable numbers of amino-terminal Mets motifs and sequence homology to both *S. cerevisiae* Ctr1 and Ctr3 (2, 16–18). Both *S. cerevisiae* Ctr1 and Ctr3 are thought to self-associate (12, 15); however, whether the copper transporters are present in a high affinity copper transport complex with other functional subunits is not known.

The fission yeast *Schizosaccharomyces pombe* is also of particular interest in studies of copper homeostasis since fission yeast exhibit similarity to mammals in several respects (19). Interestingly, a high affinity copper transport protein from *S. pombe*, Ctr4 (17), resembles a chimera between the *S. cerevisiae* Ctr1 and Ctr3 proteins at the primary sequence level. Ctr4 harbors five MX₃MX₅MX₃ repeats in the predicted amino-terminal extracellular region similar to Ctr1, but transmembrane domains homologous to Ctr3 (Fig. 1A). Like the *CTR1* and *CTR3* genes in *S. cerevisiae*, *ctr4Δ* transcription is controlled by environmental copper levels and Cuf1, a copper-sensing transcription factor functionally analogous to Mac1 in *S. cerevisiae* (17, 20–23).

In this work we report the identification of a new *S. pombe* gene encoding a transmembrane high affinity copper transporter protein, denoted *ctr5*Δ. We demonstrate that like Ctr4, Ctr5 is essential for high affinity copper transport and is subject to transcriptional control by the copper-sensing transcription factor Cuf1. Furthermore, Ctr4 and Ctr5 both localize to the plasma membrane, exhibit an interdependence for this localization, and are present in a complex. These data describe the first example of a heteroprotein complex that forms a high affinity copper transport activity at the cell surface.

**Experimental Procedures**

**Strains, Plasmids, Media, and Reagents—**The wild type *S. pombe* strain used in this study was FY254 (Δcan1–1 leu-32 ade6-M210 ura4-D18). The isogenic *ctr4Δ* (HZY3), *ctr5Δ* (HZY2), and *ctr4Δctr5Δ* (HZY4) strains were constructed by replacing the coding region of each gene with a hisG-URA4-hisG cassette through homologous recombination as described (24). The genotypes of disruption strains were confirmed by polymerase chain reaction, using gene-specific primers. The *can1Δ S. pombe* strain SPY1 has been described previously (17). The *S. cerevisiae* *ctr1Δctr3Δ* strain MPY17 was described previously (25). *S. pombe* cells were grown at 30 °C in rich media (YES), or synthetic medium is thought to be reduced to Cu(I) by cell surface Fe³⁺ due to its powerful genetics (2, 6). Cu(II) in yeast growth is also an essential metal ion for life. Due to its ability to adopt both oxidized (Cu(II)) and reduced (Cu(I)) states, copper is an important redox co-factor for many copper-dependent enzymes, including cytochrome oxidase, copper/zinc superoxide dismutase, ceruloplasmin, and lysyl oxidase (1–3). Many human genetic diseases have been linked to aberrant copper homeostasis, including Menkes syndrome, Wilson disease, neurodegenerative diseases such as amyotrophic lateral sclerosis, and iron deficiency anemia (4, 5). Consequently, it is important to identify the components, and understand the mechanisms for how organisms acquire, distribute, and utilize copper.

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In this work we report the identification of a new *S. pombe* gene encoding a transmembrane high affinity copper transporter protein, denoted *ctr5Δ*. We demonstrate that like Ctr4, Ctr5 is essential for high affinity copper transport and is subject to transcriptional control by the copper-sensing transcription factor Cuf1. Furthermore, Ctr4 and Ctr5 both localize to the plasma membrane, exhibit an interdependence for this localization, and are present in a complex. These data describe the first example of a heteroprotein complex that forms a high affinity copper transport activity at the cell surface.
media (EMM) as described (26). The nonfermentable carbon source media YES-EG was made by substituting the glucose in YES with 2% glycerol/3% ethanol. *S. cerevisiae* cells were grown at 30 °C in rich media (YPD) or synthetic media (SC), as previously described (27). YPEG media was made by substituting the glycerol in YPD with 2% glycerol/8% ethanol.

The multicopy *S. pombe* plasmids pSP1 and pSP2 (28) were used to express *ctr4*′ and *ctr5*′. The coding regions of these genes, with ~1 kilobase pair of 5′ upstream sequences, were amplified from FY254 genomic DNA, cloned into pSP1 and pSP2, and sequenced. The expression of *ctr4* and *ctr5* in *S. cerevisiae* was from plasmids p423GPD and p426GPD (29), respectively, under the control of the strong constitutive GPD promoter. The GFP, myc, and FLAG epitope tags were added to the carboxyl termini of Ctr4 and Ctr5, as described previously (15, 17).

Isolation of the *ctr5*′ Gene—*E. coli* strain BNN132 was transformed with an *S. pombe* cDNA library in *AYE* phage vector (ATCC no. 87284, deposited by S. Elledge). The resulting yeast expression library generated by self-recombination was retrieved by a large scale plasmid preparation from 432,000 independent transformants. *S. cerevisiae* strain MPY17 harboring plasmid *ctr4*′-p413GPD was transformed with the *S. pombe* cDNA library and plated on SC-His-Ura medium. A total 360,000 transformants were pooled and re- plated on YPEG plates. Among hundreds of colonies growing on YPEG, 24 were isolated, their phenotype verified, plasmids retrieved, and inserts classified by restriction endonuclease digestion and agarose gel electrophoresis. The cDNAs from eight representative isolates were sequenced and were all found to contain the same open reading frame, designated *ctr5*′. The flanking sequences of the *ctr5*′ gene were obtained from the *S. pombe* genome data base at the Sanger Center (Hinxton Hall, United Kingdom).

Fluorescence Microscopy—For localization of Ctr4-GFP fusion proteins in *S. cerevisiae*, MPY17 cells harboring a *ctr4*′-GFP-p423GPD plasmid were grown in SC-His to early log phase. Cells were suspended in SC-His with 100 μM bathocuproinedisulfonate (BCS) to Aοω = 2, and immobilized on slides with 1% low melting agarose. Ctr4-GFP was visualized under a Zeiss Axioskop photomicroscope with filters observing green fluorescence. Photos were taken on Kodak Tmax 400 film and digitalized to CD-ROM, then processed by Adobe Photoshop 5.5 software. For Ctr4-GFP and Ctr5-GFP localization in *S. pombe*, cells were grown in EMM and treated as above. The fluorescence signal was visualized by a Nikon Eclipse E800 fluorescent microscope with a Hamamatsu ORCA-2 cooled CCD camera. Images were obtained using ESee and ISee software from Innovision (Raleigh-Durham, NC) and then processed with Photoshop 5.5 software.

RESULTS

The *S. pombe* Ctr4 High Affinity Copper Transporter Is Nonfunctional and Mislocalized in *S. cerevisiae*—Due to the structural similarity between the *S. pombe* Ctr4 high affinity copper transporter and the *S. cerevisiae* Ctr1 and Ctr3 proteins (Fig. 1A, and Ref. 17), we assessed the ability of Ctr4 to complement the inability of an *S. cerevisiae* ctr1Δctr3Δ strain to grow on nonfermentable carbon sources due to copper insufficiency. The *S. pombe* *ctr4*′ open reading frame was placed under the control of the strong GPD promoter (29) and *S. cerevisiae* *ctr1Δctr3Δ* cells expressing *S. pombe* Ctr4 were tested for growth on the respiratory carbon sources glycerol/ethanol, for which high affinity copper transport is required for delivery of copper to cytochrome oxidase. As shown in Fig. 1B, although *S.
Copper Transport Complex in S. pombe

**A**

|          | WT | ctr1Δctr3Δ |
|----------|----|------------|
| Glucose  | -  | -          |
| Ctr4     | +  | +          |
| Ctr5     | -  | +          |

**B**

- **GFP Nomarski**
  - Ctr4-GFP +vector
  - Ctr4-GFP +Ctr5

Fig. 2. Expression of *S. pombe* Ctr5 complements *S. cerevisiae* *ctr1Δctr3Δ* growth defects and localizes Ctr4-GFP to the plasma membrane. A, Ctr4 and Ctr5 demonstrate co-dependence for complementation in *S. cerevisiae*. MPY17 (ctr1Δctr3Δ) was transformed with a combination of ctr4Δ-p426GPD, ctr5Δ-p426GPD, or vector alone. Growth was tested on SC-His-Ura (glucose) for 2 days or YPEG (glycerol/ethanol) for 5 days at 30 °C. WT, wild type. B, localization of Ctr4-GFP in the presence and absence of Ctr5. MPY17 was co-transformed with ctr4Δ-GFP-p423GPD and either ctr5Δ-p426GPD or the empty vector p428GPD and images captured as described for Fig. 1.

cerevisiae *ctr1Δctr3Δ* cells expressing *S. pombe* Ctr4 were able to grow on glucose, they were unable to utilize glycerol/ethanol carbon sources. To explore the underlying reasons for the inability of Ctr4 to complement the *S. cerevisiae* copper uptake defect, a Ctr4-GFP fusion protein that is functional in *S. pombe* (Fig. 6A and Ref. 17) was localized in *S. cerevisiae* *ctr1Δctr3Δ* cells by fluorescence microscopy. As shown in Fig. 1C, the Ctr4-GFP fusion protein was not localized to the *S. cerevisiae* plasma membrane, but rather appeared to be trapped within cells in both perinuclear and other regions that might correspond to the secretory compartments. As a control, the *S. cerevisiae* Ctr1-GFP fusion protein was localized to the plasma membrane in these cells (Fig. 1C). These observations suggest that at least one reason for the inability of the *S. pombe* Ctr4 protein to function in high affinity copper transport in *S. cerevisiae* is due to mislocalization.

Identification of the *S. pombe* Copper Transport Co-factor Ctr5—It is possible that *S. pombe* Ctr4 is mislocalized when expressed in *S. cerevisiae* due to incorrect folding, the presence of an inhibitory activity in *S. cerevisiae*, or the lack of a functionally interacting partner protein in *S. cerevisiae* whose association is important for Ctr4 folding or trafficking through the secretory pathway. To test this, we devised a genetic screen for potential co-factor(s) that could facilitate *S. pombe* Ctr4 function in *S. cerevisiae* cells lacking high affinity copper transporters. *S. cerevisiae* *ctr1Δctr3Δ* cells were co-transformed with a high copy plasmid in which the *S. cerevisiae* copper transporter coding region was expressed from the GPD promoter, and an *S. pombe* cDNA library under the control of the *S. cerevisiae* ADH1 promoter. Plasmids harboring *S. pombe* cDNAs were recovered from eight glycerol/ethanol-positive colonies and the cDNA inserts sequenced. Of these eight independent isolates, all harbored a cDNA encoding a single open reading frame, hereafter referred to as ctr5+. Inspection of the *S. pombe* genome data base indicates that, although the *ctr4+* gene is located on chromosome 3, the *ctr5+* gene is located on chromosome 1. As shown in Fig. 2A, although expression of either *S. pombe* Ctr4 or Ctr5 alone in *S. cerevisiae* *ctr1Δctr3Δ* cells did not restore high affinity copper transport, as ascertained by growth on glycerol/ethanol media, co-expression of Ctr4 and Ctr5 allowed growth under these conditions. Furthermore, although the *S. pombe* Ctr4-GFP fusion protein was mislocalized in these cells, co-expression of *S. pombe* Ctr5 facilitated the localization of the Ctr4-GFP fusion protein to the *S. cerevisiae* plasma membrane (Fig. 2B). These data support the notion that *S. pombe* Ctr5 is a co-factor required for Ctr4 localization to the plasma membrane and function in high affinity copper transport in *S. cerevisiae*.

Ctr5 Has Structural and Regulatory Properties Consistent with a Role in High Affinity Copper Transport—The amino acid sequence encoded by the *ctr5+* open reading frame (Fig. 3A) predicts a small protein (173 amino acids) with three potential transmembrane domains (Fig. 3, B and C) and 41% amino acid sequence identity with Ctr4 throughout the two proteins. Interestingly, Ctr5 has a predicted membrane topology that is similar to other microbial and metazoan copper transporters (Fig. 1A). The Ctr5 amino acid sequence also predicts, in the amino-terminal putative extracellular domain, the presence of two copies of partially overlapping MXMXXM sequences ("Mets" motif), a putative copper binding motif that occurs in the amino-terminal predicted extracellular domain of many high affinity copper transporters identified to date. A similar sequence (CXXMX) lies downstream of this region, with a cysteine, another potential copper ligand, replacing methionine in the motif (Fig. 3, A and C).

In addition to the shared structural homology of fungal high affinity copper transport proteins, *S. cerevisiae* CTR1 and CTR3, and *S. pombe* *ctr4+* gene expression is regulated as a function of copper availability by the copper-sensing transcription factors Mac1 and Cuf1, respectively (17, 20–23). To ascertain the potential role of the *ctr5+* gene in *S. pombe* copper homeostasis, we examined its possible regulation by extracellular copper concentrations. As shown in Fig. 4, *ctr5+* mRNA was co-regulated with that of *ctr4+* in response to copper availability. Both *ctr4+* and *ctr5+* had low basal levels of mRNA expression in rich media, which was further repressed by the addition of 10 μM CuSO₄ to wild type *S. pombe* cells. In contrast, addition of the copper chelator BCS to 100 μM significantly induced the expression of both genes. In a *ctr4Δ* strain, which had previously been shown to be copper-deficient (17), basal levels of *ctr5+* mRNA expression were dramatically increased and as much as 100 μM CuSO₄ was required to significantly extinguish *ctr5+* mRNA levels to that found at 10 μM in wild type cells.

The *S. pombe* *cuf1+* gene encodes a nuclear copper-sensing transcription factor that activates *ctr4+* gene expression under conditions of copper limitation (17). In a *cuf1Δ* strain, both *ctr4+* and *ctr5+* gene expression was severely diminished, regardless of the exogenous copper levels (Fig. 4), indicating tight co-regulation of both genes by Cuf1. Taken together, both protein structural features and regulation by copper and the copper-sensing transcription factor Cuf1 suggest that Ctr5 plays a role in copper homeostasis in *S. pombe*.

Inactivation of the Ctr5 Gene Results in Defective Copper Transport—The functional interactions of Ctr4 and Ctr5 in *S. cerevisiae*, together with copper dependent regulation of gene expression in *S. pombe*, all point to a role for Ctr5 in copper acquisition in *S. pombe*. To ascertain whether Ctr5 plays a role in copper transport in *S. pombe*, the single chromosomal *ctr5+* gene was deleted by homologous recombination and growth on nonfermentable carbon sources and high affinity copper uptake evaluated. As shown in Fig. 5 (A and B), the isogenic parental wild type strain transported ⁶⁴Cu with high affinity and was able to utilize nonfermentable carbon sources for growth. In contrast, deletion of either the *ctr4+* or *ctr5+* gene resulted in a...
dramatic reduction in high affinity copper transport and, consistent with the 64Cu transport defects observed in these mutants, neither strain was able to grow on medium containing glycerol/ethanol as sole carbon sources (Fig. 5B). Furthermore, an isogenic strain harboring a deletion of both the ctr4 and ctr5 genes exhibited equivalent defects in high affinity 64Cu uptake as compared with either single deletion, suggesting that the Ctr4 and Ctr5 proteins function in the same pathway for copper acquisition in S. pombe.

Copper Transport Complex in S. pombe

Ctr4 and Ctr5 Are Co-dependent for Localization to the Plasma Membrane—The structural, functional, and regulatory features of Ctr5 suggest that it participates in the same pathway as the Ctr4 protein in high affinity copper transport. Since previous studies (17) established that Ctr4 resides on the plasma membrane, we envision at least two possibilities for the functional relationship between Ctr4 and Ctr5 in copper uptake in S. pombe. First, both proteins could be present in a high affinity copper transport complex, the presence of both of which is needed for the proper function and or trafficking of the complex to the plasma membrane. Alternatively, Ctr5 could be a resident in the secretory pathway and facilitate the folding of Ctr4 and or its packaging into transport vesicles. To distin-

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**FIG. 3.** The amino acid sequence of Ctr5 and its predicted topology. A, the amino sequence of Ctr5. The two partially overlapped M2M2 motifs and a similar CXXM sequence are shown in bold letters. Three predicted transmembrane domains are boxed. B, the hydrophobicity plot of Ctr5. The Toppred program was used to generate the plot using the GES scale. The lines labeled Certain and Putative indicate the possibility of the existence of transmembrane domains. C, a topological model for Ctr5 predicted by Toppred. The positions of the “Mets” motifs and the CXXM sequence are indicated with rectangles. N and C indicate amino and carboxyl termini, respectively.

**FIG. 4.** The ctr4 and ctr5 genes are co-regulated by copper and the copper-sensing transcription factor Cuf1. S. pombe strain FY254 (WT), HZY3 (ctr4Δ), and SPY1 (ctr5Δ) were grown in YES medium to A595 = 0.8. One hour before harvesting, CuCl2 or BCS was added to the culture to the indicated final concentration. Total RNA was extracted and fractionated on a 1.2% agarose gel, and RNA blot analysis was performed using the indicated cDNAs as probes. The positions of the S. pombe ctr4, ctr5, and act1 mRNAs are shown with arrows.

**FIG. 5.** Ctr4 and Ctr5 are required for growth on nonfermentable carbon sources and high affinity 64Cu transport. A, in vivo copper uptake by S. pombe cells. FY254 (wt), HZY3 (ctr4Δ), HZY2 (ctr5Δ), and HZY4 (ctr4Δctr5Δ) cells were grown in YES medium to A595 = 0.6–1.0. 64CuCl2 was added to the culture to a final concentration of 10 μM, and cultures were incubated either at room temperature or on ice for 10 min. The values were normalized to culture density, and counts on ice were subtracted from the counts from room temperature incubation to give the final values. B, S. pombe cells defective for ctr4 or ctr5 are defective for growth on nonfermentable carbon sources. Cells were spotted on YES (glucose) or YES-EG (glycerol/ethanol), incubated for 5 or 7 days, respectively, and photographed.
guish between these two possibilities, we ascertained the localization of the Ctr5 protein in S. pombe. Functional Ctr4-GFP and Ctr5-GFP fusion proteins were expressed in S. pombe cells from multicopy plasmids under their native promoters (Fig. 6A), and the localization of each GFP fusion was determined by fluorescence microscopy. As shown in Fig. 6B (panel 1), the Ctr4-GFP fusion was present on both the plasma membrane, and in a perinuclear region that might coincide to the early secretory compartment. Because episomal plasmids in S. pombe are present in multiple copies with nonequivalent segregation to progeny cells (28), cell variability in Ctr4-GFP expression is likely to underline its presence in both plasma membrane and perinuclear locations in many, but not all, cells. A similar perinuclear and plasma membrane distribution was observed for the Ctr5-GFP fusion in wild type S. pombe cells (Fig. 6B, panel 4).

Because the levels of Ctr4-GFP protein expressed from high copy plasmids in these cells are likely to be much higher than Ctr5 expressed from the single endogenous gene, the partial mislocalization of Ctr4-GFP could be due to inadequate levels of Ctr5 to act in concert with Ctr4. To test this possibility wild type S. pombe cells were co-transformed with multicopy plasmids expressing both the Ctr4-GFP fusion and the wild type Ctr5 protein. As shown in Fig. 6B (panel 2), in the presence of elevated levels of Ctr5, the bulk of Ctr4-GFP fusion protein was found at the plasma membrane, with little remaining in the perinuclear region. Similarly, in the presence of elevated levels of Ctr4, the Ctr5-GFP fusion protein was predominantly localized to the plasma membrane, with only a small amount of fluorescence in the perinuclear compartment (Fig. 6B, panel 5). These results suggest that specific levels of both Ctr4 and Ctr5 may be required for their proper localization in S. pombe. To further test this hypothesis, the ctr5Δ gene was insertionally inactivated in a wild type S. pombe strain by homologous recombination. As shown in Fig. 6B (panel 3), ctr5Δ cells accumulated the Ctr4-GFP fusion protein almost exclusively in intracellular compartments, with little or none detected on the plasma membrane. This effect appears to be specific, since an S. pombe lid1-GFP fusion protein (31) was localized mainly to the plasma membrane in both wild type and ctr5Δ cells (Fig. 6C). Furthermore, as shown in Fig. 6B (panel 6), ctr4Δ cells accumulated Ctr5-GFP fusion protein in a perinuclear compartment, and with punctate distribution, with little if any detected on the plasma membrane. Taken together, these observations suggest that the Ctr4 and Ctr5 proteins are interdependent for localization to the plasma membrane in S. pombe.

Copper Transport Complex—The observations that both Ctr4 and Ctr5 are essential for high affinity copper transport, localize to the plasma membrane, and exhibit an interdependence for this localization suggest that these two proteins function in the same copper acquisition pathway, perhaps as a complex. To test the possibility that Ctr4 and Ctr5 may form a functional copper transport complex at the plasma membrane, we first assessed whether Ctr4 and Ctr5 are integral membrane proteins as predicted by the presence of three potential membrane-spanning domains within their primary sequences (Fig. 3). Two copies of the FLAG epitope were added to the Ctr4 coding region, and Ctr5 was tagged with four tandem copies of the myc epitope to generate functional Ctr4 and Ctr5 epitope-tagged derivatives (Fig. 6A). As shown in Fig. 7A, functional epitope-tagged versions of both Ctr4 and Ctr5 were detected by immunoblotting, in the total cell lysate (lane 1), but not in the soluble fraction after centrifugation of native membrane preparations at 100,000 × g (lane 2). Furthermore, Ctr4 and Ctr5 were efficiently extracted by 1% Triton X-100 from the membrane fraction (lane 8), a nonionic detergent that solubilizes mem-

![Fig. 6. S. pombe Ctr4 and Ctr5 co-localized to the plasma membrane in an interdependent manner. A, the epitope-tagged Ctr4 and Ctr5 proteins are functional. Strains with indicated genotypes were transformed with plasmids expressing the indicated Ctr4 and Ctr5 epitope-tagged proteins and grown either 5 days on EMM-Leu (glucose) medium or 7 days on YES-EG (glycerol/ethanol) medium and photographed. B, localization of Ctr4-GFP and Ctr5-GFP in wild type and ctr4Δ or ctr5Δ cells. FY254 (wild type (WT)), HZY2 (ctr5Δ), and HZY3 (ctr4Δ) cells were transformed with ctr4Δ-GFP-pSP1 (Ctr4-GFP), ctr4Δ- pSP2 (Ctr5-GFP), or ctr4Δ-pSP1 (Ctr4), as indicated. Nuclei were stained by Hoechst 33342. C, the deletion of Ctr5 does not affect the localization of an unrelated membrane protein, Lid1, fused to GFP. FY254 and HZY2 were transformed with pTE667 (Lid1-GFP) and photographed as described under “Experimental Procedures.”]
Copper Transport Complex in S. pombe.

**Fig. 7.** Ctr4 and Ctr5 are integral membrane proteins found in a complex. A, Ctr4 and Ctr5 are integral membrane proteins. FY254 (wild type) S. pombe cells were transformed with ctr4-FLAG-pSP1 and ctr5-myc-pSP2 plasmids. Cell lysate was made by vortexing the cells with glass beads and centrifugation was performed by 100,000 × g for 30 min. The supernant was loaded in lane 2, and the pellet was re-suspended and treated with buffer, 0.2 M Na₂CO₃, or 1% Triton X-100 for 30 min on ice. After re-centrifugation by 100,000 × g for 2 h, each of the pellets were re-dissolved and loaded with supernatants as indicated (P indicates pellet and S supernatant). 20 μg of protein was loaded for each lane except lane 4, where all protein was loaded. B, co-immunoprecipitation of Ctr4 and Ctr5. FY254 was transformed with the indicated combination of ctr4-FLAG-pSP1, ctr5-myc-pSP2 (as indicated by + above the corresponding lanes) or control vectors (indicated by −). Cells were harvested and Triton X-100-solubilized membrane preparations subjected to immunoprecipitation (lanes marked IP) and immunoblotting as described under “Experimental Procedures.” 15 μg of total lysate protein was loaded as comparison (lanes labeled Total). Immunoprecipitation and immunoblotting were performed using 9E10 anti-c-myc or M2 anti-FLAG antibody, as indicated. Numbers on the left indicate molecular size markers (in kDa). Asterisks indicate the position of the immunoglobulin heavy chain.

Because both Ctr4 and Ctr5 are integral membrane proteins that co-localize to the plasma membrane in an obligate fashion and likely function in the same pathway for copper uptake, we ascertained whether Ctr4 and Ctr5 are physically associated. S. pombe cells expressing epitope-tagged Ctr4 and Ctr5 were used to prepare Triton X-100-solubilized total cell extracts. The data in Fig. 7B show that Ctr4-FLAG₂ and Ctr5-myc₄ were detected as a single band of ~58 kDa and a doublet of ~30 and 36 kDa in whole total extracts, respectively (Fig. 7B, lanes 2–4). Both proteins migrated in SDS-polyacrylamide gel electrophoresis at a larger apparent molecular weight than predicted by their primary sequences, possibly due to glycosylation or other post-translational modifications. Immunoprecipitation of Ctr5-myc₄ resulted in the co-immunoprecipitation of Ctr4-FLAG₂ (Fig. 7B, top panel, lane 8) and immunoprecipitation of Ctr4-FLAG₂ co-fractionated with Ctr5-myc₄ (Fig. 7B, bottom panel, lane 8). Taken together, these data support the hypothesis that S. pombe Ctr4 and Ctr5 are directly or indirectly physically associated, and form a high affinity copper transport complex at the plasma membrane.

**DISCUSSION**

Previous work on the mechanisms for copper transport across the plasma membrane has resulted in the identification of related proteins from bakers' yeast, fission yeast, mice, and humans that play a key role in this process. Although experimental evidence suggests that Ctr1 and Ctr3 from S. cerevisiae (12–15) and human Ctr1² homomultimerize when isolated from cells, no reports to date demonstrate that high affinity copper transport requires a heteromeric protein complex. In this work, we identify a new protein denoted Ctr5 that acts in concert with Ctr4 in copper uptake in S. pombe. Ctr4 and Ctr5 form a dual partner complex, and the presence of both subunits is required for appropriate localization to the plasma membrane and function of the complex in copper uptake. This represents the first such example of a copper transport complex containing at least two distinct partners. The existence of Ctr4 and Ctr5 proteins distinguishes the S. pombe copper transport machinery from that previously established in S. cerevisiae, where two high affinity transporters work independently and appear to be functionally redundant (14).

The involvement of both Ctr4 and Ctr5 in high affinity copper uptake is supported by a number of observations. First, both the ctr4−(17) and ctr5− genes are regulated by copper availability. As may be expected for proteins involved in copper uptake, these genes are activated in response to copper scarcity and repressed in response to copper excess. Both previously reported genetic experiments for ctr4− (17) and data presented here for ctr5− demonstrate that this regulation requires the function of the S. pombe copper-sensing transcription factor Cuf1. Furthermore, both the ctr4− and ctr5− promoters harbor Cuf1-responsive cis-acting DNA sequences (22). Second, the Ctr4 and Ctr5 proteins have homology throughout their primary sequence and specifically harbor amino-terminal Mets motifs that may play a role in copper uptake. Third, growth assays on respiratory carbon sources and ⁶⁴Cu uptake experiments demonstrate that both Ctr4 and Ctr5 must be functional for S. pombe cells to carry out high affinity copper transport. Finally, the Ctr4 and Ctr5 proteins are both polytopic membrane proteins that physically associate and are interdependent for co-localization to the plasma membrane. Whether Ctr4 and Ctr5 engage in direct or indirect interactions is currently unknown. Although our results suggest that specific levels of Ctr4 and Ctr5 are important for the complex to properly localize to the plasma membrane, the exact stoichiometry of these two partners in the complex is unknown. Nor is it clear yet whether there are other components in the high affinity copper transport complex.

The Ctr5 protein was identified based on its ability to alleviate a block in Ctr4 secretion to the plasma membrane, and complement high affinity copper uptake defects when expressed in S. cerevisiae cells. Although this is the first such example of a protein complex involved in high affinity copper uptake, previous studies have identified components of a high affinity iron transport complex at the plasma membrane in S. cerevisiae (32–35). Within this complex, the Fet3 protein serves as a multi-copper ferroxidase, whereas the Ftr1 protein is thought to comprise an iron-binding permease component. Indeed, similar to the features of Ctr4 and Ctr5 in S. pombe, the Fet3-Ftr1 proteins are both required for high affinity iron up-

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² J. Lee and D. J. Thiele, manuscript in preparation.

Beaudoin, J., and Labbé, S. (2001) J. Biol. Chem. 276, 15472–15480.
take, are regulated at the level of gene transcription by iron, and are interdependent for secretion to the S. cerevisiae plasma membrane. A homologous iron transport complex, containing the Fet3-Ftr1-related proteins Fet5 and Fth1, has been found on the S. cerevisiae vacuolar membrane and is thought to pump iron from the vacuolar lumen to the cytosol (36). Most relevant to the S. pombe Ctr4-Ctr5 copper transport complex, expression of the S. pombe Fet3 homologue Fio1 in S. cerevisiae fails to rescue the iron uptake defect of a fet3Δ strain. However, co-expression of Fip1, the S. pombe Ftr1 homologue, can reconstitute high affinity iron transport in fet3Δ cells (37), suggesting that, although these are functionally homologous proteins, they cannot engage in critical interactions across species. Perhaps a protein functionally, but not structurally, homologous to Ctr5 exists in S. cerevisiae, which is not capable of engaging in productive interactions with the S. pombe Ctr4 transport subunit.

The existence of Ctr4 and Ctr5 in a high affinity copper transport complex at the S. pombe plasma membrane raises a number of interesting questions. First, what are the respective roles of these two proteins in the complex? Thus far we have not identified any functional domains in Ctr4 or Ctr5 except the potential copper-binding Mets motifs. However, it is possible that Ctr4 and Ctr5 serve different functional roles in the complex during copper uptake, in addition to their interdependent roles in protein trafficking. Second, are there homologous subunits in a high affinity copper transport complex in other eukaryotes? Indeed, data base searching with both Ctr4 and Ctr5 from S. pombe has revealed the presence of multiple open reading frames, in several organisms, with varying degrees of homology to both proteins. However, whether homologous subunits exist in other eukaryotes will require a structure-function analysis of Ctr4 and Ctr5 to identify critical functional residues that may be conserved across species, as well as in vivo functional assays for copper transport. Furthermore, it will be important to ascertain whether there are additional components present in the Ctr4-Ctr5 complex, to determine the precise role of each component in the function and regulation of high affinity copper transporter, and to explore the possibility that defects in these subunits are associated with pathophysiological states in humans.

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