

# Biochemical and Genetic Analyses of Yeast and Human High Affinity Copper Transporters Suggest a Conserved Mechanism for Copper Uptake\*

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**The redox active metal copper is an essential cofactor in critical biological processes such as respiration, iron transport, oxidative stress protection, hormone production, and pigmentation. A widely conserved family of high affinity copper transport proteins (Ctr proteins) mediates copper uptake at the plasma membrane. However, little is known about Ctr protein topology, structure, and the mechanisms by which this class of transporters mediates high affinity copper uptake. In this report, we elucidate the topological orientation of the yeast Ctr1 copper transport protein. We show that a series of clustered methionine residues in the hydrophilic extracellular domain and an MXXXM motif in the second transmembrane domain are important for copper uptake but not for protein sorting and delivery to the cell surface. The conversion of these methionine residues to cysteine, by site-directed mutagenesis, strongly suggests that they coordinate to copper during the process of metal transport. Genetic evidence supports an essential role for cooperativity between monomers for the formation of an active Ctr transport complex. Together, these results support a fundamentally conserved mechanism for high affinity copper uptake through the Ctr proteins in yeast and humans.**

Copper is a transition metal present as a catalytic cofactor in many enzymes including cytochrome *c* oxidase, Cu,Zn-superoxide dismutase, lysyl oxidase, dopamine  $\beta$ -hydroxylase, tyrosinase, or multicopper ferroxidases such as ceruloplasmin in mammals or Fet3 in *Saccharomyces cerevisiae*. However, with excess accumulation, copper generates hydroxyl radicals that damage cells at the level of nucleic acids, proteins, and lipids (1). Therefore, cells have developed sophisticated homeostatic mechanisms to control intracellular copper accumulation and distribution (2–4). In humans copper imbalances are manifest in severe genetic disorders such as Menkes syndrome and Wilson disease, which are characterized by the inappropriate distribution of copper to cells and tissues (5, 6).

The bakers' yeast *S. cerevisiae* is an excellent model system

to study copper transport and distribution in cells. In yeast, extracellular Cu(II) is reduced to Cu(I) by cell surface metallo-reductases (7, 8) and is transported into the cell by two independent high affinity copper transporter (Ctr)<sup>1</sup> proteins, Ctr1 and Ctr3 (9, 10). Copper distribution to specific target enzymes is mediated by at least three independent copper chaperones (for review, see Refs. 4, 11, and 12). The Atx1 metallochaperone mediates copper delivery to proteins localized in the secretory pathway such as Fet3, a multicopper ferroxidase essential for high affinity iron uptake (13, 14). Ccs is a specific chaperone responsible for copper insertion into Cu,Zn superoxide dismutase (15). Cox17 targets copper to the mitochondria, where it is utilized by cytochrome *c* oxidase (16). Therefore, cells deficient in high affinity copper uptake show phenotypes associated with all three copper delivery pathways: lack of growth in copper- or iron-limited conditions, oxidative stress sensitivity, and respiratory deficiency (9, 17).

Genes encoding high affinity ( $K_m$  1–5  $\mu$ M) copper transport proteins have been identified in *S. cerevisiae*, *Schizosaccharomyces pombe* (18, 19), mammals (20, 21), and plants (22). With the exception of yeast Ctr3, all Ctr family members are rich in methionine residues within the amino-terminal hydrophilic portion of the protein (18). It has been suggested that these residues, arranged as MXXXM or MXM (Mets motifs), could be involved in extracellular copper binding (10), but experimental evidence supporting a role for these methionine-rich sequences in copper transport is currently lacking. According to computer algorithm analyses, Ctr family members contain three membrane-spanning domains, within which most of protein sequence similarity lies. Biochemical and microscopy experiments in yeast and human cells have suggested that Ctr family members are integral membrane proteins that assemble as multimers and localize to the plasma membrane (17, 23, 24).

Given the recent demonstration of the essentiality of mouse Ctr1 for embryonic development (25, 26) and the conservation of Ctr1 primary sequence from yeast to humans, it is important to characterize the structure and function of this conserved family of high affinity copper transport proteins to gain insight into their mechanism of action. In the current study we elucidate the topological orientation of the *S. cerevisiae* Ctr1. We also identify conserved methionine residues in both yeast and mammalian Ctr1 which are essential for copper import. Interestingly, specific amino acid substitutions strongly suggest that these methionine residues may coordinate copper during the process of copper transport. The results of these studies support a similar mechanism for copper transport for members of the Ctr family of copper transporters from yeast to humans.

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<sup>1</sup> The abbreviations used are: Ctr, copper transporter; BCS, bathocuproine disulfonic acid; GFP, green fluorescent protein; HEK, human embryonic kidney; TMD, transmembrane domain; wt, wild type.

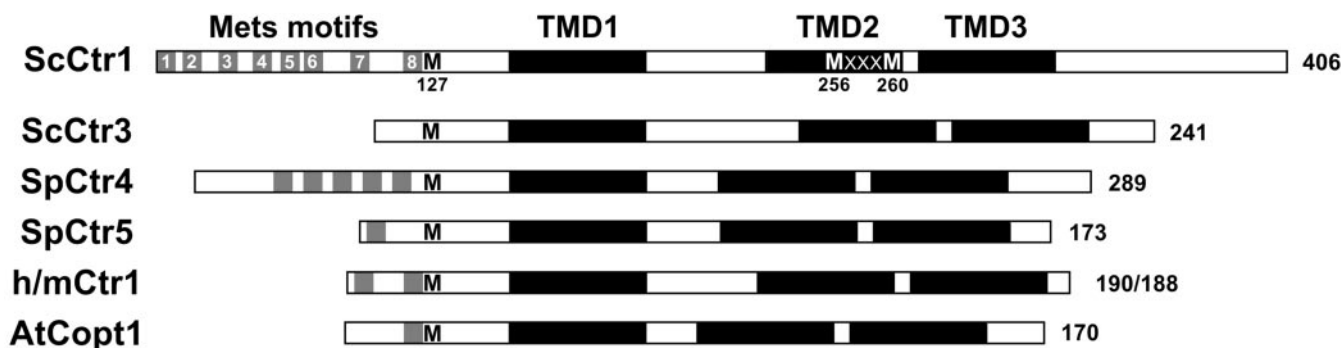


FIG. 1. Model for the primary structure of Ctr high affinity copper transport proteins. Alignment of copper transport proteins from *S. cerevisiae* (ScCtr1, ScCtr3), *S. pombe* (SpCtr4, SpCtr5), human and mouse (h/mCtr1), and *A. thaliana* (AtCopt1). Conserved features in the primary structure are represented from the amino terminus (left) to carboxyl terminus (right). All proteins contain three putative transmembrane domains (TMD1–3) shown in black. With the exception of yeast Ctr3, all members of the Ctr family of copper transporters contain putative copper binding motifs, called Mets motifs (gray boxes), consisting of 3–5 methionine residues arranged as MXXXM and/or MXM. Yeast and human Ctr1 proteins contain eight and two Mets motifs, respectively. Other conserved features, Ctr1 Met-127 and the MXXXM motif in TMD2, are represented in black and white characters, respectively. The length of each protein in amino acids is shown on the right.

#### EXPERIMENTAL PROCEDURES

**Yeast Strains and Growth Conditions**—Yeast strain MPY17 (27), lacking both the Ctr1 and Ctr3 high affinity copper transporters, was used for functional complementation and yeast  $^{64}\text{Cu}$  uptake experiments. Yeast strain 66 (23), a kind gift from Andrew Dancis (University of Pennsylvania), was used for yeast Ctr1 topological studies. Yeast strains were transformed with centromeric plasmids and maintained in minimal selective medium (SC) with adequate requirements. Media were solidified with 1.5% agar.

**Human Embryonic Kidney (HEK293) Cell Culture and Transient Transfections**—HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum under 5%  $\text{CO}_2$ . Cells were transfected with the pcDNA3.1(+) empty vector or the same vector carrying the wild type or mutant hCtr1 alleles under control of the cytomegalovirus promoter. Transfections were performed using FuGENE 6 (Roche) according to the manufacturer's instructions. 24 h after transfection, cells were collected and divided evenly for copper uptake assays and Western blotting analysis.

**Plasmids**—Wild type yeast *CTR1* and *CTR3* open reading frames and yeast *CTR* gene mutant alleles containing either amino-terminal deletions (*CTR1* $\Delta$ M genes) or site-specific mutations (*CTR1*M or *CTR3*M genes) were amplified by PCR with *Pfu* Turbo DNA polymerase (Stratagene). *Sma*I and *Bam*HI sites were used to clone the PCR product in the corresponding vector, p416GPD (see Fig. 2) or p416TEF (see Figs. 3–5). For coexpression of wild type and mutant *CTR1* or *CTR3* (see Fig. 6), genes were subcloned into the p413TEF plasmid by using *Bam*HI-*Xho*I restriction sites. The hCtr1 cDNA subcloned into the pcDNA 3.1(+) (Invitrogen) or p413GPD expression vectors was described previously (21, 24). For amino-terminal epitope tagging of hCtr1, the upstream primer containing sequences encoding the c-Myc epitope after the translation initiation codon was used for PCR amplification of the hCtr1 open reading frame. PCR products were inserted into the *Eco*RI and *Xho*I sites in the pcDNA3.1(+) and p413GPD vectors for the expression of hCtr1 in HEK293 cells and in *S. cerevisiae*, respectively. Site-directed mutagenesis of the *S. cerevisiae* *CTR1* and *CTR3* and human Ctr1 genes was performed by the overlap extension method (28).

**Functional Complementation of *S. cerevisiae* *ctr1* $\Delta$ *ctr3* $\Delta$  Mutants**—The function of the mutated CTR alleles was compared with the wild type allele by complementation studies in *S. cerevisiae* strain MPY17 harboring deletions of the *CTR1* and *CTR3* high affinity Ctr genes (27). MPY17 cells transformed with plasmids expressing wild type or mutant alleles of yeast *CTR1*, *CTR3*, or human Ctr1 were grown to exponential phase ( $A_{600\text{ nm}} = 0.8\text{--}1.2$ ) in selective media. Cells were plated as drops ( $A_{600\text{ nm}} = 0.1$ , and serial dilutions in 10-fold increments) on selective media, ethanol/glycerol (YPEG: 1% yeast extract, 2% Bactopeptone, 2% ethanol, 3% glycerol), without or with 100  $\mu\text{M}$   $\text{CuSO}_4$  (YPEG + Cu) or with 20–200  $\mu\text{M}$  bathocuproine disulfonic acid (BCS) (YPEG + BCS). Plates were incubated for 3–6 days at 30  $^\circ\text{C}$  and photographed. The function of the epitope-tagged Ctr alleles was compared with the wild type allele by complementation studies in the *S. cerevisiae* strain MPY17 and by  $^{64}\text{Cu}$  uptake assays in transformed yeast cells or transfected HEK293 cells.

**$^{64}\text{Cu}$  Uptake Assays**—For copper uptake measurements in yeast MPY17, cells expressing yeast *CTR1* or *CTR3* genes under the control

of the constitutive TEF promoter (p416TEF and/or p413TEF vectors) were grown in selective media (SC-ura, or SC-ura-His) to exponential phase ( $A_{600\text{ nm}} = 0.5\text{--}1.5$ ). Radioactive  $^{64}\text{Cu}$  (specific activity of 15–30 mCi/ $\mu\text{g}$  of  $\text{CuCl}_2$ , Mallinckrodt Institute of Radiology at Washington University, Saint Louis) was added to 1 ml of cell culture to a final concentration of 5  $\mu\text{M}$   $\text{CuCl}_2$ , and cells were incubated at room temperature or on ice for 10 min. Time course experiments showed that the rate of copper uptake was linear for at least 20 min. Samples were quenched by adding ice-cold EDTA (10 mM final concentration), filtered, and washed twice with quenching buffer (10 mM EDTA in 0.1 M Tris-succinate, pH 6.0). Cell-associated  $^{64}\text{Cu}$  was quantified in a  $\gamma$ -counter (Packard Cobra II) and calibrated to a standard curve. Values obtained on ice were subtracted from room temperature values and normalized to cell number. Experiments were repeated independently at least twice in triplicate and the S.D. represented.  $^{64}\text{Cu}$  uptake assays in HEK293 cells expressing hCtr1 were performed as described previously (24).

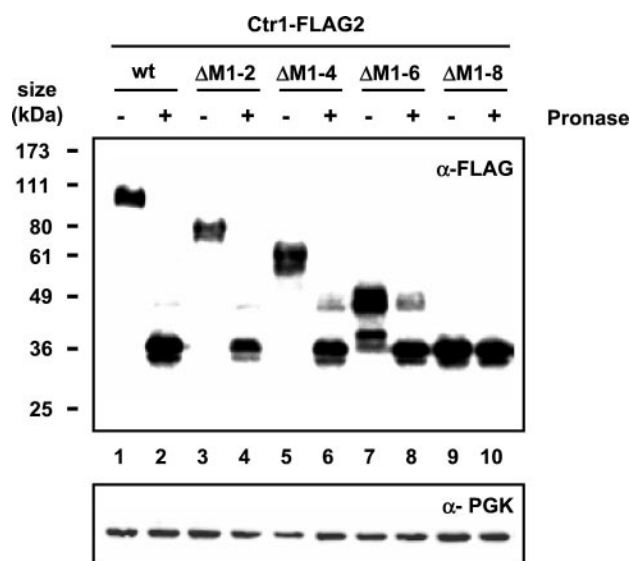
**Western Blotting Analysis**—Total protein was extracted from HEK293 cells 2 days after transfection with the c-Myc epitope-tagged hCtr1 expression plasmid. Cells were washed in phosphate-buffered saline and resuspended in lysis buffer (phosphate-buffered saline, pH 7.4, containing 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and protease inhibitor mixture (Roche)). Cells in lysis buffer were incubated at 4  $^\circ\text{C}$  for 20 min, and the supernatant was taken after centrifugation at 4  $^\circ\text{C}$  for 20 min at 16,000  $\times g$ . 50  $\mu\text{g}$  of protein was heated in SDS sample buffer at 37  $^\circ\text{C}$  for 10 min and fractionated by 15% SDS-PAGE. Monoclonal anti-c-Myc antibody (Roche) was used to detect hCtr1-Myc protein by immunoblotting.

**Protease Shaving Experiments**—The treatment of intact yeast cells with Pronase protease (Calbiochem-Novabiochem) and subsequent protein extraction were performed as described previously (29). Samples were separated in 10% SDS-PAGE, and Ctr1 protein was visualized by immunoblotting with anti-FLAG antibody (Sigma). As a control for cell integrity during exposure to Pronase, protein extracts were monitored for Pronase accessibility to phosphoglycerate kinase protein by immunoblotting with anti-phosphoglycerate kinase antibody (Molecular Probes).

**Fluorescence Microscopy**—Indirect immunofluorescence to localize c-Myc epitope-tagged hCtr1 was performed as described previously (24). For subcellular localization in yeast, Ctr1 and Ctr3 proteins were tagged with GFP at the carboxyl terminus as described previously (17). MPY17 cells expressing the GFP fusion proteins were grown in selective media to exponential cell phase. Fluorescence signals were visualized using a Nikon Eclipse E800 fluorescence microscope equipped with a Hamamatsu ORCA-2 cooled CCD camera. Images were processed using Adobe Photoshop 5.5 software.

#### RESULTS

**Common Features of High Affinity Copper Transport Proteins**—An alignment of copper transport proteins including *S. cerevisiae* Ctr1 and Ctr3, *S. pombe* Ctr4 and Ctr5, human Ctr1 (hCtr1), mouse Ctr1 (mCtr1), and *Arabidopsis thaliana* COPT1 (AtCopt1) shows several conserved structural features summarized in Fig. 1. Most of the primary structural similarity among Ctr family members lies in two regions: the conserved trans-



**FIG. 2. Topological orientation of the yeast Ctr1 protein.** Serial amino-terminal deletions of yeast Ctr1 were constructed by removing 25 ( $\Delta M1-2$ ), 66 ( $\Delta M1-4$ ), 101 ( $\Delta M1-6$ ), or 126 amino acid residues ( $\Delta M1-8$ ). Ctr1wt and Ctr1 amino-terminal deletion mutant alleles were epitope-tagged at the carboxyl terminus with two copies of the FLAG epitope. *S. cerevisiae* strain 66 expressing Ctr1wt or mutant alleles fused to the FLAG2 epitope under the control of the constitutive GPD promoter was grown to exponential phase, and cells were treated (+) or not (-) with Pronase protease (Calbiochem-Novabiochem). After protein extraction and SDS-PAGE, Ctr1 protein was detected by immunoblotting with anti-FLAG antibody. Protein extracts were immunoblotted with anti-phosphoglycerate kinase ( $\alpha$ -PGK) antibody as a control for protein loading and cell integrity. Molecular mass standards are indicated on the left.

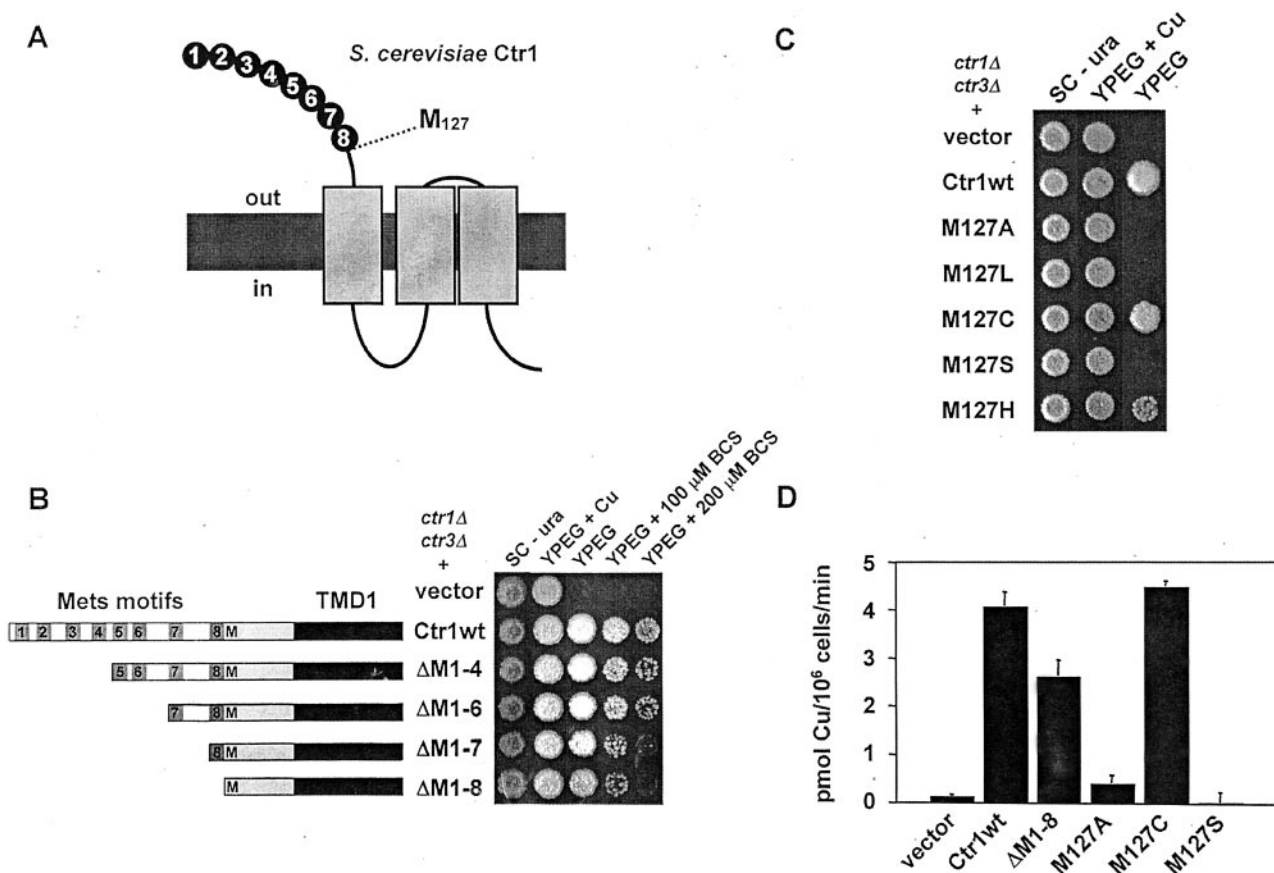
membrane domains 2 and 3 (Fig. 1, TMD2 and TMD3), with 20–40% identity) and a more variable region including transmembrane domain 1 (Fig. 1, TMD1) and the 20 preceding residues (Fig. 1, region from the Ctr1 Met-127 residue to the beginning of TMD1), with 15–50% identity. With the exception of Ctr3, the amino-terminal region of Ctr proteins is rich in motifs containing 3–5 methionine residues arranged as MXXM and MXM, where M represents methionine, and X is any amino acid (Fig. 1, Mets motifs shown in gray). Interestingly, 3 methionine residues are conserved in all known Ctrs: yeast Ctr1 Met-127 residue (Fig. 1, M127 in black characters), which is the last methionine in the Ctr1 Mets motifs and is located 20 amino acids upstream from the putative start of TMD1; and an MXXXM motif (where X represents a hydrophobic residue) located in TMD2 (Fig. 1, MXXXM).

**Proteolytic Mapping of *S. cerevisiae* Ctr1 Topology**—Based on computer algorithm analysis and electron microscopic localization of a carboxyl-terminal tagged version of yeast Ctr1 (10), the topological structure proposed for the Ctr family of copper transporters would locate the amino-terminal domain rich in methionine residues outside of the cell and the carboxyl-terminal region in the cytosol (4). To test this prediction experimentally we carried out protein shaving experiments with Pronase, a protease that enters the yeast periplasmic space but does not traverse the plasma membrane. Yeast Ctr1 protein was tagged at its carboxyl terminus with two copies of the FLAG epitope. Ctr1-FLAG2 protein was able to complement the respiratory deficiency of a *ctr1Δctr3Δ* yeast strain completely (data not shown). Cells expressing Ctr1-FLAG2 were grown to exponential phase, whole cells were treated with Pronase, proteins extracted, and Ctr1 detected by SDS-PAGE and immunoblotting with anti-FLAG antibody. In the absence of Pronase treatment, Ctr1 protein migrates with a molecular mass of ~100 kDa (Fig. 2, lane 1). After treatment with Pronase, the Ctr1

band shifted to an apparent molecular mass of ~35 kDa (Fig. 2, lane 2), indicating that a portion of the protein was accessible to proteolysis and demonstrating that the FLAG epitope-tagged Ctr1 carboxyl terminus is located in the cytosol and protected from degradation with an extracellular protease. The levels of phosphoglycerate kinase, an intracellular enzyme, were comparable in the absence or presence of Pronase, indicating the integrity of the cells used in these experiments.

Although many distinct yeast Ctr1 alleles with different carboxyl-terminal epitope tags were functional as assessed by complementation studies, none of the amino-terminal tags assayed was able to complement a *ctr1Δctr3Δ* mutant or was properly localized to the plasma membrane (data not shown). Thus, we used a different strategy to ascertain the localization of the yeast Ctr1 amino terminus with respect to the plasma membrane. Yeast Ctr1 protein migrates with an apparent molecular mass of ~100 kDa, considerably larger than the predicted size of 46 kDa. Biochemical and genetic studies suggested that this aberrant migration is a consequence of *O*-glycosylation (23). A possible Ctr1 target for *O*-linked sugar modification is the amino-terminal portion of the protein which is rich in serine residues. A series of amino-terminal deletions of Ctr1 was constructed by removing 25 ( $\Delta M1-2$ ), 66 ( $\Delta M1-4$ ), 101 ( $\Delta M1-6$ ), or 126 residues ( $\Delta M1-8$ ), and proteins were epitope tagged at the carboxyl terminus with two copies of the FLAG sequence. As described previously for Ctr1-FLAG2, cells were treated (+) or not (-) with Pronase, and Ctr1 was detected by immunoblotting with anti-FLAG antibody. Deletion of amino-terminal residues decreased the size of the protein (Fig. 2, lanes 3, 5, 7, and 9); however, the reduction in protein size was greater than expected, based on the number of amino acid residues removed. The Ctr1 $\Delta M1-8$  protein decreased ~65 kDa in apparent size (from 100 kDa for wild type to 35 kDa; Fig. 2, lanes 1 and 9), instead of 13 kDa as expected for the 126-amino acid deletion. This suggests that the yeast Ctr1 amino terminus is glycosylated and, accordingly, localized outside of the cell. Additionally, the molecular mass for Ctr1 $\Delta M1-8$  (35 kDa) is similar to the protease-protected band obtained for both wild type Ctr1 and amino-terminal deletions (Fig. 2, lanes 2, 4, 6, 8, 9, and 10), indicating that the amino-terminal region removed in Ctr1 deletions is an extracellular domain accessible to proteolysis. From these results we can conclude that the amino terminus of yeast Ctr1 is located outside of the cell. Furthermore, the fluorescence signal of an amino-terminally c-Myc epitope-tagged human Ctr1 protein, expressed in HEK293 cells, was observed at the periphery of both permeabilized and nonpermeabilized cells, suggesting that the amino-terminal region in human Ctr1 protein is also exposed to the extracellular face of the plasma membrane in transfected HEK293 cells (data not shown).

**Requirement for Yeast Ctr1 Methionine-rich Amino Terminus under Conditions of Copper Limitation**—Yeast Ctr1 and other members of the Ctr family of copper transporters are rich in methionine residues, arranged as MXXM and MXM motifs containing 3–5 methionine residues (Mets motifs) within the amino-terminal domain (Fig. 1). Based on homology with motifs present in bacterial copper homeostasis proteins such as *Enterococcus hirae* CopB (30), *Pseudomonas syringae* CopA (31), or *Escherichia coli* CutE (32), it is possible that these Mets motifs could play a role in copper binding (10, 33). To ascertain the function of the extracellular amino-terminal portion of yeast Ctr1 containing eight Mets motifs (Fig. 3A), we tested alleles encoding serial amino-terminal deletions of the protein: Ctr1 $\Delta M1-4$  (deletion of 66 amino-terminal residues), Ctr1 $\Delta M1-6$  (deletion of 101 amino-terminal residues), Ctr1 $\Delta M1-7$  (deletion of 118 amino-terminal

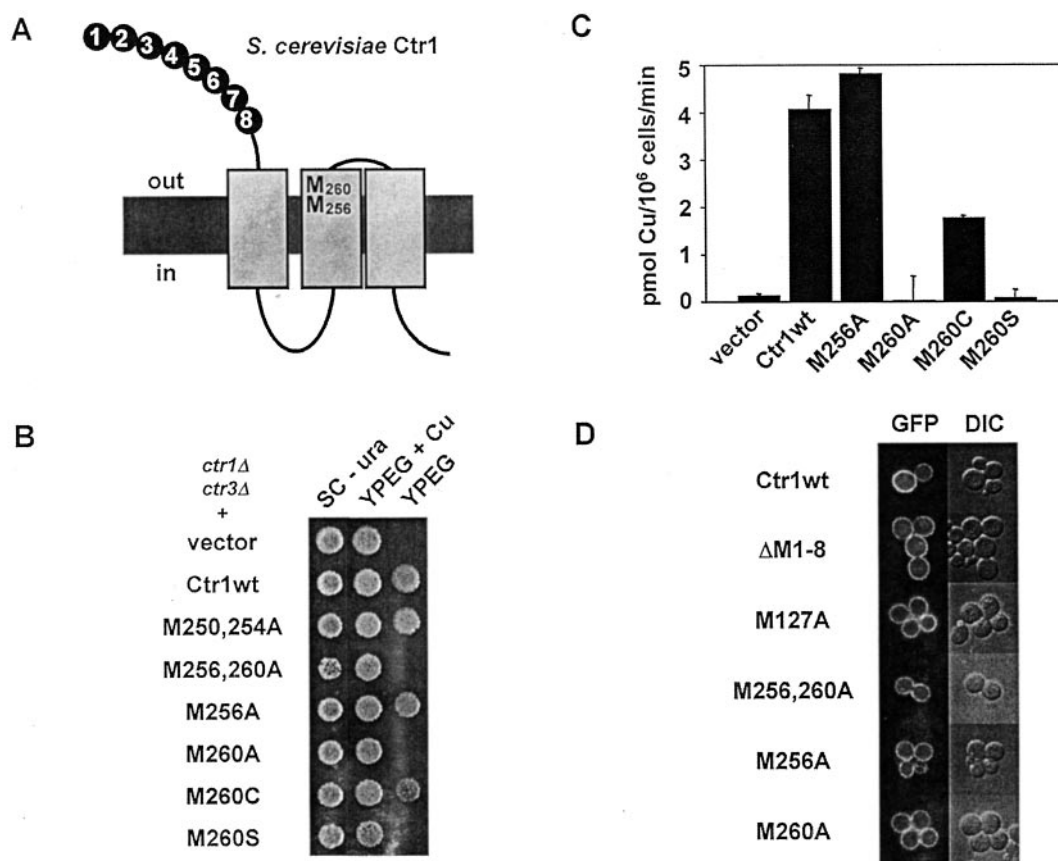


**FIG. 3. Conserved amino-terminal methionine residues in yeast Ctr1 are essential for copper transport.** *A*, proposed topological model for yeast Ctr1 protein. The amino-terminal domain containing eight Mets motifs (black circles) and Met-127 is located outside the cell. The carboxyl-terminal domain and loop between TMD1 and TMD2 are cytosolic. *B*, the yeast Ctr1 amino-terminal domain, rich in methionines, is required under copper-limiting conditions. MPY17 (*ctr1Δctr3Δ*) cells constitutively expressing different Ctr1 amino-terminal deletion mutants or containing p416TEF plasmid alone (vector) were grown in SC-ura to exponential phase and spotted onto SC-ura, YPEG + 100  $\mu$ M CuSO<sub>4</sub>, YPEG, YPEG + 100  $\mu$ M BCS, or 200  $\mu$ M BCS plates. The amino-terminal portion of each mutant is represented on the left. Cells were grown for 3 days at 30 °C. *C*, yeast Ctr1 Met-127 is essential for growth in ethanol/glycerol. MPY17 cells containing p416TEF (vector) or constitutively expressing Ctr1wt or Ctr1 Met-127 mutant alleles to alanine (M127A), leucine (M127L), cysteine (M127C), serine (M127S), and histidine (M127H) were assayed for growth on YPEG for 5 days at 30 °C and 3 days in SC-ura and YPEG + Cu. *D*, yeast Ctr1 Met-127 is essential for copper transport. MPY17 cells expressing Ctr1 mutant alleles were assayed for <sup>64</sup>Cu uptake. The rate of copper uptake is represented as pmol of Cu/10<sup>6</sup> cells/min. Uptake experiments were performed independently at least twice in triplicate, and the S.D. values are shown above the bars.

residues), and Ctr1ΔM1–8 (deletion of 126 amino-terminal residues), containing four, two, one, or no Mets motifs, respectively (Fig. 3B). Yeast cells lacking high affinity copper transporters (*ctr1Δctr3Δ*) cannot grow on nonfermentable carbon sources such as ethanol and glycerol because of functional defects in the mitochondrial copper-requiring enzyme cytochrome *c* oxidase in the respiratory chain and Fet3 ferroxidase activity, which is essential for high affinity iron transport. Consequently, a *ctr1Δctr3Δ* strain constitutively expressing these Ctr1 amino-terminal deletions was assayed for respiratory competence compared with the wild type Ctr1 (Ctr1wt) gene or vector alone (p416TEF) (Fig. 3B). Surprisingly, growth of cells expressing Ctr1 without any Mets motif (Ctr1ΔM1–8), in ethanol/glycerol, was indistinguishable from cells expressing Ctr1wt (Fig. 3B, YPEG), suggesting that the amino-terminal region of Ctr1 is not absolutely essential for the function of the transporter. Interestingly, the growth of cells lacking Ctr1 amino-terminal Mets motifs (Ctr1ΔM1–8) was defective when extracellular bioavailable copper was severely limited by the addition of the Cu(I)-specific extracellular chelator BCS (Fig. 3B, YPEG + 100 or 200  $\mu$ M BCS). The severity of this phenotype in copper-limiting conditions correlated directly with the extent of the amino-terminal deletion (Fig. 3B, compare wild type protein with deletion of four (Ctr1ΔM1–4), seven (Ctr1ΔM1–7), and eight

(Ctr1ΔM1–8) Mets motifs). As controls, cells were grown in selective medium lacking uracil (Fig. 3B, SC-ura) and in ethanol/glycerol medium containing 100  $\mu$ M CuSO<sub>4</sub> (Fig. 3B, YPEG + Cu), which restored growth of all of the strains. In terms of copper transport capacity, yeast cells expressing Ctr1ΔM1–8 showed an approximately one-third reduction in the rate of uptake compared with those expressing Ctr1wt (Fig. 3D, Ctr1wt compared with ΔM1–8). Importantly, all of the Ctr1 mutant proteins tagged with GFP at the carboxyl terminus were localized to the plasma membrane (Fig. 4D for Ctr1ΔM1–8-GFP), and immunoblotting demonstrated that all deletion mutants were expressed at similar levels (Fig. 2). Supporting the physiological relevance of these observations, a strain expressing Ctr1ΔM1–8 protein under the control of the *CTR1* promoter, where expression is induced in low copper media, also showed a defect in growth on YPEG under copper-limiting conditions (data not shown). These results indicate that the extracellular amino-terminal domain of Ctr1, harboring eight Mets motifs, plays an important role in copper uptake under copper-limiting conditions.

**Conserved Methionine 127 Is Essential for Yeast Ctr1 Function**—The Ctr family of copper transporters contains a highly conserved methionine residue in the amino-terminal portion of the protein (Fig. 1). In *S. cerevisiae* Ctr1, Met-127 is the last methionine of Mets motif 8 and is located 20 amino acids from



**FIG. 4. Yeast Ctr1 Met-260 in the conserved MXXXM motif is essential for copper transport.** *A*, proposed topological model for yeast Ctr1 protein. The amino-terminal domain containing eight Mets motifs (black circles) and the M<sup>256</sup>XXXM<sup>260</sup> motif located in the carboxyl portion of TMD2 are represented. *B*, growth of MPY17 (*ctr1Δctr3Δ*) cells containing p416TEF (*vector*), expressing Ctr1wt, or Ctr1 mutant alleles M250A, M254A and M256A, M260A and M256A, M260A, M260C, and M260S were assayed for growth in ethanol/glycerol (YPEG) plates. Spotted cells were grown on SC-ura and YPEG + 100  $\mu$ M CuSO<sub>4</sub> for 3 days at 30 °C and for 4 days in the case of YPEG. *C*, MPY17 cells expressing Ctr1wt or the mutant alleles shown were assayed for <sup>64</sup>Cu uptake. *D*, Ctr1wt, Ctr1 $\Delta$ M1–8, Ctr1 M127A, Ctr1 M256A, M260A, Ctr1 M256A, and Ctr1 M260A carboxyl-terminal GFP-tagged proteins are localized properly to the plasma membrane. MPY17 cells constitutively (p416TEF vector) expressing carboxyl-terminal GFP-tagged wild type and mutant Ctr1 proteins were grown in selective media to exponential phase and visualized by fluorescence microscopy. *DIC*, differential interference microscopy.

the beginning of TMD1. Yeast cells possess two methionine aminopeptidases that specifically remove the first methionine of a protein depending on the penultimate residue in the sequence (34). In the case of a small and uncharged penultimate residue, such as glycine, alanine, serine, cysteine, tryptophan, proline, or valine, the first methionine is cleaved and removed from the mature protein. Otherwise, the mature protein maintains its first methionine residue (35). Ctr1 $\Delta$ M1–8 truncated protein, which is able to complement the respiratory deficiency of a *ctr1Δctr3Δ* strain on YPEG medium, contains the conserved Met-127 as the first encoded residue of the protein (Fig. 3A). According to this aminopeptidase rule, Ctr1 $\Delta$ M1–8 protein, which contains a large asparagine residue as its penultimate amino acid, would maintain the first methionine in the mature protein. Interestingly, mutation of the penultimate residue of Ctr1 $\Delta$ M1–8 protein to glycine or valine, but not to leucine, abolished its ability to complement the copper transport deficiency of the *ctr1Δctr3Δ* strain functionally, suggesting an essential role for the first methionine of Ctr1 $\Delta$ M1–8 truncated protein (data not shown). To ascertain more clearly the role of Met-127 in Ctr1 function, we used site-directed mutagenesis of full-length Ctr1wt protein to convert Ctr1 Met-127 to alanine (M127A), leucine (M127L), cysteine (M127C), serine (M127S), or histidine (M127H). Plasmids expressing the mutant Ctr1 alleles were transformed into a *ctr1Δctr3Δ* strain and tested for growth in ethanol/glycerol and for their ability to drive <sup>64</sup>Cu uptake compared with Ctr1wt or the empty vector

(p416TEF). Cells expressing Ctr1 M127A were unable to grow in YPEG (Fig. 3C), and <sup>64</sup>Cu transport experiments showed a dramatic reduction in the rate of copper transport in cells expressing the Ctr1 M127A mutant allele compared with Ctr1wt (Fig. 3D). The Ctr1 M127A mutant was epitope-tagged with GFP at the carboxyl terminus, and the protein was properly localized, as ascertained by fluorescence microscopy, to the plasma membrane (Fig. 4D, M127A). The same results were obtained for a leucine replacement of Met-127 (Fig. 3C, data not shown). These data indicate that Met-127, a methionine residue that is positionally conserved in virtually all known Ctrs, is essential for Ctr1 function as a high affinity copper transport protein.

What could be the role for a conserved methionine in the mechanism of copper transport? Our results in this report suggest that yeast Ctr1 methionine-rich regions (Mets motifs) could be important for extracellular copper binding prior to the transport of the metal inside the cell. To test whether Met-127 could function as a copper ligand, we mutationally altered the Met-127 residue to cysteine or serine. Cysteines, but not serine residues, are widely found as copper ligands in many proteins involved in copper homeostasis (36, 37). The *ctr1Δctr3Δ* strain expressing the Ctr1 M127C mutant protein was able to grow in ethanol/glycerol medium and transported <sup>64</sup>Cu at a rate similar to that of the Ctr1wt protein (Fig. 3, C and D). This complementation of respiratory deficiency and copper transport capacity was not observed in the case of the Ctr1 M127S mutant.

These results suggest that yeast Ctr1 protein requires a copper ligand in position 127 such as methionine or cysteine, rather than similar residues such as serine, unable to coordinate copper, or bulkier hydrophobic residues such as leucine. Additionally, replacement of Met-127 by histidine (M127H), another potential copper ligand, partially rescued the *ctr1Δctr3Δ* respiratory deficiency (Fig. 3C). Taken together, these results strongly suggest that yeast Ctr1 Met-127 coordinates copper in the process of metal transport across the plasma membrane.

**Transmembrane Methionine Residues in the Conserved MXXXM Motif Are Essential for the Copper Transport Activity of Ctr Proteins**—Alignment of Ctr proteins from different organisms demonstrates the presence of a conserved MXXXM motif in the carboxyl-terminal portion of TMD2 (Fig. 1). According to yeast Ctr1 topology predictions, the MXXXM motif is located closer to the extracellular milieu than to the cytosolic face of the plasma membrane (Fig. 4A). To address a possible role for this motif in copper transport, we replaced each conserved methionine residue in yeast Ctr1, Met-256 and Met-260, with alanine (Ctr1 M256A, M260A). *ctr1Δctr3Δ* yeast cells expressing this double mutant allele were unable to grow in ethanol/glycerol medium (Fig. 4B, M256A, M260A), suggesting an important role for the MXXXM motif in yeast Ctr1 function. A carboxyl-terminal GFP-tagged version of this protein was localized properly to the plasma membrane when expressed in *ctr1Δctr3Δ* cells (Fig. 4D). Ctr1 protein also contains an additional MXXXM motif in TMD2, which does not align with other Ctr proteins. Mutagenesis of both methionines in this motif (Met-250 and Met-254) to alanine did not affect the function of Ctr1 protein, as shown by complementation of the respiratory deficiency in the *ctr1Δctr3Δ* strain (Fig. 4B, M250A, M254A).

To address the individual roles of Met-256 and Met-260 in the Ctr1 conserved MXXXM motif, we expressed single mutants in the *ctr1Δctr3Δ* strain and tested for function. Although expression of Ctr1 M256A mutant protein restored the respiratory deficiency of *ctr1Δctr3Δ* cells, expression of Ctr1 M260A did not allow growth in ethanol/glycerol medium (Fig. 4B, M256A and M260A). This result was corroborated by <sup>64</sup>Cu uptake experiments, in which the Ctr1 M256A mutant showed rates of uptake similar to Ctr1wt (Fig. 4C). On the other hand, Ctr1 M260A was not active in <sup>64</sup>Cu transport assays (Fig. 4C), whereas both proteins fused to GFP were properly localized to the plasma membrane (Fig. 4D). These results demonstrate that Met-260 in yeast Ctr1 is essential for function in copper transport.

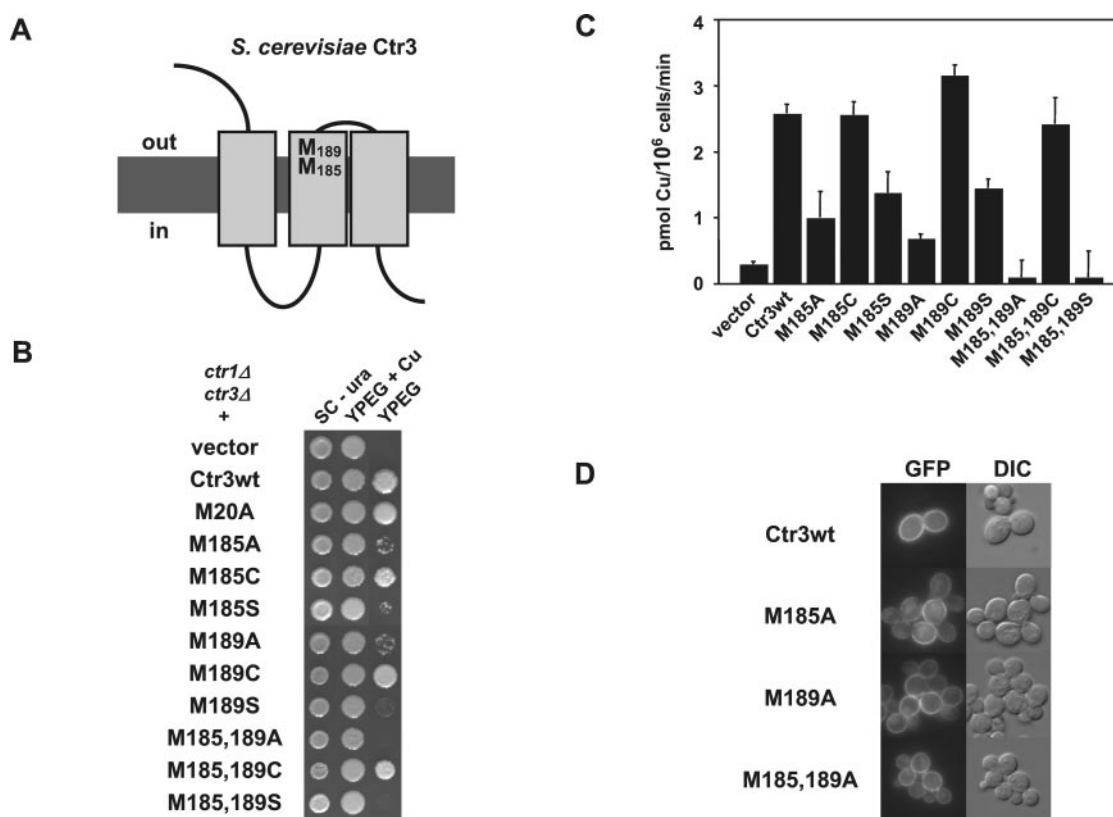
To gain insight into the potential role played by Ctr1 residue Met-260 in the mechanism of copper uptake, this residue was mutationally altered to cysteine or serine. Expression of the Ctr1 M260C mutant partially rescued both growth in ethanol/glycerol and the copper uptake defects of *ctr1Δctr3Δ* cells (Fig. 4, B and C, M260C). Similar to Met-127, mutagenesis of Met-260 to serine completely abolished Ctr1 function as a copper transporter (Fig. 4, B and C, M260S). These results strongly suggest that Ctr1 Met-260, predicted to lie within TMD2, may coordinate copper in the process of metal translocation through the plasma membrane.

*S. cerevisiae* encodes two independent plasma membrane high affinity copper transporters, Ctr1 and Ctr3 (9, 10). Despite these proteins being functionally redundant, their sequence similarity is very limited (9). Most homology lies within TMD2 and TMD3, including the MXXXM motif. The amino-terminal portion of Ctr3 protein is quite different from other members of the Ctr family in terms of the absence of any Mets motifs. Curiously, Ctr3 contains a methionine (Ctr3 Met-20) that aligns with yeast Ctr1 Met-127, but mutagenesis to alanine (Ctr3 M20A) and expression in *ctr1Δctr3Δ* cells did not affect

growth in ethanol/glycerol (Fig. 5B). However, mutagenesis of both methionine residues in the Ctr3 transmembrane MXXXM motif to alanine and subsequent expression in yeast cells deficient in high affinity copper transport did not support growth in ethanol/glycerol and demonstrated significant defects in the rate of <sup>64</sup>Cu uptake (Fig. 5, B and C, M185A, M189A). Expression of either individual mutant Ctr3 M185A or Ctr3 M189A in the *ctr1Δctr3Δ* strain only weakly rescued growth in YPEG and copper transport activity (Fig. 5, B and C, M185A and M189A), indicating an important role for both methionines in Ctr3 function. As observed for the Ctr1 Met-127 and Ctr1 Met-260 mutants, replacing Ctr3 Met-185 or Ctr3 Met-189 by cysteine, but not by serine, rescued growth of the yeast *ctr1Δctr3Δ* strain on YPEG (Fig. 5B) and high affinity copper transport (Fig. 5C), suggesting that both methionines in the Ctr3 MXXXM motif may coordinate copper during the process of copper transport across the plasma membrane. Mutagenesis of both Ctr3 methionines in the same molecule to cysteine, but not serine, also rescued growth on YPEG and the copper transport defect of a *ctr1Δctr3Δ* strain (Fig. 5, B and C). As shown for yeast Ctr1 mutant alleles, mutagenesis of the MXXXM motif in the context of a Ctr3-GFP fusion protein did not alter protein delivery to the cell surface (Fig. 5D). These data demonstrate that both methionines in the Ctr3 MXXXM motif play an important role in copper transport and strongly suggest, based on genetic evidence, that they function as copper ligands.

**Genetic Evidence for Cooperativity between Ctr Subunits for Copper Transport**—The hydrophobicity profile of the Ctr family of copper transport proteins predicts the presence of only 3 transmembrane domains. However, other characterized permeases and metal ion transporters for zinc, iron, and intracellular copper possess from 6 to 12 membrane-spanning domains (33, 38). Coimmunoprecipitation and, more recently, *in vitro* cross-linking experiments, have suggested that Ctr proteins oligomerize (17, 19, 23, 24). To evaluate the role in copper transport played by Met-127 and Met-260 in yeast Ctr1 protein in the context of an oligomeric complex, we cotransformed *ctr1Δctr3Δ* mutant strain with yeast Ctr1wt and mutant alleles. *ctr1Δctr3Δ* cells coexpressing Ctr1wt plus Ctr1 Met-127, or Ctr1wt plus Ctr1 Met-260 were able to grow in ethanol/glycerol medium, indicating that both Ctr1 mutations are recessive (Fig. 6A, Ctr1wt + M127A and Ctr1wt + M260A). Overexpression (p426GPD vector) of both mutant forms of yeast Ctr1 in cells expressing Ctr1wt did not affect cell growth in ethanol/glycerol (data not shown). Interestingly, coexpression of Ctr1 M127A and Ctr1 M260A, but not individual expression of these alleles, partially rescued the respiratory deficiency and <sup>64</sup>Cu uptake defect of a *ctr1Δctr3Δ* strain (Fig. 6, A and B, M127A + M260A). Additionally, coexpression of both yeast Ctr3 single mutant alleles in the MXXXM motif, M185A and M189A, in the Ctr-deficient yeast strain increased both growth on ethanol/glycerol and <sup>64</sup>Cu uptake, compared with cells expressing only one of both mutants, M185A or M189A (data not shown). These results show clear genetic evidence supporting cooperativity between the Ctr monomers in yeast copper transport.

**Function of Conserved Methionine Residues in Human Ctr1**—Human Ctr1 protein (hCtr1) contains two Mets motifs, M1 (M<sup>7</sup>GMSYM<sup>12</sup>) and M2 (M<sup>40</sup>MMMPM<sup>45</sup>), and two histidine-rich regions, H1 (H<sup>3</sup>SHH<sup>6</sup>) and H2 (H<sup>22</sup>HH<sup>24</sup>) in the hydrophilic amino-terminal region of the protein (for a topological representation, see Fig. 7A). We investigated the potential role of these residues in copper transport by human Ctr1. All histidine residues in the H1 or H2 motifs, or methionine residues in the M1 or M2 motifs, were mutationally altered to alanine, and the function of the mutated proteins was tested by expression in *ctr1Δctr3Δ* yeast cells. Although the wild type hCtr1, h1,



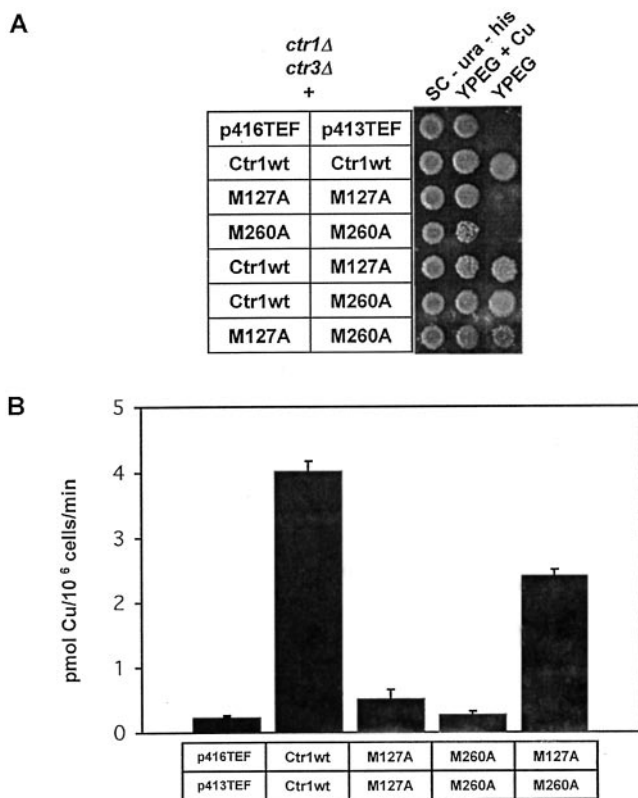
**FIG. 5. The yeast Ctr3 MXXXM motif is essential for copper transport.** *A*, proposed topological model for yeast Ctr3 protein with the M<sup>185</sup>XXXM<sup>189</sup> motif indicated. *B*, growth of MPY17 (*ctr1Δctr3Δ*) cells containing p416TEF (*vector*), expressing Ctr3wt or Ctr3 mutant alleles M20A, M185A, M185C, M185S, M189A, M189C, M189S, and M185A,M189A, M185C,M189C, and M185S,M189S were assayed for growth in ethanol/glycerol (YPEG) plates. Cells were grown for 4 days at 30 °C on SC-ura and YPEG + 100 μM CuSO<sub>4</sub> and for 6 days on YPEG. *C*, <sup>64</sup>Cu uptake for the strains shown in *B*. *D*, Ctr3wt, Ctr3 M185A, Ctr3 M189A, and Ctr3 M185A,M189A are localized properly to the plasma membrane. MPY17 cells constitutively (p416TEF vector) expressing carboxyl-terminally GFP-tagged wild type and mutant Ctr3 alleles were grown on selective media (SC-ura) to exponential phase and visualized by fluorescence microscopy. *DIC*, differential interference microscopy.

h2, and m1 mutant alleles can function in yeast cells lacking high affinity copper transporters, expression of the m2 mutant (M<sup>40</sup>MMMPM<sup>45</sup> to A<sup>40</sup>AAAPA<sup>45</sup>) does not complement the yeast growth defect on YPEG medium (Fig. 7*B*, left panel). We have reported that expression of the human Ctr1 copper transporter in HEK293 cells strongly stimulates the initial rate of copper uptake over vector-transfected control cells (24). To test the function of mutant forms of hCtr1 protein in human cells, hCtr1 mutant alleles were expressed in HEK293 cells by transient transfection and copper transport activity measured. In accordance with yeast complementation assays, the expression of the m2 mutant only modestly stimulated <sup>64</sup>Cu uptake in human cells, compared with hCtr1wt and h1, h2, or m1 alleles (Fig. 7*B*, right panel). These results strongly support an important role for the TMD1-proximal Mets motif M2 in hCtr1 copper transport function.

The last methionine in the M2 Mets motif, Met-45, is positionally conserved with respect to the beginning of the TMD1 among other identified Ctr copper transporters (Fig. 1, yeast Ctr1 Met-127-like residue), which suggests the possibility that this residue functions in hCtr1-mediated copper uptake. We tested whether the conserved hCtr1 Met-45 is sufficient for copper uptake and whether other nearby methionines are able to replace its function, by using the yeast cell growth assay and <sup>64</sup>Cu uptake in HEK293 cells (Fig. 7*C*). Although any single methionine in the M2 region is sufficient for complementation of the YPEG growth defect of the *ctr1Δctr3Δ* yeast strain, the 4th or 5th methionine residue in the M2 region (Met-43 and Met-45, respectively) is critical under copper limitation conditions generated by adding 20 μM BCS to YPEG medium (Fig.

7*C*, YPEG + BCS). Furthermore, expression of mutant alleles retaining either the Met-43 or Met-45 residue in the M2 Mets motif (A<sup>40</sup>AAMPA<sup>45</sup> and A<sup>40</sup>AAAPM<sup>45</sup>, respectively) was sufficient for stimulation of copper uptake in human cells to a level approaching that observed in cells expressing hCtr1wt protein. However, other hCtr1 mutant alleles carrying only the 1st, 2nd, or 3rd methionine residue in the M2 region only modestly stimulated copper uptake (Fig. 7*C*, right panel). These results obtained from copper transport assays in HEK293 cells parallel those observed with the yeast cell complementation assays in YPEG medium under normal and copper-limiting conditions. The copper uptake rates observed in hCtr1 mutant alleles carrying methionine in the 1st, 2nd, or 3rd residue of the M2 region appears to be sufficient for allowing yeast cells to grow in YPEG medium without further copper starvation by the addition of BCS. Taken together, these results suggest that the M2 methionine residues at positions 43 and 45 are critical for hCtr1-mediated copper transport activity under conditions of copper limitation.

Two other methionine residues in the predicted second transmembrane domain of hCtr1 protein, Met-150 and Met-154, are also conserved among Ctrs (Figs. 1 and 7*A*, MXXXM motif). To test the roles of these residues in copper transport by the hCtr1 protein, each methionine was mutated to leucine (M150L and M154L), and the function of these mutated proteins was tested in the yeast cell growth assay. As shown in Fig. 7*D* (left panel), both methionine residues are critical for hCtr1 to complement yeast cells defective in high affinity copper transport functionally. Additionally, expression of the hCtr1 M150L and M154L mutant alleles completely abolished the stimulation of copper



**FIG. 6. Yeast Ctr monomers cooperate in copper transport.** MPY17 strain was cotransformed with the p416TEF and p413TEF vectors alone or Ctr1wt, Ctr1 M127A, Ctr1 M260A, Ctr1wt + M127A, Ctr1wt + M260A, M127A + M260A. Cells were grown on selective media (SC-ura-His) and assayed for growth in ethanol/glycerol (A) or  $^{64}\text{Cu}$  uptake (B). Cells were incubated on SC-ura-His and YPEG + 100  $\mu\text{M}$   $\text{CuSO}_4$  plates for 3 days at 30 °C and for 6 days in the case of YPEG plates.

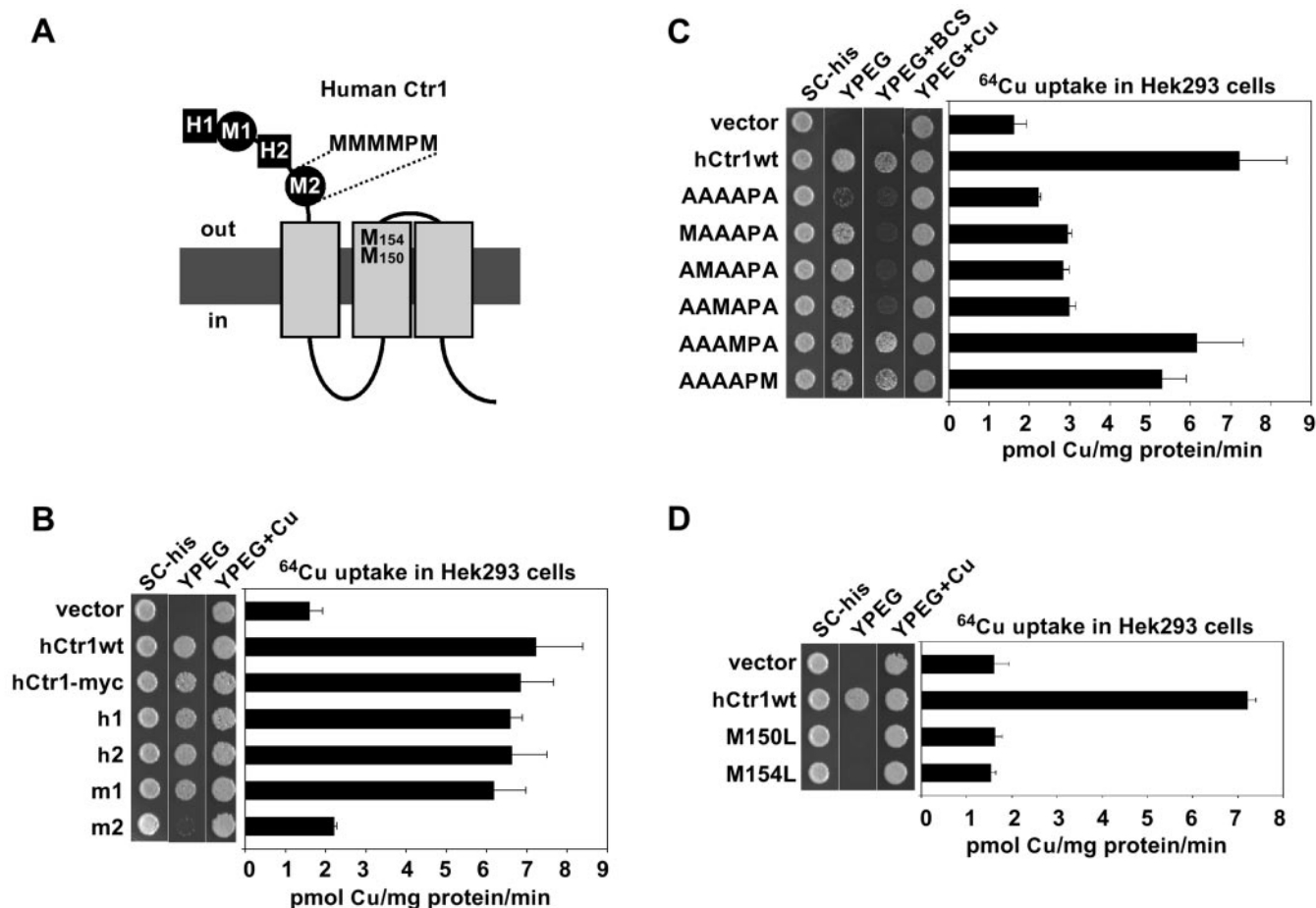
uptake observed in HEK293 cells expressing hCtr1wt (Fig. 7D). Therefore, similar to yeast Ctr proteins, methionine residues within the transmembrane MXXXM motif of human Ctr1 protein are important for copper transport function. It is necessary to demonstrate that the defective function of hCtr1 mutant proteins is the result of mutations in amino acid residues that are critical for copper transport, rather than a change in protein stability or localization induced by the mutation. Amino-terminal c-Myc epitope-tagged wild type or mutant hCtr1 was expressed in HEK293 cells by transient transfection. Amino-terminal c-Myc epitope-tagging of hCtr1wt did not alter hCtr1 protein function in either the yeast cell growth assay or the HEK293 cell  $^{64}\text{Cu}$  uptake assay (Fig. 7B). Furthermore, there was no significant difference in expression levels of any of the hCtr1 mutant proteins as ascertained by immunoblotting, or in their localization to the plasma membrane as determined by indirect immunofluorescence using anti-Myc antibody (data not shown). Taken together, these results indicate that human Ctr1 contains regions that play an Met-127-like (hCtr1 Met-43 and Met-45) and an MXXXM motif function, and are required for high affinity copper transport in human cells.

#### DISCUSSION

A number of primary structural features among the Ctr family of high affinity copper transporters are conserved from yeast to humans. The amino-terminal region that is rich in methionine residues arranged as MXXXM and MXM is reminiscent of those found previously in bacterial proteins involved in copper homeostasis, suggesting a possible function in copper binding (10). Based on our analyses, the topological

structure we propose would place the Ctr1 amino-terminal Mets motifs exposed to the extracellular environment. Deletion of these motifs from yeast Ctr1 protein does not affect proper localization of Ctr1 to the cell surface, but it decreases by one-third the wild type copper uptake rate. This defect in copper transport capacity is crucial for growth under conditions of severe copper limitation, which is precisely when yeast copper transporter genes are transcriptionally induced by the Mac1 copper metalloregulatory factor (10, 39, 40). Specific amino acids, peptides, and proteins have been proposed as putative extracellular copper ligands that directly affect cellular copper uptake (41). Additionally, cells possess cell surface metalloredutases that increase copper bioavailability by reduction to Cu(I) (7, 8), the presumed substrate transported by Ctr proteins (24). The Ctr1 Mets motifs could provide an elevated concentration of extracellular methionyl sulfur groups that could facilitate the release of copper from competent extracellular ligands and would coordinate Cu(I) to render it available for transport across the membrane channel. A similar function has been proposed for the cytosolic amino-terminal metal binding domains in the copper-transporting P-type ATPases Ccc2/ATP7A/B, which are multimer-spanning proteins localized in the *trans*-Golgi network and responsible for copper translocation into the lumen of the secretory pathway (42). Human ATP7A, or MNK protein, which is defective in patients with Menkes syndrome, contains six metal binding domains that bind Cu(I) (43–46) and are absolutely required under the low bioavailable copper concentrations of the cytosol, but apparently dispensable for the *in vitro* catalytic activity of the protein (47). Copper binding studies and structural data will be important to test a copper binding model for the Ctr1 Mets motif function. Alternatively, the Ctr1 extracellular domain could play a role in protein-protein interactions with, for example, the Fre1–7 metalloredutases.

In this study we have identified and demonstrated a role for a conserved extracellular methionine (yeast Ctr1 Met-127-like residue) in copper transport by Ctr proteins. This methionine residue is equidistant (20 residues) from the beginning of the first putative TMD in all known Ctrs and could potentially coordinate copper close to the plasma membrane as a prerequisite for transmembrane transport. Mutagenesis of this methionine residue to alanine or leucine in yeast Ctr1 protein demonstrates that it is essential for high affinity copper uptake. Substitution by cysteine and histidine strongly suggests that coordination of copper to this soft Lewis base is critical for copper import. Our results are consistent with a similar mechanism also functioning in human cells, where the Met-127-like role is performed by either hCtr1 Met-43 or hCtr1 Met-45 residues. Other methionine residues (Met-35 through Met-37) contained in the second hCtr1 Mets motif M2 are also able to complement the yeast *ctr1Δctr3Δ* growth defect in ethanol/glycerol. However, the rate of copper uptake in HEK293 cells is decreased significantly as a consequence of these mutations, indicating that these residues are not completely able to perform an Met-127-like function. Amino-terminal metal binding domains in mammalian CPx-type ATPases ATP7A (MNK protein) and ATP7B (WND protein) are thought not to be functionally equivalent in copper transport. The metal binding domains located closer to the plasma membrane appear to play a more crucial role in copper transport and copper-induced relocalization of the proteins (48–52). Similarly, both yeast Ctr1 Met-127 and human Ctr1 Met-43 and Met-45 residues are contained in the Mets motif closest to the Ctr first TMD, which could help to form a transmembrane channel for copper transport.



**FIG. 7. Conserved methionine residues are critical for human Ctr1 copper transporter function.** A, proposed topological model for human Ctr1 protein. The amino-terminal domain containing two histidine-rich regions H1 and H2 (black squares) and two Mets motifs M1 and M2 (black circles) is predicted to be located outside the cell. The M2 (M<sup>40</sup>MMMPM<sup>45</sup>) and M<sup>150</sup>XXXM<sup>154</sup> motifs are represented in detail. B, C, and D, complementation assays in *ctr1Δctr3Δ* yeast cells (left panels) and <sup>64</sup>Cu transport assays in HEK293 cells (right panels) containing vector alone (p413GPD or pcDNA3.1(+), respectively) or expressing the hCtr1wt open reading frame (*hCtr1wt*), the hCtr1 tagged with one c-Myc epitope at the amino terminus (*hCtr1-myc*), H<sup>3</sup>SHH<sup>6</sup> (H1) to A<sup>3</sup>SAA<sup>6</sup> mutated hCtr1 allele (*h1*), H<sup>22</sup>HH<sup>24</sup> (H2) to A<sup>22</sup>AA<sup>24</sup> mutated hCtr1 allele (*h2*), M<sup>7</sup>GMSYM<sup>12</sup> (M1) to A<sup>7</sup>GASYA<sup>12</sup> mutated hCtr1 allele (*m1*), M<sup>40</sup>MMMPM<sup>45</sup> (M2) to A<sup>40</sup>AAAPA<sup>45</sup> mutated hCtr1 allele (*m2*) (B); hCtr1 alleles carrying single methionine residue in the M<sup>40</sup>MMMPM<sup>45</sup> (M2) domain including M<sup>40</sup>AAAPA<sup>45</sup>, A<sup>40</sup>MAAPA<sup>45</sup>, A<sup>40</sup>AMAPA<sup>45</sup>, A<sup>40</sup>AAMPA<sup>45</sup>, and A<sup>40</sup>AAAAPM<sup>45</sup> (C); and methionine residues in the M<sup>150</sup>LIFM<sup>154</sup> sequence at the putative second TMD in hCtr1 protein were mutated to leucine (M150L, M154L) (D). Yeast cells were grown on SC-His, YPEG, and YPEG supplemented with 100 μM CuSO<sub>4</sub> (YPEG + Cu) or 20 μM BCS (YPEG + BCS) for 3–6 days at 30 °C. <sup>64</sup>Cu uptake in HEK293 cells was measured by incubating cells with 2 μM <sup>64</sup>Cu for 5 min in cell culture medium with 10% FBS. Each data point represents the mean of at least three experiments (±S.D.).

Many *S. cerevisiae* strains express another high affinity copper transporter, Ctr3, which lacks amino-terminal Mets motifs (9). Additionally, mutagenesis of the Ctr3 Met-20 residue, which aligns with yeast Ctr1 Met-127, also suggests that this residue is not absolutely required for Ctr3 protein to allow growth in ethanol/glycerol media. However, addition of low concentrations of the Cu(I)-specific chelator BCS decreased the growth of *ctr1Δctr3Δ* cells expressing the Ctr1 M20A mutant allele compared with cells expressing the wild type protein (data not shown). This result suggests a possible involvement of this methionine residue in the process of copper transport mediated by Ctr3. The presence in yeast of two proteins, Ctr1 and Ctr3, with a slightly different mechanism of copper uptake could be advantageous. Accordingly, we observed previously that the expression of both yeast Ctr1 and Ctr3 proteins is required for maximal copper transport rates and more efficient growth under copper-limiting conditions (9).

An absolutely conserved feature among the Ctr family of high affinity copper transporters is the MXXXM motif located in the second putative TMD. Substitution of both methionines by cysteine in yeast Ctr1 transporters suggests that copper coordination to these residues is required for the process of

copper translocation. According to the topological structure proposed for yeast Ctr1, the MXXXM motif is located closer to the extracellular face of the plasma membrane than to the cytosol, suggesting a copper coordination role in the initial steps of copper transport through the permease channel. An analogous role has been suggested for the methionine 1393 residue located within the TMD8 of the human AYP7A/MNK protein. This transmembrane methionine residue is highly conserved and is essential for the catalytic function of this copper-translocating ATPase (47). Additional methionine residues located within the putative TMDs of copper P-ATPases are also highly conserved (e.g. ATP7A/MNK methionine 746). These data suggest that coordination of copper by transmembrane methionine residues could play an essential role in metal translocation across membranes.

Proteins involved in copper distribution, such as copper metallochaperones Atx1/Atox1 or Ccs, and CPx-type ATPases involved in copper translocation to the lumen of the *trans*-Golgi network (Ccc2 in *S. cerevisiae*, and ATP7A/B in human cells) or copper export (ATP7A in human cells, CopB in *E. hirae* and other bacteria), have conserved CxxC motifs containing 2 cysteine residues that coordinate Cu(I) in an exchangeable but

relatively stable form (37, 53). Cysteine-rich metallothioneins, such as Cup1 in yeast, sequester several atoms of Cu(I) per molecule in a nonexchangeable form largely by trigonal coordination to cysteine thiolates, protecting cells against copper ion toxicity and oxidative damage (54). Cuproenzymes such as multicopper ferroxidases Fet3 and ceruloplasmin, cytochrome *c* oxidase, Cu,Zn-superoxide dismutase, or plastocyanin contain a wider range of copper ligands that allow binding of Cu(II) and redox chemistry (55). Therefore, the specific characteristics of the copper coordination environment determine the biochemical properties of cuproproteins. The stability of binding is highly dependent on the metal oxidation state and nature of the coordinating ligands. Cu(I), as a soft Lewis acid, coordinates preferentially to thiolates provided by the amino acids cysteine and methionine. It is therefore feasible that Ctrs utilize methionine residues, which coordinate Cu(I) with lower affinity than cysteine residues, to facilitate translocation of copper through the membrane channel and further delivery to intracellular copper chaperones en route to cuproproteins.

We have ascertained that other Ctr1-related proteins such as yeast Ctr2 (22), and human Ctr2 (19), also contain domains homologous to the Ctr family of transporters (the Met-127-like methionine residue and the MXXXM motif). However, expression of either gene in a *S. cerevisiae* *ctr1Δctr3Δ* strain does not reestablish high affinity copper transport. Yeast Ctr2 has recently been localized to the vacuolar membrane, suggesting a potential role in copper export from the vacuole (56). This observation suggests that Ctr2 proteins are not able to foster high affinity copper uptake because of their intracellular localization, but a Ctr1-like mechanism could also mediate copper translocation across intracellular membranes.

We and others have recently demonstrated that the mammalian Ctr1 high affinity copper transporter is essential for embryonic development (25, 26). Therefore, we expect that mutations in critical methionine residues described in this work would be lethal, and mutations in other residues may have more subtle consequences. Further genetic, biochemical, and structural studies on both the yeast and mammalian Ctr1 proteins will be required to elucidate the precise mechanism for how cells are able to transport and distribute this important trace element.

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