

Characterization of Mouse Embryonic Cells Deficient in the Ctr1 High Affinity Copper Transporter

IDENTIFICATION OF A Ctr1-INDEPENDENT COPPER TRANSPORT SYSTEM*

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The trace metal copper is an essential cofactor for a number of enzymes that have critical roles in biological processes, but it is highly toxic when allowed to accumulate in excess of cellular needs. Consequently, homeostatic copper metabolism is maintained by molecules involved in copper uptake, distribution, excretion, and incorporation into copper-requiring enzymes. Previously, we reported that overexpression of the human or mouse Ctr1 copper transporter stimulates copper uptake in mammalian cells, and deletion of one Ctr1 allele in mice gives rise to tissue-specific defects in copper accumulation and in the activities of copper-dependent enzymes. To investigate the physiological roles for mammalian Ctr1 protein in cellular copper metabolism, we characterized wild type, Ctr1 heterozygous, and Ctr1 homozygous knock-out cells isolated from embryos obtained by the inter-cross of Ctr1 heterozygous mice. Ctr1-deficient mouse embryonic cells are viable but exhibit significant defects in copper uptake and accumulation and in copper-dependent enzyme activities. Interestingly, Ctr1-deficient cells exhibit ~30% residual copper transport activity that is saturable, with a K_m of ~10 μM , with biochemical features distinct from that of Ctr1. These observations demonstrate that, although Ctr1 is critical for both cellular copper uptake and embryonic development, mammals possess additional biochemically distinct functional copper transport activities.

harbor mechanisms for homeostatic copper metabolism with respect to uptake, excretion, and distribution. The Ctr1 copper transporter was first cloned from bakers' yeast (4, 5), and other Ctr family copper transporters have been identified from fission yeast, lizard, mouse, and human based on their functional complementation of yeast Ctr1 copper transporter mutants or structural similarities to yeast Ctr1 (6–11). Once copper is transported across the plasma membrane, cytoplasmic metallochaperones including CCS, Cox17, and Atox1 deliver copper to specific cellular targets or compartments (12–23). CCS delivers copper to Cu,Zn-SOD via direct physical interactions as a CCS-SOD heterodimer (12–15). Cox17 is required for incorporation of copper into cytochrome *c* oxidase in the mitochondria (16–19), and Atox1 interacts with trans-Golgi network membrane copper transporting P-type ATPases to deliver copper into the lumen of the secretory compartment where it is loaded onto proteins such as the Fet3 iron transport subunit in yeast and ceruloplasmin in mammals (20–23).

Saccharomyces cerevisiae cells deficient in the Ctr1 high affinity copper transporter exhibit phenotypes including respiratory deficiency, oxidative stress sensitivity, and iron deficiency that occur as a consequence of copper-dependent defects in cytochrome *c* oxidase, Cu,Zn-SOD, and the Fet3 multicopper ferroxidase, respectively (4, 5). These results demonstrate that the bakers' yeast Ctr1 high affinity copper transport system provides copper to each of the three characterized copper chaperone pathways. Ctr1 copper transporters from a variety of eukaryotes possess similar structural features including three transmembrane domains and conserved residues required for copper transport activity (24, 25). The expression of human or mouse Ctr1 by mammalian cell transfection was shown to increase both steady state levels of copper and the rate of ^{64}Cu uptake with an apparent K_m of ~1–2 μM (26–29). Human Ctr1-mediated ^{64}Cu transport is an energy-independent process and is stimulated by extracellular acidic pH and high K^+ concentrations (27). The strong inhibition by Ag(I) in Ctr1-mediated ^{64}Cu uptake suggests that Ag(I), which is isoelectric to Cu(I), can be transported by human Ctr1 and that reduced monovalent copper is a preferred substrate for human Ctr1 (27). Consistent with this observation, ascorbate treatment to reduce Cu(II) to Cu(I) also enhanced human Ctr1-mediated copper uptake (27), and metalloreductase genes are important for high affinity copper transport in *S. cerevisiae* (30, 31).

Although overexpression of human or mouse Ctr1 enhances copper uptake, targeted disruption of one allele of the mouse Ctr1 gene results in tissue-specific reductions in copper accumulation and in the activities of copper-dependent enzymes (28, 32). For example, brain and spleen copper levels in Ctr1 heterozygous mice were ~50% that of copper levels found in wild type littermates, and cuproenzyme activities including

Copper is an essential cofactor for enzymes including cytochrome *c* oxidase, copper, zinc superoxide dismutase (Cu,Zn-SOD),¹ tyrosinase, dopamine β -monooxygenase, peptidylglycine α -amidating monooxygenase, and ceruloplasmin (1, 2). Consequently, copper metabolism is an important underlying component for cellular energy generation, defense against oxidative damage, pigmentation, neurotransmitter generation and maturation, and iron metabolism. However, with excess accumulation, copper generates hydroxyl radicals that damage virtually all cellular components (3).

Consistent with the essential yet toxic nature of copper, cells

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¹ The abbreviations used are: Cu,Zn-SOD, copper, zinc superoxide dismutase; HBSS, HEPES-buffered salt solution; Pipes, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid.

Cu,Zn-SOD and cytochrome *c* oxidase were reduced by ~20%. However, there were no significant differences in liver and kidney copper levels between wild type and Ctr1 heterozygous mice. The mechanisms underlying tissue-specific defects in copper accumulation in Ctr1 heterozygous mice are not yet clearly understood. This could reflect low levels of Ctr1 expression in the brain and spleen (9) or the presence of as yet uncharacterized tissue-specific copper uptake compensatory mechanisms. Furthermore, mice deficient for both alleles of the Ctr1 gene exhibit profound growth and developmental defects and die *in utero* in mid-gestation, underscoring the critical function for Ctr1 in embryonic development (28, 32).

To further investigate the roles for Ctr1 protein in cellular copper metabolism, we generated and characterized mouse cells from wild type, Ctr1 heterozygous, and Ctr1 homozygous knock out embryos obtained from the inter-cross of Ctr1 heterozygous mice. Mouse embryonic cells lacking Ctr1 exhibit significant defects in copper uptake and accumulation and in copper-dependent enzyme activities, demonstrating the important physiological functions of Ctr1 in copper uptake in mammalian cells. Interestingly, Ctr1-deficient cells express residual copper transport activity that is saturable and is characterized by a K_m for copper of ~10 μM . Unlike Ctr1-mediated copper uptake, the Ctr1-independent copper transport activity appears to be a Cu(II) transporter, as suggested by the lack of stimulated copper uptake by ascorbate and the absence of competition by silver ions. These results demonstrate that although Ctr1 is a major copper transporter that plays essential roles for copper acquisition and for mouse development, an alternative copper transporter system identified in Ctr1-deficient cells is sufficient for copper acquisition, which is required for proliferation of mouse embryonic cells *in vitro*.

MATERIALS AND METHODS

Isolation and Culture of Mouse Embryonic Cells—Embryos generated from the inter-cross of Ctr1 gene heterozygous knock-out mice were dissected out of decidua between E7 and E8 embryonic days with the removal of all maternal tissues. Total embryonic tissue was treated with 0.05% trypsin at 4 °C for 30 min and incubated at 37 °C for 1 h. Cells were cultured on a feeder layer of mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 1 \times nonessential amino acids, 55 μM 2-mercaptoethanol, and 100 units/ml penicillin and streptomycin. To facilitate mitochondrial function under potentially low intracellular copper concentrations, uridine (50 mg/liter) and pyruvate (110 mg/liter) were added to the culture medium (33). After 2–4 weeks cells were cultured without mouse embryonic fibroblast feeder cells, and the pSV3 plasmid carrying SV40 large T antigen gene (34) and the pTK-hygro-mycin plasmid (Invitrogen) were co-transfected into the cells for immortalization using the FuGENE 6 (Roche Molecular Biochemicals) reagent. Hygromycin (100 $\mu\text{g}/\text{ml}$) was used for the selection of transfected cells, and surviving cell clones were isolated and expanded.

Genotyping and RNA Analysis of Mouse Embryonic Cells—Ctr1 genotyping in embryonic cell lines was carried out by Southern blotting as described previously (28). Mouse Ctr1 expression in mouse embryonic cells of distinct Ctr1 genotypes was detected by RNA blotting with the mouse Ctr1-specific probe as described previously (28).

^{64}Cu Uptake and Accumulation Assays—Radioactive copper (^{64}Cu) was purchased from the Mallinckrodt Institute of Radiology, Washington University (Saint Louis, MO). ^{64}Cu uptake was measured with a slight modification of methods described previously (27). Briefly, HEPES-buffered salt solution (HBSS) (pH 7.5) containing 140 mM NaCl, 3 mM KCl, 1 mM Na_2HPO_4 , 1 mM CaCl_2 , 0.5 mM MgCl_2 , 5 mM glucose, and 10 mM HEPES was used for ^{64}Cu uptake assays except as indicated in figure legends. A Pipes-buffered salt solution (substitution of Pipes for HEPES (pH 6.5)) and MES-buffered salt solution (substitution of MES for HEPES (pH 5.5)) were used to ascertain pH effects on ^{64}Cu uptake. The effect of ascorbic acid on ^{64}Cu uptake was analyzed by adding ascorbic acid to 100 μM final concentrations in uptake buffer. Competition effects of other metal ions on ^{64}Cu uptake was measured by the co-incubation of a 50-fold molar excess of test metals with ^{64}Cu . The effects of metabolic inhibitors, extracellular pH, Na^+ , or K^+ on ^{64}Cu

uptake were tested as described previously (27). To measure steady state cellular copper levels, 90% confluent cells were collected and wet-digested with concentrated NH_4OH , and copper levels were analyzed by atomic absorption spectroscopy and normalized to protein concentrations as described previously (28).

Copper-dependent Enzyme Assays—For Cu,Zn-SOD activity assays, wild type, Ctr1 heterozygous, or Ctr1 homozygous knock-out cells were harvested with or without copper supplementation (50 μM CuCl_2) for 12 h. Cells were lysed by freezing and thawing in lysis buffer containing 25 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, and protease inhibitor mixture (Roche Molecular Biochemicals). Supernatants were collected by centrifugation of total cell lysates at 15,000 $\times g$ for 10 min and used for enzyme assays. Cu,Zn-SOD activity was determined as described previously (35), and protein concentrations were measured by the BCA method (Pierce). Protein extract was diluted into 1 \times SDS-PAGE sample buffer, boiled, fractionated on 15% SDS-PAGE, and probed with antibodies against human Cu,Zn-SOD (1:2000, StressGen).

For cytochrome *c* oxidase assays cells were lysed in a 10 mM Tris-HCl buffer (pH 7.0) containing 250 mM sucrose, protease inhibitor mixture (Roche Molecular Biochemicals), and 1% Tween 80 (Sigma). Total cell lysate was centrifuged at 16,000 $\times g$ at 4 °C for 20 min, and supernatants were used for enzyme assays and immunoblotting analysis. Cytochrome *c* oxidase activity was detected with the method described previously (36) and 15% SDS-PAGE, and immunoblotting was carried out using antibodies against cytochrome *c* oxidase subunit I (1:2000, Molecular probes). Anti-actin antiserum (1:1000, Santa Cruz Biotechnology) was used to detect actin as a loading control.

For tyrosinase assays Ctr1-deficient cells were cultured overnight to 80% confluency and transiently transfected with the pcTyr human tyrosinase cDNA expression plasmid (provided by Dr. Richard Spritz) in combination with either the hCtr1-myc expression plasmid (27) or the pcDNA3.1 empty vector. Cells were cultured for 48 h in basal medium or in medium containing 30 μM CuCl_2 , harvested by scraping into phosphate-buffered saline, pelleted, and sonicated in a buffer containing 2% SDS, 62.5 mM Tris-Cl (pH 6.8), protease inhibitor mixture (Roche Molecular Biochemicals), 1 mM ascorbate, and 1 mM bathocuproine disulfonate to chelate free copper. Cell lysates (20 μg) were fractionated by non-reducing 7.5% SDS-PAGE, and tyrosinase activity was colorimetrically determined by incubating gels for 15 min at 37 °C in 10 mM phosphate buffer (pH 6.8) containing 1.5 mM L-3,4-dihydroxyphenylalanine and 4 mM 3-methyl-2-benzothiazolone hydrazone (Sigma). The hCtr1-myc and tyrosinase proteins were detected by chemiluminescence using anti-Myc 9E10 monoclonal antibody (1:1000, Roche Molecular Biochemicals) and goat anti-tyrosinase antibodies (1:500, Santa Cruz Biotechnology).

RESULTS

Mouse Ctr1 Is Not Essential for Cell Viability—We previously generated Ctr1-deficient mice as one approach to understand roles for Ctr1 in copper metabolism. Ctr1 heterozygous mice (Ctr1+/-) manifest tissue-specific defects in copper accumulation, and Ctr1-deficient mice (Ctr1-/-) die during mid-gestation with severe growth and developmental phenotypes (28, 32). To further investigate the function of mammalian Ctr1 in copper metabolism at the cellular level, we isolated wild type, Ctr1 heterozygous, and Ctr1 homozygous knock-out cells from embryos between E7 and E8 embryonic days obtained from the inter-cross of Ctr1 heterozygous mice. Total embryonic tissue was trypsinized and cultured, and cells were immortalized by expression of the SV40 large T antigen. Cell clones carrying wild type (Ctr1+/+), Ctr1 heterozygous (Ctr1+/-), and Ctr1 homozygous knock-out (Ctr1-/-) alleles were identified by Southern blotting analysis, which we previously described for the genotyping of Ctr1 knock-out mice (28). As shown in Fig. 1A with probes specific for the Ctr1 5'-region (*upper panel*) and 3'-region (*lower panel*), respectively, we detected 3.2- and 6.0-kilobase fragments from the wild type Ctr1 locus and 4.0- and 5.4-kilobase fragments, indicative of the Ctr1 knock-out allele. Furthermore, wild type, Ctr1+/-, and Ctr1-/- cells exhibit Ctr1 gene dosage-dependent Ctr1 mRNA levels, as ascertained by RNA blotting analysis with a mouse Ctr1-specific probe (Fig. 1B). Therefore, the characterization of cells carrying Ctr1 wild type and heterozygous and homozy-

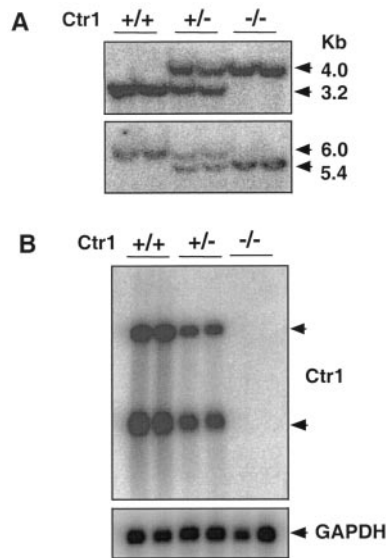


FIG. 1. Isolation of Ctr1 knock-out mouse embryonic cells. Embryos generated from the inter-cross of Ctr1 heterozygous knock-out mice were dissected out of decidua between E7 and E8 embryonic days. Trypsin-digested embryos were cultured, and cell clones were isolated and expanded. *A*, wild type (Ctr1+/+) and Ctr1 heterozygous (Ctr1+/-) and homozygous (Ctr1-/-) knock-out cell lines were identified by Southern blotting analysis with a 5' Ctr1 genomic locus specific (*upper panel*) or 3'-specific (*lower panel*) probe as described previously (28). Genomic DNA isolated from two representative cell lines was digested with *Xba*I (*upper panel*) or *Bam*HI (*lower panel*) restriction enzymes. 3.2- and 6.0-kilobase (Kb) fragments indicate the wild type Ctr1 locus, and 4.0- and 5.4-kilobase fragments indicate the Ctr1 knock-out locus. *B*, Northern blotting analysis confirmed Ctr1 gene dosage-dependent Ctr1 mRNA expression levels. 20 μ g of total RNA isolated from two independent cell lines of each genotype was separated on a 1% formaldehyde-agarose gel. A mouse Ctr1-specific DNA probe was used to detect mouse Ctr1 mRNA. A glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific probe was used as a loading control as shown in the *lower panel*.

gous deletion alleles and loss of Ctr1 mRNA clearly demonstrate that we could isolate viable Ctr1-deficient cells. However, the precise lineage of these cells and whether it is common for all cell types is currently unknown.

We initially supplemented high concentrations of copper (50 μ M) in culture medium with the expectation of copper uptake through alternative mechanisms by which Ctr1-deficient cells may acquire copper required for cell growth. However, even without copper supplementation we did not observe significant growth defects in the Ctr1-deficient cells compared with wild type cells isolated from littermate embryos (data not shown). These results demonstrate that, although Ctr1-mediated copper uptake is critical for embryonic development, Ctr1 is dispensable for cell viability at least for specific cells proliferating under these culture conditions.

Mammalian Ctr1 Functions as a Copper Transporter in Mouse Embryonic Cells—Previous studies demonstrated that Ctr1 overexpression in mammalian or insect cell lines stimulates copper uptake (26–29) and that Ctr1+/- mice manifest tissue-specific defects in copper accumulation (28, 32). These results suggest that Ctr1 is an important component for mammalian copper acquisition. Therefore, we examined whether Ctr1-deficient cells exhibit defects in the rate of 64 Cu uptake or in steady state cellular copper levels. The initial rate of copper uptake was measured in wild type (Ctr1+/+), Ctr1 heterozygous (Ctr1+/-), and Ctr1-deficient (Ctr1-/-) cells by incubation of cells in HBSS with 2 μ M 64 Cu for 5 min, conditions under which copper uptake by Ctr1 is linear (27). As shown in Fig. 2A, the rate of 64 Cu uptake in these cells was Ctr1 dosage-dependent, with Ctr1+/- cells exhibiting 64 Cu uptake rates between

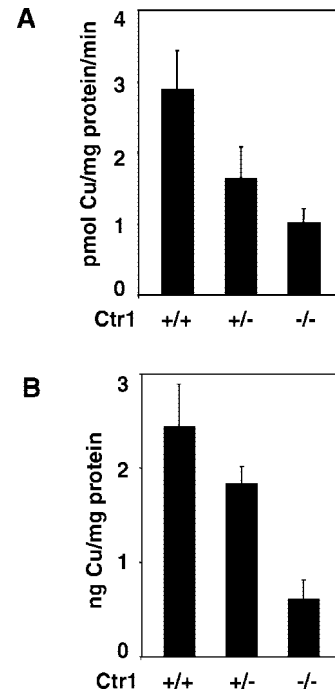


FIG. 2. Defects in copper transport and accumulation in mouse cells deficient for the Ctr1 copper transporter. *A*, 2 μ M 64 Cu was added to the culture medium for 5 min. 64 Cu accumulation was measured using a γ -counter and normalized to protein concentrations of cell lysates. *B*, cell-associated copper levels (ng of copper/mg of protein) were analyzed by atomic absorption spectrophotometry. Each point represents the mean \pm S.D. of three experiments from two independent wild type (Ctr1+/+), Ctr1 heterozygous (Ctr1+/-), and homozygous (Ctr1-/-) knock-out cell lines.

that observed for wild type and Ctr1-/- cells. Consistent with the changes observed for copper uptake rate, cellular copper accumulation, measured by atomic absorption spectrophotometry, was also significantly decreased and displayed a Ctr1 gene dosage-dependent profile (Fig. 2B). These results demonstrate that Ctr1 is a copper transporter at physiological levels, which constitutes a major component of cellular copper transport activity in these cell lines. Interestingly, Ctr1-deficient cells possess \sim 30% of 64 Cu transport activity that is observed in Ctr1+/+ cells, as measured by both 64 Cu uptake rates and total copper accumulation, suggesting the existence of a Ctr1-independent route for copper uptake.

Mouse Ctr1 Functions in Copper Delivery to Three Metallochaperone Pathways—Once transported into cells copper is delivered by target-specific metallochaperones, CCS, Cox17, and Atox1, to Cu,Zn-SOD, mitochondrial cytochrome *c* oxidase, and the secretory compartment, respectively (12–23). We tested whether and to what extent the Ctr1-mediated copper transport system in mouse cells provides copper to all three known copper distribution pathways by measuring cellular copper-dependent enzyme activities. As shown in Fig. 3A Cu,Zn-SOD activity is decreased \sim 50% in Ctr1-/- cells compared with wild type cells, without a significant decrease in activity in Ctr1+/- cells. Consistent with similar steady state levels of the Cu,Zn-SOD monomer in all cell lines as determined by immunoblotting (Fig. 3A), copper supplementation completely restored Cu,Zn-SOD activity levels in Ctr1-/- cells to that found in wild type cells. As shown in Fig. 3B, both cytochrome *c* oxidase enzyme activity and steady state levels of the copper-containing subunit 1 of cytochrome *c* oxidase are clearly decreased in the Ctr1-/- cells and modestly though significantly decreased in the Ctr1+/- cells. Consistent with a copper deficiency in the Ctr1-deficient cells, copper supplement-

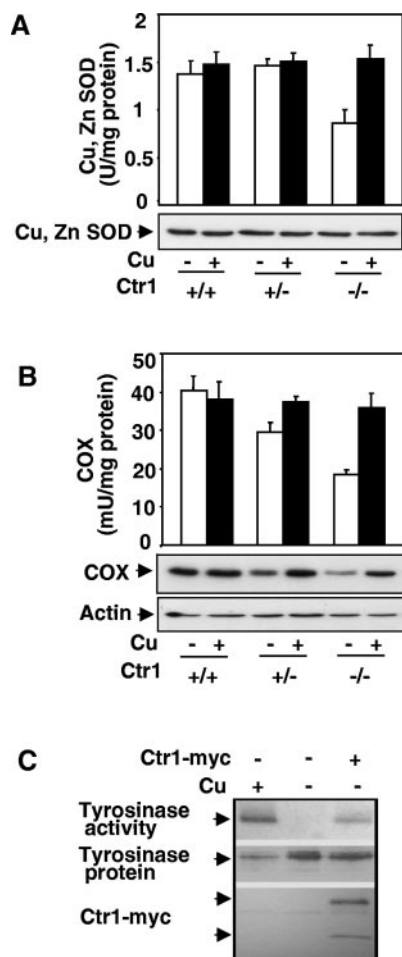


FIG. 3. Activities and expression levels of copper-dependent enzymes in mouse cells deficient in the Ctr1 copper transporter. Cu,Zn-SOD (A) and cytochrome *c* oxidase (COX) (B) activities were measured from cytosolic fractions and total cell extract, respectively. Cells were cultured for 12 h with (Cu+) or without (Cu-) 50 μ M copper supplementation and extensively washed before harvesting cells. Each point represents the mean \pm S.D. of three experiments from two independent wild type (Ctr1+/+) and Ctr1 heterozygous (Ctr1+/-) and homozygous (Ctr1-/-) knock-out cell lines. Immunoblots are shown below enzyme assay data, indicating the steady state levels of Cu,Zn-SOD and cytochrome oxidase subunit 1, respectively. C, expression of Ctr1 is required for the activation of tyrosinase. Ctr1 homozygous knock-out cells were transiently transfected with the tyrosinase expression plasmid in combination with either the hCtr1-myc plasmid or the pcDNA3.1 vector and grown for 48 h in basal media (Cu-) or 30 μ M copper (Cu+), as indicated. Tyrosinase activity was determined colorimetrically in polyacrylamide gels, as described under "Materials and Methods." The lower panels show the corresponding levels of tyrosinase and hCtr1-myc proteins as determined by immunoblotting experiments.

tation in the culture medium (50 μ M for 12 h) restored cytochrome *c* oxidase enzyme activity and steady state levels of cytochrome *c* oxidase subunit 1 (Fig. 3B). Furthermore, as shown in Fig. 3C, Ctr1-deficient cells display severe defects in ectopically expressed tyrosinase activity, which is corrected by either copper supplementation to the growth medium or co-expression of a functional c-Myc epitope-tagged allele of human Ctr1 (27). These results clearly demonstrate that an adequate supply of copper to all known intracellular copper delivery pathways is dependent on the levels of the Ctr1 copper transporter.

Ctr1-/- Cells Harbor a Time-dependent and Saturable Copper Transport Activity—Ctr1-/- cells exhibit severe reductions in copper transport, accumulation, and delivery to the three characterized pathways of intracellular copper routing.

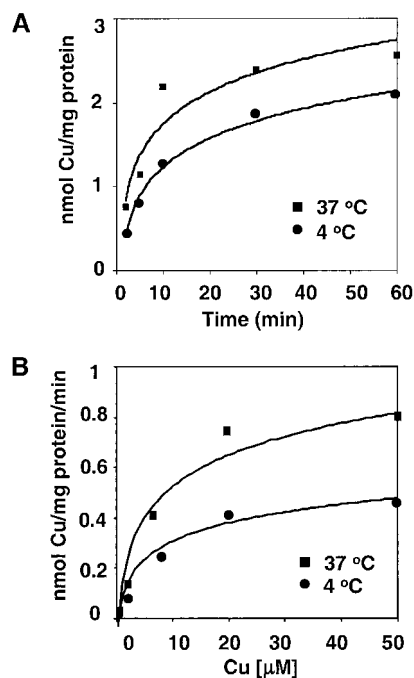


FIG. 4. Time-dependent and saturable ^{64}Cu transport activity in Ctr1-deficient cells. A, time dependence for ^{64}Cu uptake. 2 μM ^{64}Cu was added to HBSS copper uptake buffer, and cells were incubated for 2–60 min at 37 °C or 4 °C. B, concentration dependence for ^{64}Cu uptake. 0.5, 2, 8, 20, or 50 μM ^{64}Cu was added to HBSS uptake buffer for 5 min. To measure ^{64}Cu uptake at 4 °C, cells were preincubated for 15 min at 4 °C before adding ^{64}Cu and cultured at 4 °C during the uptake period. ^{64}Cu uptake was quantitated and normalized to protein concentrations of cell lysates. Each data point represents the mean of four experiments.

However, as shown in this work, Ctr1-/- cells possess significant residual ^{64}Cu transport activity. To begin to characterize this alternative cellular copper uptake pathway, we tested whether copper transport in Ctr1-deficient cells is a carrier-mediated process or a consequence of simple diffusion. ^{64}Cu transport activity in the Ctr1-/- cells was measured both as a function of time and copper concentration. As shown in Fig. 4A, Ctr1-/- cells exhibited time-dependent ^{64}Cu uptake that was linear for ~ 10 min. Consequently, the initial rate of ^{64}Cu uptake at 5 min was measured in a copper dose-response analysis and demonstrated that the residual copper transport activity in Ctr1-/- cells is concentration-dependent and saturable (Fig. 4B). Furthermore, this activity was significantly reduced at 4 °C (Fig. 4, A and B). Analysis of these data indicates that, compared with the low micromolar (~ 1 μM) K_m for Ctr1-mediated copper transport, the Ctr1-independent copper transport activity shows Michaelis-Menten kinetics with an apparent K_m for copper of ~ 10 μM . These results strongly suggest that the Ctr1-independent copper transport activity is a carrier-mediated and thermal energy-requiring process that has relatively low affinity compared with Ctr1-mediated copper transport.

Metal Specificity of the Ctr1-independent Copper Transport Activity—We previously demonstrated that Ctr1-mediated copper transport is highly specific for copper, since of the several metals tested, only copper or silver could effectively compete for ^{64}Cu uptake (27). Consequently, we examined whether the Ctr1-independent copper transport activity is specific for transporting copper rather than other metals under our standard ^{64}Cu transport conditions. A 50-fold molar excess of copper or other metal including zinc, silver, iron, manganese, or cadmium was individually added to ^{64}Cu uptake medium to test for competition by these metals in ^{64}Cu transport. As shown in

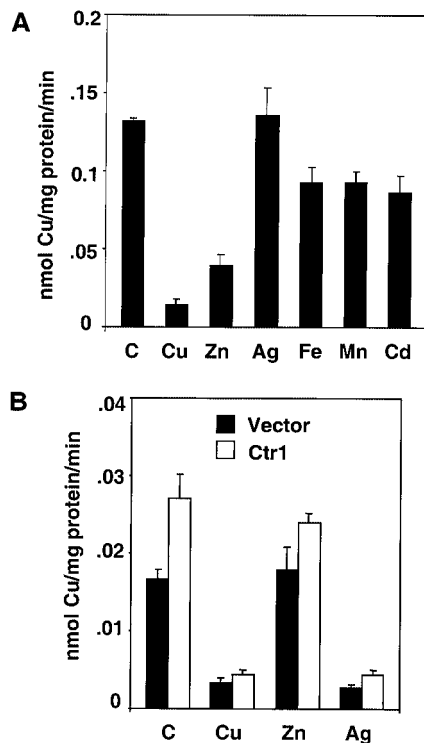


FIG. 5. **Metal specificity for ⁶⁴Cu uptake.** A, effects of other metal ions on ⁶⁴Cu uptake in Ctr1-deficient cells. A 50-fold molar excess of copper (CuCl₂), zinc (ZnCl₂), silver (AgNO₃), iron (FeCl₂), manganese (MnCl₂), or cadmium (CdSO₄) was added to HBSS uptake buffer with 2 μM ⁶⁴Cu for 5 min. ⁶⁴Cu uptake was measured and compared with control cells incubated in the absence of other metals. B, a mouse Ctr1 expression vector or empty vector was transiently transfected into Hek293 cells. ⁶⁴Cu uptake experiments were performed 2 days after transfection. Effects of copper, zinc, or silver were analyzed with coinubation of a 50-fold molar excesses of the metals with 2 μM ⁶⁴Cu for 5 min. ⁶⁴Cu uptake was quantitated and normalized to protein concentrations of cell lysates. Each data point represents the mean of four experiments ± S.D.

Fig. 5A, co-incubation with a 50-fold molar excess of nonradioactive copper inhibited ~90% of ⁶⁴Cu uptake. Among other metals tested, zinc inhibited ~70% of ⁶⁴Cu transport, and iron, manganese, or cadmium inhibited ~25% ⁶⁴Cu transport. This trend of competition by copper and other metals in ⁶⁴Cu uptake by Ctr1-deficient cells is similar to that of human Ctr1-mediated copper transport (27), the human counterpart of mouse Ctr1. However, in contrast to Ctr1-mediated ⁶⁴Cu uptake, silver did not detectably inhibit ⁶⁴Cu transport by the Ctr1-independent activity. Furthermore, to test whether there might be differences in the metal specificity profile between human and mouse Ctr1, we confirmed the competitive effect of silver on mouse Ctr1-mediated copper transport by expressing mouse Ctr1 in Hek293 cells (Fig. 5B). Because of the electronic similarity between silver and reduced copper (Cu(I)), these and other previous observations suggest that Ctr1 transports Cu(I). However, these studies and data shown below suggest that the Ctr1-independent activity may transport another form of copper, perhaps Cu(II).

Biochemical Characterization of the Ctr1-independent Copper Transport Activity—We further tested several biochemical features of the copper transport activity in Ctr1^{-/-} cells to compare it with known features of Ctr1-mediated copper transport. The primary sequences of the Ctr1 family of copper transporters do not possess recognizable ATP-binding domains (2, 24). Moreover, Ctr1-mediated ⁶⁴Cu uptake was not inhibited in cells depleted of ATP (27), suggesting that this process is not ATP-dependent. We tested the effects of the metabolic inhibi-

tors antimycin A, oligomycin, and sodium azide on the rate of Ctr1-independent copper uptake but observed no significant difference in ⁶⁴Cu uptake under the conditions of metabolic inhibitor treatment (data not shown), during which we previously demonstrated that ATP-dependent bile acid transport is clearly inhibited (27).

We also tested the effects of extracellular pH, Na⁺, or K⁺ concentrations on ⁶⁴Cu uptake in Ctr1^{-/-} cells. We previously demonstrated that Ctr1-mediated ⁶⁴Cu uptake is stimulated by both low extracellular pH and high extracellular K⁺ (27). As shown in Fig. 6A, we found that ⁶⁴Cu uptake in the Ctr1^{-/-} cells was stimulated in uptake buffer at pH 5.5 compared with the pH 6.5 or 7.5 uptake buffers (Fig. 6A). However, in contrast to Ctr1-mediated ⁶⁴Cu uptake, the K⁺ concentration in the medium did not effect ⁶⁴Cu uptake by the Ctr1-independent activity (data not shown).

It is believed that copper in mammalian circulation is bound to ligands such as albumin and histidine and that these chelate complexes may function in cellular copper uptake (1). However, we previously showed that Ctr1-mediated copper uptake is inhibited by supplementation of the assay medium with albumin or histidine (27). As shown in Fig. 6B, we tested the effects of copper ligands on ⁶⁴Cu transport in the Ctr1^{-/-} cells and observed that, like Ctr1-mediated copper uptake, both histidine and albumin have inhibitory effects on ⁶⁴Cu uptake.

Both mutant analysis and biochemical inhibitor studies have demonstrated that the high affinity plasma membrane copper uptake system in *S. cerevisiae* is dependent on Cu(II) reductases (30, 31). Indeed, by treating cells with ascorbate, which reduces Cu(II) to Cu(I), copper uptake can occur in the absence of the reductase activities (30). We previously demonstrated that human Ctr1-mediated copper uptake is also stimulated by treatment with ascorbate (27), suggesting that human Ctr1 transports Cu(I). We tested the effects of ascorbate treatment on ⁶⁴Cu uptake in Ctr1-deficient cells. Fig. 6B shows that 100 μM ascorbate in ⁶⁴Cu uptake medium did not stimulate ⁶⁴Cu uptake in the Ctr1^{-/-} cells. In contrast, however, this concentration of ascorbate clearly stimulated both the endogenous and Ctr1-mediated ⁶⁴Cu uptake in Hek293 cells (Fig. 6C). Indeed, the absence of ascorbate-stimulated copper uptake in the Ctr1-deficient cells is consistent with the earlier result showing a lack of competition by silver for ⁶⁴Cu uptake (Fig. 4A), since silver has similar chemical properties with Cu(I). These results suggest that Cu(II) is a substrate for the copper transport activity observed in Ctr1^{-/-} cells.

DISCUSSION

Consistent with critical roles for copper as an essential cofactor for a number of key cellular enzymes and its toxicity upon over-accumulation, copper metabolism is controlled via a number of fine-tuned mechanisms through uptake, distribution, and excretion (2, 24). Studies at the cellular level in both bakers' yeast and in fission yeast demonstrate that the presence of the Ctr1 family of high affinity copper transporters is crucial to render copper available to all three copper chaperone-mediated intracellular copper delivery pathways (4, 5, 8, 10). Furthermore, the generation of Ctr1 knockout mice has revealed that Ctr1 is essential for normal embryonic development and that Ctr1 heterozygous mice, whereas viable, exhibit tissue-specific partial defects in copper accumulation and the activities of some copper-dependent enzymes (28, 32). In this present work we generated and characterized Ctr1-deficient mouse embryonic cell lines to understand the function of the Ctr1 high affinity copper transporter in copper uptake and distribution at the cellular level. The analysis of two independently derived Ctr1 wild type, Ctr1^{+/-} and Ctr1^{-/-}, cell lines clearly establishes that in these mouse embryonic cells Ctr1 is

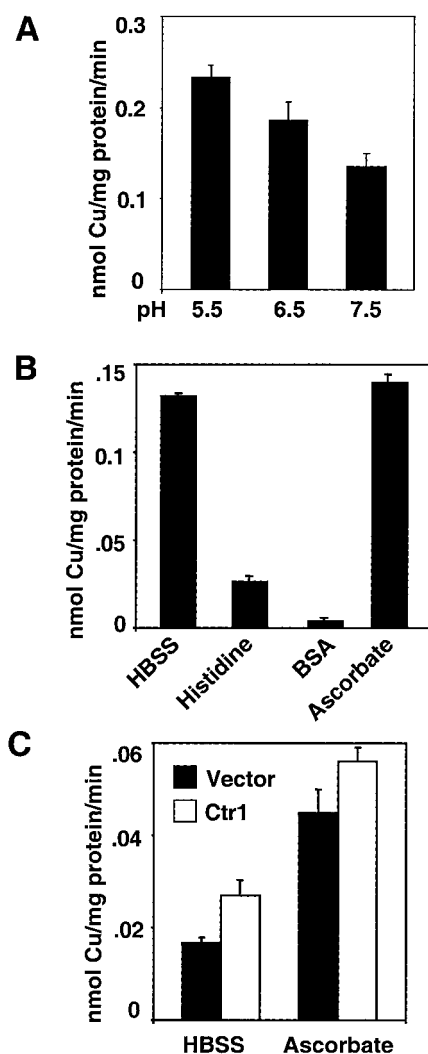


FIG. 6. Effect of extracellular pH, copper ligands, or ascorbate on ^{64}Cu uptake in Ctr1-deficient cells or Hek293 cells expressing mouse Ctr1. A, effect of pH on ^{64}Cu uptake activity in Ctr1-deficient cells was measured on cells incubated in HBSS (pH 7.5), Pipes-buffered salt solution (pH 6.5), or MBSS (pH 5.5) uptake buffer. B, Ctr1-deficient cells were incubated with $2\ \mu\text{M}$ ^{64}Cu in HBSS buffer with or without histidine ($50\ \mu\text{M}$), bovine serum albumin (BSA) (0.2%), or ascorbate ($100\ \mu\text{M}$) for 5 min. C, mouse Ctr1 expression vector (28) (white bar) or empty vector (black bar) was transiently transfected into Hek293 cells. ^{64}Cu uptake experiments were performed 2 days after transfection with or without ascorbate ($100\ \mu\text{M}$) treatment. Cells were incubated with $2\ \mu\text{M}$ ^{64}Cu for 5 min. ^{64}Cu uptake was quantitated and normalized to protein concentrations of cell lysates. Each data point represents the mean of four experiments \pm S.D.

a limiting factor for copper uptake, since these cells exhibit a Ctr1 gene dosage dependence for high affinity transport of ^{64}Cu . Although in bakers' yeast the structurally distinct Ctr1 and Ctr3 proteins are functionally redundant high affinity copper transporters localized to the plasma membrane (4, 5), both the targeted mouse Ctr1 deletion studies (28, 32) and the data described in this work clearly establish that mice do not express proteins that are functionally redundant with Ctr1.

The results of studies presented here clearly establish that mouse Ctr1 functions to provide copper to each of the three copper chaperones characterized and conserved from yeast to humans. Although Ctr1 $^{+/-}$ cells exhibited only marginal reductions in the activities of two cuproenzymes, Cu,Zn-SOD and cytochrome *c* oxidase, Ctr1 $^{-/-}$ cells displayed an $\sim 50\%$ reduction in the activities of these enzymes that was corrected by the addition of copper to the culture medium. Interestingly, the

activity levels of ectopically expressed tyrosinase were essentially undetectable in Ctr1 $^{-/-}$ cells and restored by the addition of copper to the cell culture medium or by the expression of mouse Ctr1. Currently it is unclear why the levels of tyrosinase activity in Ctr1 $^{-/-}$ cells are so exquisitely dependent on Ctr1 as compared with cytochrome oxidase or Cu,Zn-SOD. It is possible that the additional membrane barrier of the secretory compartment serves to enhance the dependence on Ctr1-mediated high affinity copper transport. Alternatively, since the cell lines we have established do not express endogenous tyrosinase, expression of the enzyme by transfection may have generated an enhanced dependence on Ctr1. It is also possible that the Atox1-dependent copper trafficking pathway to tyrosinase is unable to access the copper provided by the Ctr1-independent transporter. Nonetheless, these studies clearly demonstrate that copper transported into mammalian cells via the Ctr1 high affinity transporter is routed to all three characterized intracellular copper delivery pathways.

Previous studies demonstrate that the copper and heme A prosthetic groups in cytochrome *c* oxidase contribute not only catalytic cofactor function but also are important for stabilization of the assembled complex (16, 37–39). Mutations interfering with heme A biosynthesis reduce steady state levels of the three mitochondrially encoded subunits (37, 38), and mutations in the copper binding residues of subunit 2 destabilized the enzyme (39). Furthermore, mutation of *S. cerevisiae* Cox17, a cytoplasmic copper chaperone for cytochrome *c* oxidase, severely reduces the steady state levels of cytochrome oxidase subunits 1 and 2, the two copper-containing subunits (16). In this report we demonstrated that cytochrome *c* oxidase subunit 1 steady state levels are significantly reduced in Ctr1 $^{-/-}$ mouse cells, and these levels are recovered by copper supplementation. These results support the conclusion that Ctr1 is a physiologically important copper transporter and that copper incorporation via the Ctr1 transport pathway is critical for the stability of the cytochrome *c* oxidase complex. The effect of copper on Cu,Zn-SOD levels appears to be determined by the severity of the copper deficiency and organ specificity (41). In rats copper levels and Cu,Zn-SOD activities in the liver and heart are more sensitive to dietary copper deprivation as compared with brain and kidney. Although we observed no significant changes in Cu,Zn-SOD steady state protein levels in our cells with distinct Ctr1 alleles, the precise lineage of these cell lines is currently unknown.

Mouse Ctr1 is expressed in all tissues examined (9), and Ctr1 mRNA levels in tissues of Ctr1 heterozygous mice are uniformly reduced by 50% compared with the levels observed in the wild type (28). However, in Ctr1 $^{+/-}$ mice brain and spleen manifest a 50% reduction in total copper accumulation and $\sim 20\%$ decreases in Cu,Zn-SOD and cytochrome oxidase activity, with no significant change of copper levels in the liver and kidney (28). These observations demonstrate that both alleles of Ctr1 are required for normal copper metabolism in specific tissues and also raise questions regarding the underlying mechanisms for the tissue-specific dependence on Ctr1 levels. One possibility we previously suggested (28) is that some tissues may express Ctr1 levels in excess of need and that 50% reductions do not fall below the physiologically important threshold. Alternatively, we speculated that there may exist variable tissue-specific levels of an alternative copper transport system. Indeed, the results presented here clearly demonstrate the presence of such an alternative, Ctr1-independent, copper transport system. Although not sufficient to allow normal embryonic development in the complete absence of Ctr1, this Ctr1-independent copper uptake activity may enable some tissues to accumulate normal levels of copper, at least in the Ctr1

heterozygous state. The distinct nature of this system compared with Ctr1-mediated copper transport is further supported by the ~10-fold increase in K_m for copper, the lack of stimulation by K^+ ions or ascorbate (which reduces Cu(II) to Cu(I)), and the distinct metal competition profile, with zinc clearly competing for ^{64}Cu uptake but silver unable to compete. However, like Ctr1-mediated copper uptake, the Ctr1-independent activity is saturable, time-, temperature-, and pH-dependent, ATP-independent, and inhibited by the biological copper ligands histidine and albumin. Taken together, these data suggest that the Ctr1-independent copper transport activity we describe could function to transport Cu(II) rather than Cu(I).

What is the identity of this alternative copper transport system? Although a Ctr1-related protein, Ctr2, has been identified in yeast and mammals (6, 7), no evidence exists to support the hypothesis that Ctr2 serves to directly transport copper into cells. Biochemical and genetic evidence suggests that yeast low affinity iron transporter Fet4 is able to transport copper to the three known delivery pathways (42–44), but a protein harboring equivalent functions to Fet4 has not been identified yet in mammals. The divalent metal transporter-1 (DMT1, DCT1, or Nramp2) is an iron transporter that is thought to have a broad range of substrate specificity and was suggested to transport copper (45–47). However, the Ctr1-independent copper transport activity characterized here was not competed by a 50-fold molar excess of iron, and it has been demonstrated that although DMT1 mutant mice exhibit microcytic anemia, these mice do not display copper deficiency (48). Therefore, although copper and iron could be transported by DMT1 via distinct mechanisms, there is no evidence at present that the Ctr1-independent copper uptake activity involves DMT1. However, given that the residual ^{64}Cu transport activity observed in Ctr1-deficient cells is strongly competed by zinc and the observation that copper competes for zinc uptake through human Zip1 zinc transporter (40), we tested whether this alternative copper transport activity could be due to Zip1 or Zip2 and found no evidence for this mechanism. Therefore, it is not yet clear which system is responsible for the residual copper transport activity in Ctr1-deficient cells. Current efforts are under way to identify the components of this copper uptake pathway to gain a more detailed understanding of the mechanisms by which copper enters cells.

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