THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 6, Issue of February 8, pp. 4380–4387, 2002
Printed in U.S.A.
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Biochemical Characterization of the Human Copper Transporter Ctr1*

Received for publication, May 23, 2001, and in revised form, November 29, 2001
Published, JBC Papers in Press, December 4, 2001, DOI 10.1074/jbc.M104728200

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The trace metal copper is an essential cofactor for a number of biological processes including mitochondrial oxidative phosphorylation, free radical detoxification, neurotransmitter synthesis and maturation, and iron metabolism. Consequently, copper transport at the cell surface and the delivery of copper to intracellular proteins are critical events in normal physiology. Little is known about the molecules and biochemical mechanisms responsible for copper uptake at the plasma membrane in mammals. Here, we demonstrate that human Ctr1 (hCtr1) is a component of the copper transport machinery at the plasma membrane. hCtr1 transports copper with high affinity in a time-dependent and saturable manner and is metal-specific. hCtr1-mediated ^{64}Cu transport is an energy-independent process and is stimulated by extracellular acidic pH and high K^+ concentrations. hCtr1 exists as a homomultimer at the plasma membrane in mammalian cells. This is the first report on the biochemical characterization of the human copper transporter hCtr1, which is important for understanding mechanisms for mammalian copper transport at the plasma membrane.

Copper is a micronutrient that plays an essential role in biology, serving as a co-factor for several enzymes that include Cu,Zn-superoxide dismutase, cytochrome oxidase, lysyl oxidase, and ceruloplasmin (1, 2). Dietary copper limitation studies in animals, as well as the existence of human genetic diseases of copper homeostasis such as Menkes and Wilson disease, underscore critical roles for proper copper absorption in the intestine and distribution to the organs and tissues to serve as an essential biochemical co-factor for these enzymatic activities and other important biological processes (3–6).

At the cellular level, copper is transported at the plasma membrane and distributed to cellular proteins and compartments for the incorporation of copper into copper-dependent proteins. Studies in yeast cells first identified genes encoding high affinity copper ion transport proteins in the plasma membrane. Either prior to or concomitant with high affinity uptake, Cu(II) is reduced to Cu(I) by one or more metalloreductases encoded by the *FRE1* through *FRE7* genes (7–9). Cu(I) is delivered across the plasma membrane by the high affinity transporter Ctr1 or Ctr3 in *S. cerevisiae* and the Ctr4 and Ctr5 heteromeric complex in *Schizosaccharomyces pombe*, with K_m values in the low micromolar range (10–13). After crossing the plasma membrane, copper is delivered to the secretory compartment, mitochondria, and cytosolic enzymes by the target-specific copper chaperone proteins, Atx1/Atox1, Cox17, and CCS, respectively (14–18). Yeast cells lacking high affinity copper transporters exhibit striking defects in copper and iron uptake, mitochondrial respiration, and Cu,Zn-superoxide dismutase activity (10–13). Recently, candidates for human and murine copper transporters have been isolated by complementation of yeast Ctr mutants and by data base homology searches (19, 20). Mammalian Ctr1 mRNA is expressed in all tissues examined, with relatively high expression levels in liver and kidney and lower levels in the brain and spleen (19, 20). Murine Ctr1 displays 92% sequence identity to human Ctr1 and maps to a syntenic locus in the mouse genome (20). While mammalian Ctr1 functionally complements several growth phenotypes associated with yeast cells lacking the Ctr1 and Ctr3 high affinity copper transporters, and there are structural similarities among known copper transporters in yeast and putative copper transporters in mammals, little is known about the function and biochemical characteristics of human Ctr1 in copper uptake in mammalian cells.

We have examined the kinetics of copper transport, metal specificity, localization, oligomerization, and roles for energy and an electrochemical gradient at the plasma membrane in copper uptake by the functional expression of hCtr1[1] in a human embryonic kidney (Hek293) cell line. Our studies demonstrate that hCtr1 is a component of a plasma membrane copper transporter system. hCtr1 oligomerizes and transports copper with specificity and in a time-, concentration-, and extracellular pH- and K^+-dependent manner. Given the known importance of mouse Ctr1 in copper homeostasis and embryonic development, as demonstrated by the characterization of Ctr1 gene knock-out mice (21, 22), the biochemical properties established here are consistent with mammalian Ctr1 playing a key role in copper acquisition across the plasma membrane and provide fundamental information for understanding copper transport mechanisms by hCtr1 protein.

EXPERIMENTAL PROCEDURES

Human Ctr1 Expression Vectors—The human Ctr1 open reading frame, with Kozak translation initiation sequences in the 5′-untranslated region (23), was PCR-amplified from the hCtr1 cDNA clone (20)

* This work was supported by National Institutes of Health Grant GM62555 and a grant from the International Copper Association (to D. J. T.), National Institutes of Health Postdoctoral Fellowship 5F32GM18089 (to M. M. O. P.), and American Heart Association Postdoctoral Fellowship 9920536 (to J. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: hCtr1 and -2, human Ctr1 and -2, respectively; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hepes-buffered saline solution; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; EGS, ethylene glycol bis(succinimidylsuccinate).

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using human hCtr1-specific primer sets. For amino-terminal epitope-tagging of hCtr1, a *Not*I restriction enzyme site was generated in the upstream PCR primer just after the translation initiation codon. PCR products were inserted into the *Eco*RI and *Xho*I sites in the pcDNA3.1(+) (Invitrogen) and p413-GPD (24) vectors for the expression of hCtr1 in human embryonic kidney cells (Hek293) and in *Saccharomyces cerevisiae*, respectively. A DNA fragment encoding one c-Myc epitope was inserted into the *Not*I restriction enzyme site within the hCtr1 open reading frame. The function of the c-Myc-tagged hCtr1 allele was compared with the wild type allele by complementation studies in the *S. cerevisiae* strain MPY17 (25), harboring deletions of the *CTR1* and *CTR3* genes, in which copper transport-competent cells are able to utilize nonfermentable carbon sources for growth and by ^{64}Cu uptake assays in transfected Hek293 cells.

Cell Culture and Transient Transfections—Hek293 cells were cultured in DMEM (Invitrogen) with 10% fetal bovine serum (FBS) under 5% CO_2. Cells were transfected with the pcDNA3.1 vector or pcDNA3.1 expressing the human Ctr1 cDNA under control of the cytomegalovirus promoter. Transfections were performed using FuGENE™6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. We observed a range of transfection from 50 to 70% in Hek293 cells. 24 h after transfection, cells were collected from transfected dishes and evenly divided for copper uptake assays.

^{64}Cu Uptake Assays—Radioactive copper (^{64}Cu) was purchased from the Mallinckrodt Institute of Radiology, Washington University (Saint Louis, MO). The average specific activity of ^{64}Cu was 16 mCi/μg of copper in the form of $CuCl_2$ in 0.1 M HCl. ^{64}Cu was added to Hek293 cell culture medium 2 days after transfection with empty vector or the hCtr1 expression vector. Stimulation of copper uptake in Hek293 cells by the expression of hCtr1 was measured by incubating the cells with 2 μM ^{64}Cu in DMEM with or without 10% FBS, Hepes-buffered salt solution (HBSS) (pH 7.5), or HBSS with 0.2% bovine serum albumin or 50 μM histidine. HBSS (pH 7.5) contains 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, and 10 mM Hepes. For time course studies, cells cultured in DMEM containing 10% FBS were incubated for 2–60 min with 5 μM ^{64}Cu. For dose-response assays, cells were incubated with 0.25–25 μM ^{64}Cu for 5 min. For metal competition assays, 10- or 50-fold molar excess concentrations of nonradioactive metals ($CuCl_2$, $FeCl_2$, $ZnCl_2$, $MnCl_2$, $AgNO_3$, or $CdSO_4$) (Sigma) were added to cell growth medium with ^{64}Cu for 5 min. To study the effects of reductant on hCtr1-stimulated ^{64}Cu uptake, ascorbate (1 mM final concentration) was added to copper uptake medium simultaneously with the ^{64}Cu addition. After incubations, the uptake medium was aspirated, and copper uptake was quenched by adding ice-cold EDTA (10 mM in PBS). Cells were washed three times with ice-cold PBS and resuspended in 0.1% SDS, 1% Triton X-100, PBS buffer for cell lysis. Aliquots of cell lysate were counted using a γ-counter (Packard Cobra II). Parallel experiments were conducted at 4 °C for cell surface ^{64}Cu binding, which was subtracted from the values at 37 °C to obtain net copper uptake values. Copper uptake was calculated using a standard curve and normalized to protein concentrations of cell lysates. V_{max} and K_m were determined via extrapolation to zero in the reciprocal plot for velocity *versus* substrate concentration using GraphPad PRISM™ software.

Effect of Metabolic Inhibitors and Extracellular pH, Na^+, or K^+ on ^{64}Cu Uptake—Hek293 cells with or without hCtr1 expression plasmid transfection were preincubated for 60 min with metabolic inhibitors before ^{64}Cu uptake assays were performed. Stock solutions of antimycin A, oligomycin, or ouabain (Sigma) in 95% ethanol or sodium azide in water were prepared. Antimycin A (3.6 μM), oligomycin (4.7 μM), sodium azide (0.1 mM) or ouabain (1 mM) was added into culture medium in a volume of less that 0.5% of culture medium for a 60-min preincubation before ^{64}Cu transport was measured. The concentration of and length of preincubation with metabolic inhibitors were based on previous studies (26), and the efficacy of treatment was confirmed by the energy-dependent [^{3}H]taurocholate transport assay (see below). To test the effect of pH on copper uptake, cells were preincubated for 15 min in HBSS (pH 7.5) with or without 0.2% bovine serum albumin, Pipes-buffered salt solution (PBSS; substitution of Pipes for Hepes of HBSS, pH 6.5), or MES-buffered salt solution (MBSS; substitution of MES for Hepes of HBSS, pH 5.5), and then 2 μM ^{64}Cu was added to the buffer for 5 min. To investigate the effects of sodium or potassium content of the buffer on ^{64}Cu uptake, NaCl in HBSS was replaced by KCl or choline chloride, and ^{64}Cu uptake assays were performed. Valinomycin (1.4 μM), a K^+-selective ionophore, was dissolved in glacial acetate, diluted in water, and added in uptake buffer in a volume of less than 0.5% of culture medium for 30 min before ^{64}Cu uptake measurements. All control experiments were performed with the addition of the same amount of solvents used for dissolving chemicals.

Energy-dependent [^{3}H]Taurocholate Transport Assay in Hek293 Cells—The ileal bile acid transporter expression plasmid (27) or empty vector was transfected into Hek293 cells by the method as described for hCtr1 expression. Two days after transfection, cells were incubated with oligomycin (4.7 μM) or ouabain (1 mM) in cell culture medium (DMEM with 10% FBS) for 1 h, and then [^{3}H]taurocholate was added to a final concentration of 250 μM for 5 min. After incubation, the medium was removed, and cells were washed three times with ice-cold PBS containing 1 mM cold taurocholate (Sigma). Cells were solubilized, and aliquots were taken to determine radioactivity and protein concentration. [^{3}H]Taurocholate (74 GBq/mmol) was purchased from PerkinElmer Life Sciences.

Subcellular Fractionation and Western Blotting Analysis—Total protein was extracted from Hek293 cells 2 days after transfection with the Myc epitope-tagged hCtr1 expression plasmid. Cells were washed in PBS and resuspended in homogenization buffer (10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 2 mM EDTA, protease inhibitor mixture (Roche Molecular Biochemicals)). Cells were then homogenized by 15 passages through a 23-gauge needle and 40 strokes with a Dounce homogenizer. The cell homogenate was centrifuged at 20,000 × *g* for 2 min at 4 °C, and the supernatant was collected for total protein extract. Total protein extract was further centrifuged at 100,000 × *g* for 30 min, the supernatant (cytosolic fraction) was collected, and the pellet (membrane fraction) was resuspended in buffer (10 mM Tris (pH 7.4)/2 mM EDTA), 0.2 M sodium carbonate (pH 11), or 1% Triton X-100 at a final concentration of less than 1 mg of protein/ml. The suspensions were incubated for 30 min on ice and centrifuged at 100,000 × *g* for 30 min. The supernatant containing remaining soluble and detached peripheral membrane proteins was precipitated in trichloroacetic acid (10%), washed twice with acetone, and resuspended in SDS buffer. After incubation at 37 °C, the samples were analyzed by SDS-PAGE and immunoblotting. Monoclonal anti-c-Myc antibody (Roche Molecular Biochemicals) was used to detect hCtr1-Myc protein. G protein β subunit antibody (Upstate Biotechnology, Inc., Lake Placid, NY) was used as a control to demonstrate that peripheral membrane proteins are released by treatment of the membranes with 0.2 M sodium carbonate, pH 11.

Indirect Immunofluorescence—Hek293 cells were harvested 2 days after transfection with the c-Myc epitope-tagged hCtr1 expression vector and seeded onto a polylysine-coated cover glass. Cells were cultured for 24 h, washed with PBS, fixed with 4% paraformaldehyde, and permeabilized by treatment with 0.1% Triton X-100 for 10 min at room temperature. Cells were treated with 4′,6-diamidino-2-phenylindole for nuclear DNA staining and anti-c-Myc monoclonal antibody (Roche Molecular Biochemicals) followed by goat anti-mouse IgG (H + L)-conjugated fluorescein (Oregon Green) (Molecular Probes, Inc., Eugene, OR) to detect hCtr1-Myc. Cover glasses were mounted on slides with mounting medium (Molecular Probes) and 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.

In Vitro Cross-linking—Total protein extracts were obtained from Hek293 cells expressing c-Myc epitope-tagged hCtr1 harvested 2 days after transfection, washed two times with PBS, and lysed with buffer (PBS, pH 7.4, containing 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and protease inhibitor mixture (Roche Molecular Biochemicals)). 30 μg of protein were incubated for 30 min at room temperature with increasing concentrations (0, 0.25, 0.5, 1.0, 2.0, and 3.0 mM) of ethylene glycol bis(succinimidylsuccinate) (EGS) from stock solutions in Me_2SO. The volume of Me_2SO in each reaction was no more than 10% of the total reaction volume. Cross-linking reactions were quenched with 45 mM Tris-HCl, pH 7.5, followed by incubation at room temperature for an additional 30 min. The cross-linked products were analyzed by SDS-PAGE and immunoblotting using anti-c-Myc monoclonal antibody.

RESULTS

Expression of hCtr1 Stimulates ^{64}Cu Uptake in Human Cells—Previously, we and others isolated a human cDNA that encodes a protein which functionally complements phenotypes associated with *S. cerevisiae* cells defective in high affinity copper transport (19, 20). Based on these functional observations, the similar predicted topological arrangements and primary structural homology to the *S. cerevisiae* high affinity copper transport proteins Ctr1 and Ctr3 and the *S. pombe* copper transporters Ctr4 and Ctr5, human Ctr1 has been postulated to encode a mammalian high affinity copper trans-

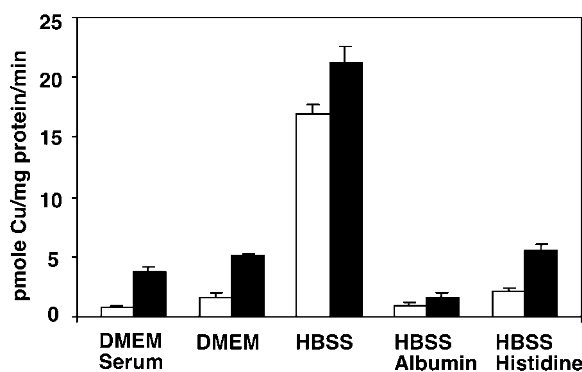

FIG. 1. **Human Ctr1 expression in Hek293 cells stimulates ^{64}Cu uptake.** Initial rates of ^{64}Cu uptake in Hek293 cells were measured 2 days after transfection of an empty vector (*white bar*) or hCtr1 expression vector (*black bar*). Cells were incubated with 2 μM ^{64}Cu in DMEM with or without 10% FBS, HBSS (pH 7.5), or HBSS with 0.2% bovine serum albumin or 50 μM histidine for 5 min. Copper uptake was quantitated and normalized to protein concentrations of cell lysates. Each data point represents the mean of four experiments ± S.D.

porter (19, 20, 28). To ascertain whether hCtr1 plays a role in copper transport in human cells, hCtr1 protein was expressed in Hek293 cells by transient transfection of an expression plasmid carrying the hCtr1 open reading frame under the control of the cytomegalovirus promoter. The initial rate of copper uptake was measured in Hek293 cells expressing hCtr1 by incubation of the cells with 2 μM ^{64}Cu for 5 min, and the effects of potential copper ligands in serum, such as albumin and histidine, on hCtr1-stimulated ^{64}Cu uptake were examined. ^{64}Cu accumulation in the vector control cells indicates the presence of a low level of copper uptake in Hek293 cells (Fig. 1), consistent with low but detectable levels of hCtr1 mRNA detected in these cells by RNA blotting analysis (data not shown). hCtr1 expression stimulates the initial rate of ^{64}Cu uptake 3–7-fold over vector-transfected control cells, depending on the transfection efficiency in each experiment (Fig. 1). Interestingly, stimulation of copper uptake by hCtr1 expression is more obvious when ^{64}Cu uptake is measured in DMEM cell culture medium rather than HBSS (Fig. 1). It has been reported that copper in mammalian serum is bound to ligands such as albumin and histidine; thus, these serum ligands may play an important role in the copper uptake process (1). We examined potential roles of serum components by measuring copper uptake in DMEM with or without 10% serum and in HBSS with or without albumin and histidine. ^{64}Cu uptake is significantly higher in HBSS compared with DMEM with or without serum, which suggests that components of DMEM and serum, such as amino acids and albumin, inhibit copper uptake (Fig. 1). Furthermore, since copper uptake in HBSS is inhibited by supplementation with albumin or histidine (Fig. 1), copper may be dissociated from these ligands for hCtr1-mediated transport.

Human Ctr1-stimulated ^{64}Cu Uptake in Hek293 Cells Is Concentration-dependent and Saturable—^{64}Cu accumulation was examined in Hek293 cells both as a function of time and copper concentration (Fig. 2). copper accumulation was measured by incubating cells with ^{64}Cu in DMEM culture medium with 10% serum, because we could clearly observe stimulation of ^{64}Cu uptake in the Hek293 cells expressing hCtr1 under these conditions compared with other uptake buffer conditions (Fig. 1). ^{64}Cu uptake in cells transfected with the hCtr1 expression plasmid was stimulated, in a time-dependent manner, with a greater than 30-fold stimulation over vector-transfected cells at 60 min (Fig. 2*A*). A copper dose-response analysis demonstrated that copper uptake is concentration-dependent and saturable (Fig. 2*B*). Further analysis of these data (see

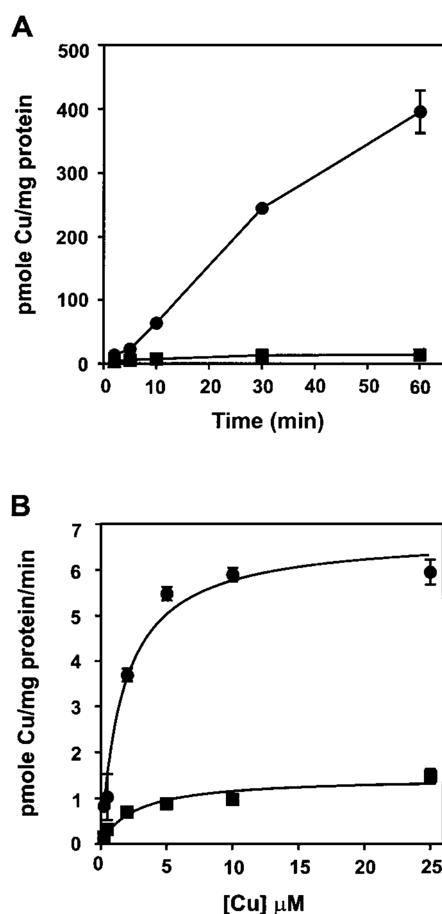

FIG. 2. **Stimulation of copper transport by hCtr1 expression is time-dependent and saturable.** *A*, time dependence for ^{64}Cu uptake in Hek293 cells. 5 μM ^{64}Cu was added to Hek293 cell culture medium 2 days after transfection with an hCtr1 expression vector (*black circles*) or empty vector (*black squares*). Cells were incubated for 2–60 min at 37 °C, and ^{64}Cu uptake was measured. *B*, concentration dependence for ^{64}Cu uptake. 0.25, 1, 2, 5, 10, or 25 μM ^{64}Cu was added to Hek293 cell culture medium 2 days after transfection with the hCtr1 expression vector (*black circles*) or empty vector (*black squares*) and incubated for 5 min. Copper uptake was quantitated and normalized to protein concentrations of cell lysates. Each data point represents the mean of four experiments ± S.D.

"Experimental Procedures") indicates an apparent K_m for copper uptake in vector-transfected Hek293 cells under these experimental conditions of 2.56 ± 1.04 μM and a $V_{\max}$ of 1.45 ± 0.17 pmol of Cu/min/mg of protein. Transfected cells expressing hCtr1-Myc manifests a K_m of 1.71 ± 0.39 μM, similar to the endogenous system, and a $V_{\max}$ of 6.76 ± 0.39 pmol of Cu/mg of protein/min, as expected for increased hCtr1 expression as compared with wild type levels indicated by RNA blotting experiments (data not shown). These results demonstrate that hCtr1 stimulates high affinity copper transport when expressed in cultured human cells.

hCtr1 Transporter Metal Specificity—We examined whether the endogenous copper uptake activity in Hek293 cells, and cells expressing transfected hCtr1, transport other metals or whether these are relatively specific copper transport activities. 10- or 50-fold molar excesses of copper or five other nonradioactive metals (iron, zinc, manganese, silver, and cadmium) were independently added to the ^{64}Cu uptake medium to test whether these metals could compete for ^{64}Cu uptake. Co-incubation with a 10-fold molar excess of nonradioactive copper inhibited more than 90% ^{64}Cu uptake for both the endogenous (Fig. 3*A*) and hCtr1-transfected cells (Fig. 3*B*). Among the other metals tested, only Ag(I) displayed similar

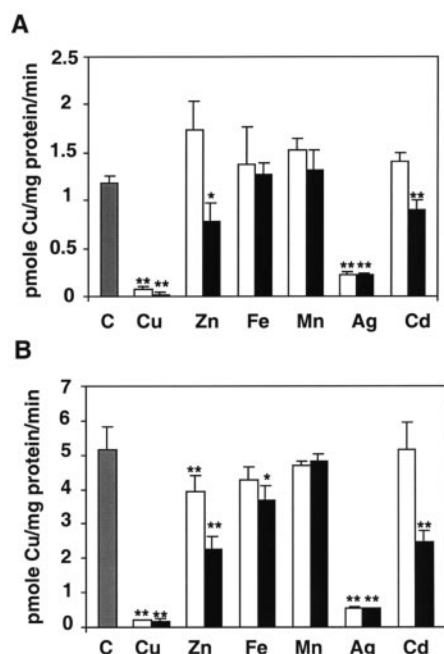

FIG. 3. **Competition of endogenous and hCtr1-stimulated copper uptake by other metals.** The effects of other metal ions on ^{64}Cu uptake by the endogenous system (*A*) or hCtr1 (*B*) in Hek293 cells are shown. 10-fold (*white bar*) or 50-fold (*black bar*) molar excesses of copper, zinc, iron, manganese, silver, or cadmium were added in uptake medium with 5 μM ^{64}Cu. ^{64}Cu accumulation was measured and compared with untreated control cells (*gray bar*). Copper uptake was normalized to protein concentrations of cell lysates. Each point represents the mean of four experiments ± S.D. The *asterisks* indicate significant differences (*, $p < 0.05$; **, $p < 0.01$) by Student's *t* test.

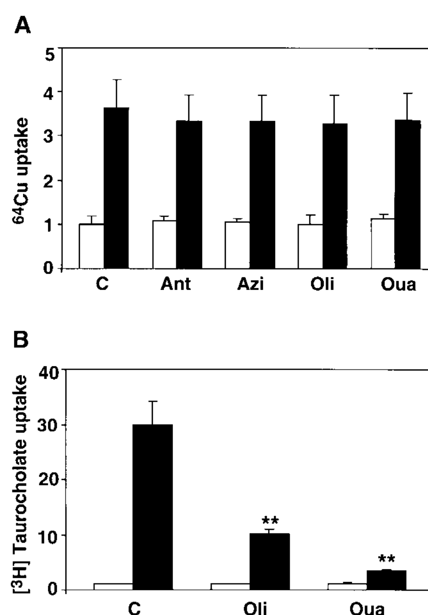

FIG. 4. **Effects of metabolic inhibitors on hCtr1-mediated ^{64}Cu uptake in Hek293 cells.** *A*, the effect of metabolic inhibitors on copper uptake was evaluated by preincubation of Hek293 cells with antimycin A (*Ant*) (3.6 μM), oligomycin (*Oli*) (4.7 μM), sodium azide (*Azi*) (0.1 mM), or ouabain (*Oua*) (1 mM) for 1 h. ^{64}Cu transport was measured by incubation of cells in DMEM with 5 μM ^{64}Cu for 5 min. Experiments were performed with both vector transfected cells (*white bars*) and hCtr1 expression vector transfected cells (*black bars*). The concentration of and length of preincubation with metabolic inhibitors was based on previous studies (26), and the efficacy of treatment was confirmed by the energy-dependent [^{3}H]taurocholate transport assay in Hek293 cells transfected with the ileal bile acid transporter expression vector (*B*). The values of ^{64}Cu uptake or [^{3}H]taurocholate uptake are relative ratios of the uptake value in vector-transfected control cells without treatment of metabolic inhibitor. Each data point represents the mean of four experiments ± S.D. The *asterisks* indicate significant difference (**, $p < 0.01$) from Student's *t* test.

inhibitory potency as copper on ^{64}Cu uptake, suggesting that Ag(I) may also be a substrate for transport by hCtr1 and that Cu(I) is transported by hCtr1. Consistently, supplementation of ascorbic acid (1 mM) in DMEM plus 10% FBS to reduce Cu(II) to Cu(I) enhances ^{64}Cu uptake approximately 2-fold (data not shown). 50-fold molar excesses of zinc, iron, or cadmium also significantly inhibited ^{64}Cu uptake, and we could not observe a significant effect of ascorbate on the competition by these metals (data not shown). Therefore, iron, zinc, and cadmium either may be low affinity substrates for the copper transport system or may simply inactivate the endogenous and hCtr1-mediated copper transport activities at very high concentrations. To investigate these possibilities, we treated cells for 5 min with 50-fold molar excesses of competitor metals, washed cells to remove competitor, and then measured ^{64}Cu uptake, but we observed no effect of iron, zinc, manganese, or cadmium pretreatment on ^{64}Cu uptake (data not shown). We did, however, observe an ~10–15% inhibition of ^{64}Cu uptake by pretreatment with copper or silver (data not shown), suggesting that these metals were either incompletely washed away or that there was some as yet uncharacterized form of regulation by these specific metals. The lack of strong competition by metals except copper and silver at a 10-fold molar excess is probably not due to chelation of these metals by components of the culture medium, since we observe the same pattern of metal-specific competition for ^{64}Cu uptake in HBSS (data not shown). Therefore, we suggest that zinc, iron, and cadmium may be either reversible inhibitors or low affinity substrates of hCtr1 protein. Taken together, these results suggest that both the endogenous copper transport system and hCtr1 comprise rather metal-specific high affinity copper transport activities.

Effects of Energy Generation and Extracellular pH, Na^+, and K^+ on Copper Uptake—Mammalian and yeast Ctr family copper transporter proteins do not possess an obvious ATP-binding domain, suggesting that copper uptake by the Ctr1 protein may not be energy-dependent. However, it is possible that ATP hydrolysis is required for high affinity copper transport and that other components of a functional copper transporter complex may hydrolyze ATP. We tested this hypothesis by pretreatment of cells with metabolic inhibitors to decrease ATP levels and carried out copper uptake experiments. Treatment of cells with antimycin A, oligomycin, or sodium azide did not inhibit ^{64}Cu uptake in Hek293 cells by either the endogenous system or in hCtr1-transfected cells (Fig. 4*A*). The efficacy of metabolic inhibitor treatment under these conditions was evident by the demonstration that oligomycin inhibited the activity of the ATP-dependent bile acid transporter, expressed in Hek293 cells, while there is no inhibition of hCtr1-mediated ^{64}Cu transport (Fig. 4*B*). These data strongly suggest that hCtr1-mediated copper transport is not an energy-dependent process.

Previous studies have shown that a number of nutrient and ion transporters, including DCT1 (Nramp2, DMT1), a divalent metal transporter that has a broad substrate range including iron and copper, mediate active transport that is proton-coupled and dependent on cell membrane potential (29, 30). We tested whether either endogenous or hCtr1-stimulated copper transport in 293 cells may be mediated by an electrochemical gradient by conducting ^{64}Cu transport assays in buffers of different pH. Indeed, copper accumulation in Hek293 cells was significantly increased at pH 6.5 and 5.5 compared with pH 7.5 (Fig. 5*A*), for hCtr1-transfected cells, although the endogenous activity was not as strongly stimulated at low pH.

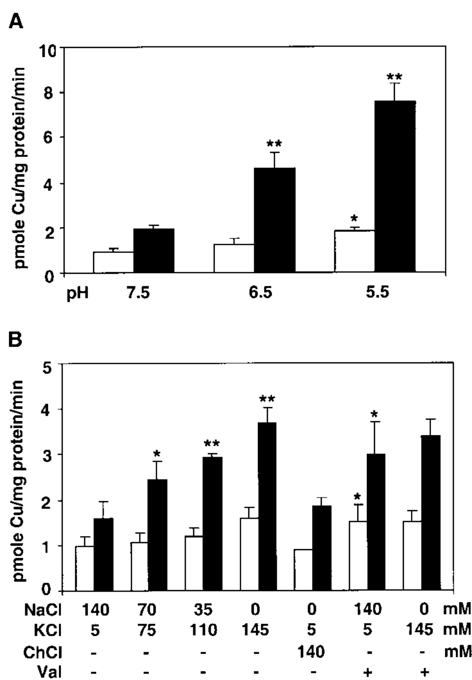

FIG. 5. **Effects of extracellular pH and K^+ on endogenous and hCtr1-mediated ^{64}Cu uptake in Hek293 cells.** *A*, effect of pH on copper uptake activity was measured on cells incubated in HBSS (pH 7.5), PBSS (pH 6.5), or MBSS (pH 5.5) containing 0.2% bovine serum albumin. Hek293 cells with or without transfection of the hCtr1 expression plasmid were preincubated for 15 min with uptake buffer, and ^{64}Cu accumulation was measured by adding 2 μM ^{64}Cu in the uptake buffer for 5 min. *White bars* and *black bars* represent endogenous and hCtr1-mediated ^{64}Cu uptake activities, respectively. Each value obtained from endogenous and hCtr1- mediated ^{64}Cu uptake at pH 5.5 or pH 6.5 was compared with the control value obtained from cells incubated in pH 7.5 buffer. *B*, to investigate the effects of sodium or potassium content of the buffer on the ^{64}Cu uptake, NaCl in HBSS was successively replaced by KCl or choline chloride (*ChCl*), and then ^{64}Cu uptake was assayed by adding 2 μM ^{64}Cu in the uptake buffer for 5 min. Each value obtained from endogenous or hCtr1- mediated ^{64}Cu uptake obtained in higher K^+ concentration buffer was compared with its control value obtained from low K^+ buffer (140 mM NaCl, 5 mM KCl). The pretreatment with 1.4 μM valinomycin (*Val*) (K^+ ionophore) for 30 min before measuring ^{64}Cu accumulation was indicated. ^{64}Cu uptake with valinomycin treatment was compared with the value obtained from the same buffer condition without treatment of valinomycin. *White bars* and *black bars* represent endogenous and hCtr1-mediated ^{64}Cu uptake activities, respectively. Each value represents the mean of four experiments ± S.D. The *asterisks* indicate significant difference (*, $p < 0.05$; **, $p < 0.01$) from Student's *t* test.

It is well established that some transport activities, such as the glucose transporter, are monovalent cation-dependent processes (31). As shown in Fig. 5*B*, we have tested whether the alteration of extracellular Na^+ or K^+ concentrations affect hCtr1-mediated copper uptake. Incubation of Hek293 cells with high K^+ buffer (115 mM) significantly increased copper accumulation compared with low K^+ buffer (5 mM), and the use of choline chloride indicates that this is not due to decreased Na^+ concentrations (Fig. 5*B*). Furthermore, pretreatment of cells with ouabain, a potent Na^+-K^+-ATPase inhibitor, to increase intracellular Na^+ concentrations did not alter copper accumulation in Hek293 cell by the endogenous transporter or in hCtr1 expression vector-transfected cells (Fig. 4*A*). However, the K^+ ionophore valinomycin increased copper accumulation, although, as expected, K^+ inonophore treatment in a high extracellular K^+ buffer did not further increase intracellular copper accumulation (Fig. 5*B*). These results suggest that copper transport at the plasma membrane is a Na^+-independent but K^+-stimulated process.

Human Ctr1 Is an Integral Membrane Protein Localized to the Plasma Membrane—The amino acid sequence of hCtr1 protein has homology with copper transporters from yeast, plants, and mice and is predicted to contain three transmembrane regions (28). To ascertain whether hCtr1 is an integral membrane protein, c-Myc epitope-tagged hCtr1 was expressed in Hek293 cells by transient transfection, extracts were prepared by differential centrifugation, and hCtr1 was detected by SDS-PAGE and immunoblotting using anti-Myc antibody. The Myc-hCtr1 protein was judged to be functional based on its ability to stimulate ^{64}Cu transport when expressed in Hek293 cells and to complement the respiratory growth phenotype of *S. cerevisiae ctr1Δ ctr3Δ* cells (data not shown). Epitope-tagged hCtr1 was detected as an ~29-kDa protein, and variable levels of a 58-kDa species were observed in cell extracts (Fig. 6). The migration of the hCtr1-Myc protein is slightly slower than that predicted from hCtr1 coding sequences (21 kDa) fused to the Myc epitope sequences. Total cell extracts were fractionated by centrifugation (100,000 × *g*) and analyzed by immunoblotting. hCtr1 was detected in the pellet, corresponding to a membrane fraction, but little if any hCtr1 was detected in the soluble fraction (Fig. 6, *lanes 3* and *4*). The membrane fraction was incubated with 0.2 M Na_2CO_3 (pH 11), which releases peripheral membrane proteins into the soluble fraction (30). The G-protein β subunit, which is a well characterized peripheral membrane protein, was indeed released into the soluble fraction by Na_2CO_3 treatment (Fig. 6, *lower panel*, *lanes 6* and *7*). However, under these same conditions, hCtr1 was detected only in the membrane fraction (Fig. 6, *upper panel*, *lanes 6* and *7*). Incubation of the membrane fraction with Triton X-100 (1%) released nearly all detectable hCtr1 into the soluble fraction, suggesting that hCtr1 is not a component of a large protein complex that is pelleted by high speed centrifugation (Fig. 6, *lanes 8* and *9*). Taken together, these results demonstrate that a functional epitope-tagged hCtr1 protein, expressed in Hek293 cells, is an integral membrane protein.

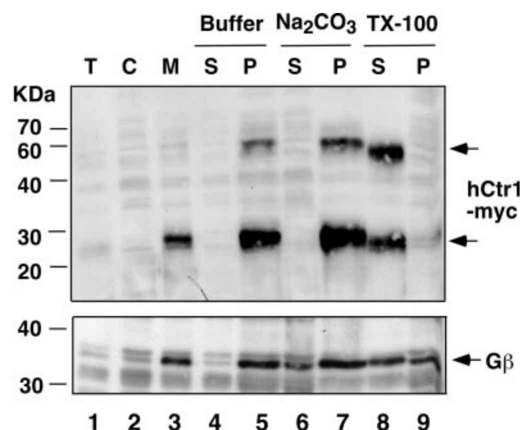

FIG. 6. **Human hCtr1 is an integral membrane protein.** Total protein was extracted from Hek293 cells 2 days after transfection with the Myc-hCtr1 expression vector. Total protein extract (*T*) was further centrifuged at 100,000 × *g* for 30 min. The supernatant (cytosolic fraction) (*C*) was collected, and the pellet (membrane fraction) (*M*) was resuspended in buffer, 0.2 M sodium carbonate (pH 11), or 1% Triton X-100. The suspensions were incubated for 30 min on ice and then centrifuged at 100,000 × *g* for 30 min. The supernatant (*S*) containing the remaining soluble and detached peripheral proteins was separated from the pellet (*P*) and precipitated in trichloroacetic acid (10%) and washed two times with acetone. Samples were resuspended in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting with anti-c-Myc antibody. Analysis of a G protein β subunit (*Gβ*) was performed as a control to demonstrate that peripheral membrane proteins are released by treatment of membranes with 0.2 M sodium carbonate (pH 11).

To further investigate the subcellular localization of hCtr1, indirect immunofluorescence microscopy was performed on the

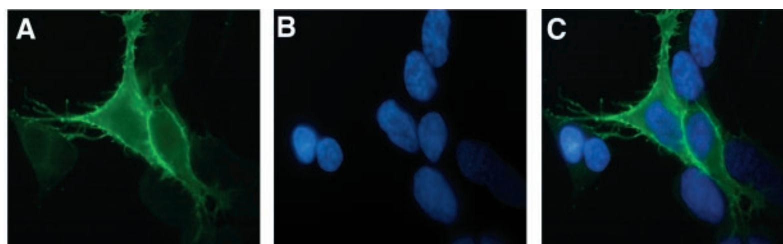

FIG. 7. **Human Ctr1 is localized to the plasma membrane in Hek293 cells.** Hek293 cells transfected with the c-Myc epitope-tagged human Ctr1 expression vector were analyzed by indirect immunofluorescence using anti-c-Myc antibody. Cells were fixed with 4% paraformaldehyde 3 days after transfection and then permeabilized by treatment with 0.1% Triton X-100. Cells were treated with 4′,6-diamidino-2-phenylindole and anti-c-Myc monoclonal antibody, washed, and then incubated with goat anti-mouse IgG (H + L)-conjugated fluorescein (Oregon Green). Coverslips were mounted on slides, and the fluorescence signal was visualized by fluorescence microscopy. Images were processed using Adobe Photoshop 5.5. c-Myc-tagged hCtr1 signal (*A*) and nuclear 4′,6-diamidino-2-phenylindole staining (*B*) were merged (*C*).

Hek293 cells expressing c-Myc-tagged hCtr1. Cells permeabilized with 0.1% Triton X-100 were treated with c-Myc antibody to detect hCtr1-Myc and treated with 4′,6-diamidino-2-phenylindole for nuclear DNA staining. As shown in Fig. 7, transfected Hek293 cells expressing Myc-tagged hCtr1 protein showed strong fluorescence at the cell periphery, which is consistent with its function in copper uptake at the plasma membrane.

Multimerization of hCtr1—All of the plasma membrane copper transporter proteins identified from yeast to humans have three putative membrane-spanning domains (28). This is a unique feature of copper transporters compared with many other membrane permeases or transporters, which have 6–12 or more predicted transmembrane domains (32). Therefore, it is possible that plasma membrane copper transporters may form homo- or heteromultimers, because several membrane-spanning domains are thought to be important for the formation of a channel for the transport process. Consistent with this hypothesis, it has been demonstrated that the *S. cerevisiae* Ctr3 or Ctr1 plasma membrane copper transporter forms a homotrimer or higher order multimers, respectively (34, 35). To examine whether hCtr1 may multimerize, Triton X-100-solubilized total cell extracts from Hek293 cells expressing Myc-hCtr1 were cross-linked *in vitro* by treatment with the multivalent cross-linker EGS. The cross-linked products were resolved by SDS-PAGE and analyzed by immunoblotting using anti-Myc antibody. In the absence of EGS, hCtr1-Myc migrated as ~29- and 58-kDa proteins, with very low levels of slower migrating species (Fig. 8, *lane 1*). As the EGS concentration was increased, hCtr1-Myc formed an ~90-kDa complex corresponding to the size expected for an hCtr1 homotrimeric form. In the presence of 2 or 3 mM EGS, the 29- and 58-kDa forms were completely shifted to the upper species, without formation of an additional higher molecular weight complex. These results suggest that hCtr1 forms a homotrimer as part of a copper transport channel at the plasma membrane.

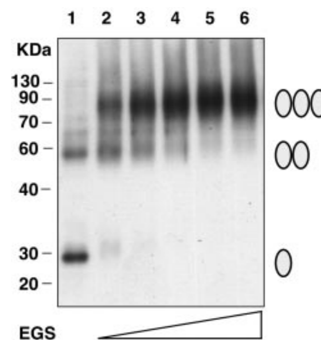

FIG. 8. **Human Ctr1 exists as a multimer.** Triton X-100 (1%)-solubilized total cellular extract was prepared from Hek293 cells transfected with hCtr1-Myc plasmid 2 days after transfection. 30 μg of protein were incubated for 30 min at room temperature with EGS. *Lanes 1–6* have 0, 0.25, 0.5, 1.0, 2.0, and 3.0 mM EGS, respectively. Reactions were quenched with 45 mM Tris-HCl, pH 7.5, followed by incubation at room temperature for an additional 30 min. The cross-linked products were analyzed by SDS-PAGE and immunoblotting with anti-c-Myc antibody. Polypeptide species consistent with the expected sizes of monomeric, dimeric, and trimeric complexes are indicated by *one*, *two*, or *three ovals*, respectively.

DISCUSSION

Copper acquisition at the plasma membrane and its subsequent distribution to subcellular locations are critical processes for providing copper to copper-requiring enzymes and proteins. Although functional complementation and structural features have suggested that hCtr1 is a human ortholog of yeast high affinity copper transporters, direct functional analysis in human cells was required to test the hypothesis that hCtr1 functions as a copper transporter. Several lines of evidence presented in this work including copper uptake kinetics, localization, and assembly of the hCtr1 protein support the hypothesis that hCtr1 functions as a high affinity copper transporter at the plasma membrane in human cells. We demonstrated that overexpression of hCtr1 in cultured human embryonic kidney cells stimulates copper uptake in a time-, temperature-, and concentration-dependent manner. Furthermore, consistent with its role in copper uptake and the localization of orthologous high affinity copper transporters in yeast, a c-Myc epitope-tagged hCtr1 protein localized to the plasma membrane in transfected Hek293 cells. Further studies with antibody directed against hCtr1 will be required to elucidate its localization in distinct cell types and tissues. The K_m for copper uptake comparing Hek293 cells expressing hCtr1 by transient transfection and endogenous copper transporter activity in Hek293 cells is similar, suggesting that hCtr1 contributes to endogenous high affinity copper transport activity in these cells. This notion is supported by RNA blotting analysis, which showed that hCtr1 is expressed in Hek293 cells. However, previous studies suggest that K_m values for copper uptake appear to be cell type-specific. It has been reported that the K_m for copper uptake in fibroblasts, isolated murine hepatocytes, and C6 rat glioma cells is ~7, 11–13, and 0.6 μM, respectively (26, 36–38). Copper may be transported by more than one transport system, which have different affinities and capacities as suggested by copper uptake experiments using rat hypothalamic tissue slices (39).

Competition experiments presented here support the hypothesis that hCtr1 is a specific metal transporter. The strong inhibition by Ag(I) in copper uptake experiments suggests that Ag(I), which is isoelectric to Cu(I), can be transported by hCtr1 and that reduced monovalent copper is a preferred substrate for the hCtr1 transporter. Consistent with this observation, ascorbate treatment to reduce Cu(II) to Cu(I) enhances copper uptake, and studies in bakers' yeast strongly suggest a role for

the Fre1 and Fre7 metalloreductases for high affinity copper transport (7–9). Furthermore, it has been shown that Cu(I) and Ag(I) are transported by the CopB-ATPase of *Enterococcus hirae in vitro* (40) and that Ag binds to the fourth metal-binding domain from the Menkes copper-transporting ATPase (41) and, like copper, silver is able to trigger the trafficking of the Menkes P-type copper-transporting ATPase from the trans-Golgi network to the plasma membrane (42). Our studies also demonstrated that much higher concentrations of zinc, cadmium, or iron inhibited copper uptake significantly; however, whether this reflects a direct competitive transport or is due to the transient rapid and reversible inactivation of hCtr1 or other copper transport components by these metals is not yet clear. With respect to zinc, it has been demonstrated that high levels of dietary zinc induce a secondary copper deficiency in mammals (43). However, the competition effects of zinc or cadmium on copper uptake are much more significant in the isolated rat hepatocytes compared with the results in our studies in Hek293 cells and by hCtr1 expression in Hek293 cells (26). Furthermore, unlike a previous study (26), we could not observe any significant competition by manganese for copper uptake. Although Ctr1 is expressed in total liver tissue (19), it is possible that it is expressed in certain cell types in the liver and that other copper transport systems operate in the liver through which cadmium, manganese, and zinc inhibit copper uptake. Therefore, future studies of this inhibitory mechanism and metal specificity, whether through hCtr1 or not, will be of importance.

It is not clear how copper is distributed in plasma and mobilized from ligands to be transported across the plasma membrane. However, it has been estimated that more than 90% of plasma copper is bound to ceruloplasmin and that the remaining 10% of plasma copper is bound to albumin and histidine (1). The effects of albumin and histidine on copper transport have been studied mainly in isolated hepatocytes or fibroblasts by the supplementation of these ligands in cell culture medium (36, 44–47). Albumin markedly inhibited initial rates of copper uptake, but histidine facilitated copper uptake when the uptake medium contained serum or albumin (36, 43). However, the histidine effects for copper uptake are cell type-specific. Histidine stimulates copper uptake in hepatocytes and trophoblasts, but albumin and histidine have additive inhibitory effect on copper transport by fibroblasts (36). We observed that both albumin and histidine inhibit copper accumulation in Hek293 cell by the endogenous transporter or in hCtr1 expression vector-transfected cells. These data suggest that histidine may mobilize copper from other complexes and that histidine-bound copper may be a better substrate for copper transport. However, it has been shown that histidine is not co-transported by copper and that histidine-copper complexes are a less favorable substrate for copper transport compared with free copper (43). Therefore, copper may be dissociated from the histidine-copper complex to be transported at the plasma membrane. However, further studies must address these specific mechanisms.

The increased copper uptake under low extracellular pH conditions is an interesting observation that may be important for understanding copper transport mechanisms. It has recently been demonstrated that copper and iron transport genes are up-regulated in the yeast *S. cerevisiae* grown in alkaline pH medium (48). Although the underlying mechanisms have not elucidated, transcriptional up-regulation of yeast copper and iron transport genes may be part of a homeostatic mechanism in response to decreased uptake of these metals at high pH. In mammals, dietary copper is mainly absorbed in the stomach and duodenum, where extracellular pH is relatively low compared with other parts of the intestine (1, 2). Therefore, increased copper uptake in the acidic buffer in our experiments may reflect modulation of transporter activity by protons, as has been observed for the GABA neurotransmitter receptor (49, 50) or copper transport by a proton co-transport mechanism. It has been reported that plasma membrane H^+-ATPase activity in the yeast *S. cerevisiae* is increased in response to copper treatment (51). The underlying mechanism for increased H^+-ATPase activity by copper treatment may be a compensatory response to the dissipation of a proton gradient generated by the proton-coupled copper transport. Additionally, a number of nutrient and ion transporters, including DCT1 (DMT1, Nramp2), which transports iron and other divalent metals, function in a pH-dependent and proton-coupled manner (29, 30). Further *in vitro* experiments will provide more insight into the precise mechanisms for copper transport by mammalian Ctr1.

Recently, we have demonstrated that mice heterozygous for a targeted deletion of the mouse Ctr1 gene display tissue-specific defects in copper accumulation and that mouse Ctr1 homozygous deletions are embryonic lethal (21, 22). These observations support dietary and genetic studies establishing a crucial role for copper acquisition by the Ctr1 protein and distribution for normal mammalian development. Furthermore, the Ctr1 knock-out experiments in mice demonstrate the lack of a functionally redundant high affinity copper transport activity in mammals. Although, based on sequence homology, a cDNA encoding a Ctr1-related protein denoted hCtr2 was identified, hCtr2 could not complement the respiratory deficiency of the yeast lacking high affinity copper transporters (19). Furthermore, we and others (52) have demonstrated that expression of hCtr2 by transient transfection in Hek293 cells did not alter the kinetics of ^{64}Cu uptake (data not shown) or export. Based on these observations, hCtr2 is not likely to function as a high affinity copper transporter in a manner analogous to hCtr1. Further evaluation of copper acquisition in cells obtained from Ctr1 knock-out mice may provide additional information about the existence of other copper transport systems that function in distinct mammalian tissues or cells.

Although here we provide fundamental information about the hCtr1 copper transporter, several questions remain to be answered regarding the mechanism of copper transport through hCtr1 and subsequent intracellular copper trafficking to targets. The yeast high affinity copper transporters and the human and mouse Ctr1 copper transporters are rich in methionine and histidine residues within an amino-terminal hydrophilic region. The methionine-rich motifs, repeated eight times in *S. cerevisiae* Ctr1 and five times in the *S. pombe* Ctr4 amino terminus, are arranged as the consensus Met-*X*-*X*-Met-*X*-Met (10, 12). Furthermore, these and a number of other conserved residues in the transmembrane domains are identified in the plasma membrane copper transporters in yeast, mice, and humans (data not shown). These conserved motifs or residues may be critical for the coordination of copper during the transport process. Once copper is transported into cells at the plasma membrane, copper is carried to intracellular compartments by copper chaperones; however, it is currently unclear how copper chaperones capture copper delivered by Ctr1. Direct copper chaperone-Ctr1 interactions or the involvement of other molecules that could serve as intracellular reservoirs are potential models for copper delivery. Further characterization of the Ctr1 copper transporter family will address these and other questions and whether defective function or regulation of hCtr1 is involved in inappropriate copper acquisition that leads to human pathophysiological states.

Acknowledgments—We are grateful to members of the Thiele laboratory and the reviewers of the manuscript for advice on experiments and critical reading of the manuscript and to Miranda Lau and Chen Kuang for excellent technical assistance. We gratefully acknowledge Dr. Jonathan Gitlin for many insightful suggestions and the Mallinckrodt Institute of Radiology at the Washington University School of Medicine (St. Louis, MO) for ^{64}Cu (supported by National Institutes of Health Resource Grant 1R24CA 86307). We thank Dr. Tohru Saeki for the ileal bile acid transporter expression vector (pZmISBT).

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