Biometals (2012) 25:697–709
DOI 10.1007/s10534-012-9528-8

Uptake of copper from plasma proteins in cells where expression of CTR1 has been modulated

Theodros Z. Kidane · Ramin Farhad · Kyoung Jin Lee · Abraham Santos · Eric Russo · Maria C. Linder

Received: 1 January 2012 / Accepted: 1 February 2012 / Published online: 22 February 2012

Abstract Plasma proteins rather than amino acid chelates are the direct sources of copper for mammalian cells. In continuing studies on the mechanisms by which albumin and transcuprein deliver copper and the potential involvement of CTR1, rates of uptake from these proteins and Cu–histidine were compared in cells with/without CTR1 knockdown or knockout. siRNA knocked down expression of CTR1 mRNA 60–85% in human mammary epithelial and hepatic cell models, but this had little or no effect on uptake of 1 μM Cu(II) attached to pure human albumin or alpha-2-macroglobulin. Mouse embryonic fibroblasts that did/did not express Ctr1 took up Cu(II) bound to albumin about as readily as from the histidine complex at physiological concentrations and by a single saturable process. Uptake from mouse albumin achieved a 2–4-fold higher Vmax (with a lower Km) than from heterologous human albumin. Maximum uptake rates from Cu(I)–histidine were >12-fold higher (with higher Km) than for Cu(II), suggesting mediation by a reductase. The presence of cell surface Cu(II) and Fe(III) reductase activity responding only slightly to dehydroascorbate was verified. Excess Fe(III) inhibited uptake from albumin–Cu(II). Ag(I) also inhibited, but kinetics were not or un-competitive. In general there was little difference in rates/kinetics of uptake in the Ctr1+/+ and −/− cells. Endocytosis was not involved. We conclude that plasma proteins deliver Cu(II) to homologous cells with greater efficiency than ionic copper at physiological concentrations, probably through the mediation of a Steap Cu(II)-reductase, and confirm the existence of an additional copper uptake system in mammalian cells.

Keywords Copper uptake · CTR1 · Plasma proteins · Copper reductase · Silver inhibition

T. Z. Kidane · R. Farhad · K. J. Lee · A. Santos · E. Russo · M. C. Linder (✉)
Department of Chemistry and Biochemistry, California State University, Fullerton, CA 92834-6866, USA
e-mail: mlinder@fullerton.edu

Introduction

Copper is an essential cofactor for many enzymes involved in many processes critical to life; but exactly how it is delivered to mammalian cells from the blood remains unclear. In the blood plasma, prevailing evidence indicates that copper destined for uptake by most cells is carried by plasma proteins and not by low molecular weight complexes like with amino acids, at least in the normal state (Linder 1991, 2010). The three known protein carriers are albumin and transcuprein (a macroglobulin; Liu et al. 2007), the main components of the exchangeable plasma copper pool, which bind Cu(II) directly and with high affinity; plus ceruloplasmin, the protein responsible for up to 70% of the copper in human and rat plasma, and somewhat less in the case of the mouse (Cabrera et al. 2008; Gray et al. 2009). Radioactive copper tracer studies in

animals and stable isotope studies in mice indicate that albumin and transcuprein are the first and probably only components to which copper ions initially bind upon entering the blood (Cabrera et al. 2008; Linder 1991, 2002, 2010; Weiss and Linder 1985; Wirth and Linder 1985). Administration of radioactive copper tracer does not result in the initial labeling of any low molecular weight components within the blood plasma. Instead it associates with the large proteins that make up the exchangeable copper pool. From there, most of the copper finds its way into cells of the liver, with a smaller proportion entering those of the kidney, before reappearing in the blood on ceruloplasmin synthesized and secreted mainly by hepatocytes (Linder 2002, 2010; Owen 1971; Scott and Turnlund 1994; Turnlund et al. 1998; Weiss and Linder 1985). Albumin and transcuprein have accessible binding sites for Cu(II) (Breslow 1964; Lau and Sarkar 1971; Masuoka et al. 1993; Moriya et al. 2008) and can rapidly exchange copper with each other (Linder 1991; Weiss and Linder 1985); ceruloplasmin-copper is buried in its structure, not dialyzable, and only extractable with disruptive procedures. Remetallation of ceruloplasmin in vitro is difficult and has only been successfully documented by one group using complex procedures (Musci et al. 1999). Intravenous infusion of ^{67}Cu-labeled ceruloplasmin produced in donor rats found its way rapidly into the cells of various organs (Campbell et al. 1981; Lee et al. 1993; Weiss and Linder 1985), indicating it too is a source of copper for cells. Moreover, rates of uptake from ^{67}Cu-ceruloplasmin were more rapid than from the exchangeable plasma copper pool (albumin and transcuprein), indicating that cells in certain organs preferred ceruloplasmin as a source (Lee et al. 1993). Inhibition of ceruloplasmin synthesis markedly reduced the rate of appearance of ^{67}Cu in these organs. Separation of radiolabeled copper-binding components of plasma or serum by size exclusion chromatography showed that following injection, radioactive tracer is not associated with low molecular weight material of the size of amino acids or metabolites (Linder 1991, 2002, 2010; Weiss and Linder 1985). Also, no "actual" stable copper in components of that size have been detected (Cabrera et al. 2008; Gercken and Barnes 1991; Gray et al. 2009; Linder 1991, 2010; Mestek et al. 2002; Shum and Houk 1993; Wirth and Linder 1985). Within cells, copper is carried on proteins (Cu-chaperones) that transfer the element to intracellular enzymes and organelles (Festa and Thiele 2011; Linder 2010; Lutsenko 2010). Although copper is bound to and carried by proteins in the mammalian organism, uptake studies with cultured cells have almost always been carried out with copper salts or the Cu(II)–histidine complex, which does not reflect "real life". We instead are studying how the plasma proteins implicated in transport may be delivering the copper.

A major question is then: To what uptake system (or systems) is the copper being delivered, and exactly how is this happening? The only recognized copper-specific transporter so far identified that normally resides in the plasma membrane is CTR1 (Festa and Thiele 2011; Kuo et al. 2001; Lee et al. 2001, 2002a; Zhou and Gitschier 1997). It takes up Cu(I) and is inhibited by Ag(I). CTR2 is also specific for Cu(I) and inhibited by Ag(I); but it is unlikely to localize to the plasma membrane (Rees and Thiele 2007; van den Berghe et al. 2007) and may be an efflux protein to the cytosol from lysosomes and endosomes. [When transfected into COS-7 cells, some was present in the plasma membrane and stimulated Cu(I) uptake (Bertinato et al. 2008). However, CTR1 and CTR2 appear to have opposing functions, knockdown of CTR1 lowering uptake of cisplatin but CTR2 knockdown enhancing it (through increased macropinocytosis) (Abada and Howell 2010; Blair et al. 2011).] In enterocytes, DMT1 may also play a role in uptake of copper from the intestinal lumen (Arredondo et al. 2003; Linder et al. 2003); but it does not appear to play a role in uptake of Cu from plasma albumin or transcuprein by hepatic and mammary epithelial cells, where this has been studied (Moriya et al. 2008), there being no inhibition of copper uptake by high concentrations of Fe(II).

CTR1 would thus appear to be the most likely transporter involved in uptake of copper by cells from albumin and transcuprein. However, in uptake studies we performed with hepatic (HepG2) and mammary (PMC42) cells, 50–200 μM Ag(I) did not inhibit copper uptake from albumin in either cell type, and only partially inhibited uptake from alpha-2-macroglobulin (a2M) in HepG2 but not PMC42 cells (Moriya et al. 2008). This implied that CTR1 was not the main transporter involved and that other uptake systems for copper must be present. Studies with mouse embryonic fibroblasts lacking Ctr1 also indicated the presence of an additional copper uptake system (Lee et al. 2002b). The objectives of the studies

reported here were to further explore the involvement or lack of involvement of CTR1 in uptake of copper from plasma proteins, using cells with knocked down and knocked out transporter expression.

Materials and methods

Protein purification and loading with ^{64}Cu tracer

These procedures were carried out as previously described (Moriya et al. 2008). Briefly, mouse and human albumin were purified from blood plasma by a combination of Affigel Blue (Cibacron blue) and size exclusion chromatography, purity determined by SDS-PAGE. a2M was purified from human plasma by the protocol from Salvatore Pizzo's laboratory (Duke University), using a combination of PEG 8000 fractionation (4–16%) and Zn-affinity chromatography. Purified proteins were stripped of endogenous Cu by dialysis against 100 μM histidine in 20 mM K phosphate, pH 7, followed by the same buffer without histidine. Proteins were loaded with ^{64}Cu-labeled Cu-nitrilotriacetate (NTA) for 1 h at 4°, prior to cell uptake studies, using a ratio of 1 Cu/molecule for albumin and 2/molecule for a2M tetramer (Moriya et al. 2008). ^{64}Cu-labeled Cu–histidine was prepared from Cu-NTA with ratios of Cu:his of 1:2–200. ^{64}Cu tracer was obtained from the Mallinkrodt Institute of Radiology at Washington University, St Louis.

Cells and culturing

Cultured cell lines used were human hepatoma cells, HepG2; a human mammary epithelial cell model, PMC42 (Moriya et al. 2008); and mouse embryonic fibroblasts expressing (Ctr1 +/+) and not expressing (Ctr1 −/−) copper transporter 1 (kindly provided by Dennis Thiele, Duke University). HepG2 and PMC42 cells were cultured in MEM (supplemented with 1 mM sodium pyruvate and 0.1 mM non-essential amino acids) and RPMI-1640, respectively, containing 10% fetal bovine serum (FBS). Fibroblasts were cultured in MEM, with 10 or 20% FBS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. Knockdown of CTR1 expression in HepG2 and PMC42 cells was performed by reverse transfection for periods spanning 48–96 h with CTR1-specific and scrambled siRNA (30–55 nM) (Dharmacon, Thermo Fisher, Lafayette CO) delivered by Lipofectamine RNAi-Max (Invitrogen, Carlsbad) at doses of 1 μl per 1.2 ml.

Cu uptake studies

Uptake studies with HepG2 and PMC42 cells were carried out in 24 well plates or 12-well Transwell plates, respectively, over 30 min using Hepes buffer (with glucose and other components) to which ^{64}Cu-labeled Cu-complexes with plasma proteins or histidine were added, as previously described (Moriya et al. 2008). Fibroblasts grown in 6 or 12-well plates were thoroughly washed 3× with warm MEM, prior to initiation of uptake studies, carried out with pure ^{64}Cu–protein complexes or Cu–his in MEM (without serum), for 7.5 or 15 min, during which accumulation in cells was linear. For all cell types, radioactivity incorporated into cells was determined after they were washed 3× with ice-cold PBS containing 10 μM histidine, lysed, and counted for radioactivity in a gamma counter, as previously described (Moriya et al. 2008). Cu uptake rates were calculated based on the % ^{64}Cu accumulating in the cells. Values are given as pmol/min/mg cell protein, the latter determined with BCA or the Bradford assay, with bovine serum albumin as standard. In some cases, silver ions (as $AgNO_3$) and/or ascorbate (1 mM) were included. Ascorbate reduced Cu(II)–Cu(I). In some cases, cells were pretreated with dehydroascorbate (DHA; 200 μM) for 4 h prior to measurements of uptake rates. Effects of endocytosis inhibitors were examined by pretreating cells with fluorosulfonylbenzoyladenosine (FSBA; 100 μM) in MEM without FBS, or nocodazole (5 μM) in MEM plus 1% FBS for 1.5 h prior to uptake studies over 15 min. Control cells were given equal volumes of the vehicle (DMSO) in which the inhibitors were dissolved (100 and 0.45 μl per ml, for FSBA and nocodazole, respectively).

Measurement of Cu(II) and Fe(III) reductase activity

Post-confluent cells in 6-well plates were assayed for reductase activities as described by Wyman et al. (2008). After washing 3× with PBS, cells were incubated in a MOPS/MES medium (25 mM MOPS, 25 mM MES, 5.4 mM KCl, 5 mM glucose, 140 mM

NaCl, 1.8 mM $CaCl_2$, 800 μM $MgCl_2$, pH 7.2) supplemented with 50 or 100 μM Cu(II)-NTA and 200 μM bathocuproinedisulfonate (BCS), or 50 or 100 μM Fe(III)-NTA and 200 μM 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid, disodium salt hydrate (ferrozine). Accumulation of Cu(I)–BCS in the medium over time (at 37°C) was followed at 482 nm, that of Fe(II)–ferrozine at 562 nm. Standard curves for the complexes were constructed to allow calculation of reductase rates in terms of nmol/min/mg cell protein, the latter after assay of cell lysates for protein by the BCA procedure.

Quantitation of mRNA

Quantitative real time PCR was performed on cDNA samples prepared from total RNA extracted from cells by the RNA-Bee RNA isolation reagent (Tel-Test; Friendswood, TX), using the BioRad iCycler, and primers and probes designed and provided by Applied Biosystems (Carlsbad, CA).

Statistics

Calculations were performed by *t* test; *P* values <0.05, for differences between means, were considered significant.

Results

Effect of CTR1 knockdown on uptake of Cu from albumin and transcuprein by HepG2 and PMC42 cells

To test whether CTR1 was involved in uptake of copper from human albumin and transcuprein (a2M), cells were pretreated with CTR1-specific siRNA during plating, and CTR1 mRNA levels were monitored over 96 h. As shown in Fig. 1a, quantitative (real time) PCR measurements of cDNA from the hepatic (HepG2) and mammary epithelial (PMC42) cells revealed that CTR1 mRNA levels were markedly reduced by CTR1-specific siRNA treatment, compared with controls treated with scrambled siRNA, and relative to 18S rRNA. Combined data from 2 to 3 experiments (each in triplicate) showed an average expression reduction of about 85% in the HepG2 cells and 65% in the PMC42 cells by 96 h. This was not accompanied by a notable decrease in rates of uptake of copper from either transcuprein or albumin. Figure 1b shows the effects of specific siRNA on rates of uptake as a percentage of those for controls receiving scrambled siRNA in the same experiment, providing Cu(II) attached to human a2M or albumin. Throughout, Cu concentrations were 1 μM, which is at the low end of the range of Cu concentrations associated with these proteins in plasma (Moriya et al. 2008). siRNA treatment did not change uptake rates in HepG2 cells provided with Cu(II)–a2M, and only decreased it 15–20% in the case of mammary epithelial cells given Cu(II)–a2M and HepG2 cells given Cu(II)–albumin. This implies that CTR1 is not the main or only physiological copper uptake system in these cells. Since most other investigators use Cu–histidine to study uptake, we also examined the potential effects of CTR1 knockdown on uptake from this complex (Fig. 1c). Uptake from the Cu(II)–dihistidine complex was not altered by knocking down expression of CTR1.

We also re-examined the effects of Ag(I), an established inhibitor of Cu(I) uptake by CTR1 (Lee et al. 2002a). A large excess of Ag(I) (200 μM) had very little effect on uptake of 1 μM Cu(II) delivered on a2M, albumin or di-histidine to hepatic and mammary cells. Multiple studies with each of these ligands and HepG2 cells gave an average inhibition by Ag(I) of 20–22% ($P < 0.01$–0.001; Mean ± SD, $N = 6$–9). With the mammary cells, no significant inhibition was observed (data not shown); all again implying CTR1 was not the main transporter involved.

Copper uptake from plasma proteins by mouse fibroblasts expressing and not expressing Ctr1

To further determine the role of CTR1 in uptake of copper from plasma proteins by various cell types, we turned to cells that clearly do and do not express this transporter, namely mouse embryonic fibroblasts kindly provided by Dennis Thiele (Duke University) and described in the publications by Lee et al. (2002a, b). In our hands, the null cells grew somewhat slower than the wild type. Both lines exhibited some toxic sensitivity to Ag(I), not tolerating exposure to concentrations above 20 μM, and particularly so in the case of the null cells. Both cell lines also tended to lift off the flasks when incubated in the Hepes-buffered saline solution normally used for copper uptake

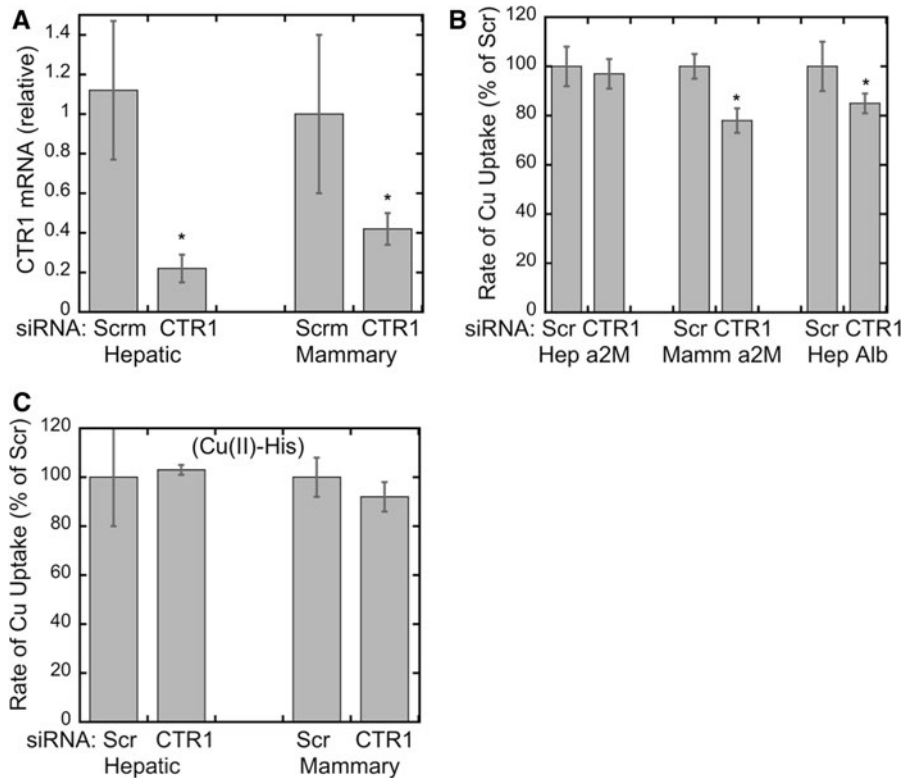

Fig. 1 Effect of CTR1 siRNA knockdown on uptake of copper from plasma proteins by human hepatic (Hep) and mammary (Mamm) cells. HepG2 and PMC42 cells were treated with CTR1-specific or scrambled (Scrm) siRNA for 96 h then analyzed for levels of CTR1 mRNA (**a**) or rates of 1 μM copper uptake from human transcuprein (alpha-2-macroglobulin; a2M) or albumin (Alb) (**b**) or Cu(II)–dihistidine (**c**). Values for CTR1 mRNA are relative to 18S rRNA levels in the same samples; rates of copper uptake are given as a percent of the average value obtained for the scrambled siRNA-treated control cells. $*P < 0.01$–0.001 for difference from the control cell results

studies in the case of HepG2 and PMC42 cells (see "Materials and methods" section). As a result, uptake studies with the fibroblasts were performed in MEM (without added serum).

Negligible levels of Ctr1 mRNA were detected in the null cells (Fig. 2a). A comparison of rates of Cu(I) uptake (1 μM) from histidine, human a2M/transcuprein, and human albumin, in null cells (Ctr1−/−) and those of the control (wild type Ctr1+/+) cells is shown in Fig. 2b. Absence of Ctr1 did not inhibit uptake of Cu(I) from these sources. Actual uptake rates from these three sources were quite similar at the 1 μM Cu concentrations tested—which are at the low end of the physiological range for Cu associated with albumin and transcuprein (Moriya et al. 2008). Clearly also in these cells, Ctr1 was not the main copper uptake system, and a robust additional system was present, as previously reported by Lee et al. (2002b).

The kinetics of uptake of Cu from histidine and the plasma proteins were then examined. Figure 3a shows the results of initial experiments for uptake of Cu(I) and Cu(II) provided as the histidine complex. These were carried out in the Ctr1−/− cells, since almost identical uptake rates were observed in the Ctr1+/+ cells, and the kinetic data would indicate something about the ionic state of copper preferred by the unknown uptake system. Clearly, rates were much higher when cuprous ions were provided (Fig. 3a, compare diamonds and triangles). Looking at individual examples of detailed curves for each copper form (Fig. 3b, c) obtained in later parallel studies, the maximal apparent rate of Cu(II) uptake (Vmax) was about 12-fold lower for Cu(II) than Cu(I) (40 vs

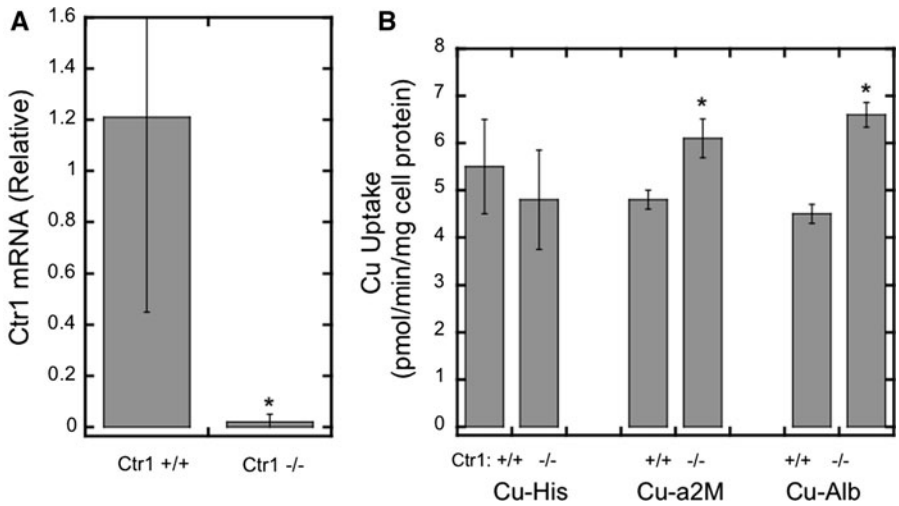

Fig. 2 Ctr1 expression and rates of copper uptake by mouse embryonic fibroblasts expressing and not expressing Ctr1. (**a**) Relative quantities of Ctr1 mRNA detected in the wild type (Ctr1+/+) and null (Ctr1−/−) cells, expressed relative to values for 18S rRNA. (**b**) Rates of 1 μM copper uptake from histidine (His) or human transcuprein (alpha-2-macroglobulin; a2M) or albumin (Alb), in the wild type and null cells. In this case, uptake was in the presence of 1 mM ascorbate, which reduced the Cu(II) to Cu(I) while still remaining bound to the proteins or histidine. All values are means ± SD ($N = 3$–6) *$P < 0.01$–0.001 for difference from the wild type

489 pmol/min/mg cell protein). This difference was replicated in several parallel studies, although there was variation in absolute values from one set of cells to another over the 15 month period involved.

There are two possible interpretations of this result. First, there could be two separate uptake systems, one for Cu(II) the other for Cu(I). Alternatively (and more likely), the unknown uptake system prefers Cu(I), and rates of Cu(II) uptake are much lower because the Cu(II) needs first to be reduced before it can enter the cell. The reduction of Cu(II) would thus be the rate limiting step for uptake, and the apparent kinetics of Cu(II) uptake determined would really be the kinetics of Cu(II) reduction. It is noteworthy that the kinetics of Cu(II) and Cu(I) uptake were very different not only in terms of Vmax, but also in terms of Km, which would be consistent with different uptake mechanisms. If the kinetics for Cu(II) reflect reductase kinetics, it is noteworthy that the affinity for Cu(II) was greater (Km about 1 μM) than that for Cu(I)–histidine (Km 6 μM or higher). The results also indicate that the main unknown copper uptake system present in the Ctr1−/− cells, like CTR1, prefers Cu(I).

To determine whether a reductase was present on the surface of these mouse embryonic fibroblasts, we applied the assay developed by Wyman et al. (2008) to measure iron and copper reductase activity (dCytb) on the surface of enterocytes, which follows the appearance of colored complexes of the reduced metal ions, over time. Figure 4 shows examples of the linear accumulation of reduced copper and iron ions in the culture medium over time when the human HepG2 cells (A) and mouse fibroblasts (B) were incubated with 50 μM concentrations of Cu(II) and Fe(III) as the NTA complexes (see "Materials and methods" section), in the presence of chelators for the reduced metal ions. Reductase activity for both metals was clearly present.

The kinetics of copper uptake from albumin (vs histidine) were then examined, first using Cu(II) bound to human albumin. The data in Fig. 5a show that the Ctr1 +/+ and −/− cells displayed similar kinetics and had maximal rates (6–7 pmol/min/mg cell protein) in the range of those for Cu(II)–his. This would fit with the concept that the Cu(II) on human albumin also needs to be reduced before it can enter the cells through the transport system present. Rates of uptake were somewhat inhibited by 5 μM Ag(I) (Fig. 5b), more so in the case of the Ctr1+/+ cells. Kinetic calculations and double reciprocal plots indicated that the inhibition was not competitive. One study with Ctr1+/+ cells suggested kinetics were uncompetitive (Fig. 5c); but with Ctr1−/− cells there were clear reductions in Vmax and less change in Km (data not shown).

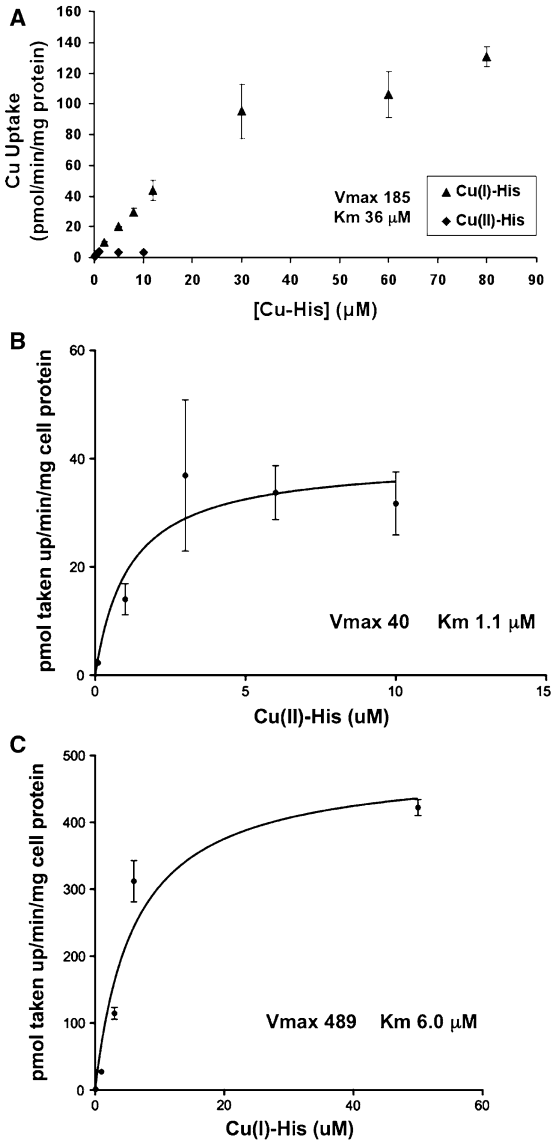

Fig. 3 Kinetics of Cu(II) and Cu(I) uptake from the histidine complex by mouse embryonic fibroblasts null for Ctr1. (**a**) Combined data for kinetic results for Cu(II) (*diamonds*) and Cu(I) (*triangles*) uptake rates at different copper concentrations. *Error bars* are SD for Means from 3–6 rates obtained at the same copper concentration in separate experiments. Vmax was 186 pmol/min/mg cell protein; Km was 36 μM. (Several data points ±SD in the range of 0.1–2 μM copper are present and hard to distinguish in this version of the figure.) (**b**, **c**) Representative examples of detailed results for Cu(II) (**b**) and Cu(I) (**c**) kinetics obtained from another set of parallel experiments. Each point represents the mean ± SD for $N = 3$

Cu(II) attached to human albumin gave somewhat lower Vmax values than Cu(II)–histidine. So we examined the question of whether binding to species–homologous albumin would provide a better rate of copper uptake than heterologous human albumin. Mouse albumin was therefore purified and used in parallel kinetic studies, after loading with Cu(II)-NTA. An example of the results is shown in Fig. 5d. In multiple studies with Ctr1 null cells over 8 months, maximal rates (Vmax values) obtained ranged from 14 to 36 pmol/min/mg cell protein; Kms were 1.0–1.3. Thus, maximal rates were much higher for uptake from homologous versus heterologous albumin, while apparent affinities were similar (Kms of 1–3 μM). This suggested either that reduction was not required when native albumin delivered the copper, or that the homologous albumin led to more efficient reduction of the albumin–Cu(II). Uptake of Cu(II) from mouse albumin in the presence of 5 μM Ag(I) changed both Vmax and Km in the Ctr1−/− fibroblasts (data not shown). These results indicate that the unknown uptake system, like CTR1, is also sensitive to silver, but that inhibition does not occur through competition for uptake by the metal ions.

Evidence for the involvement of a reductase in mediating copper uptake from albumin

Virtually all of the presently known cell surface copper reductases also reduce iron and they fall into two categories, the cytochrome b variety (Knopfel and Solioz 2002; Vargas et al. 2003; Wyman et al. 2008) and the Steap family of proteins (Knutson 2007; Ohgami et al. 2006). In general, rates of copper reduction are greater than those for iron, and we found this to be the case also for the reductase activity in the hepatic and fibroblast cells we were using (Fig. 4). To begin to identify the type of reductase involved, we checked whether the activity of the mouse fibroblasts would be enhanced by preloading them with DHA, as is the case with dCytb (where activity was enhanced 3-fourfold by this procedure) (Wyman et al. 2008). As shown in Fig. 6a, the availability of more ascorbate (produced by this treatment) did not have a marked effect on either the ferric or cupric reductase activities. It also had no effect on the rate of copper uptake from Cu(II)–mouse albumin (Fig. 6b).

If a Cu/Fe reductase is mediating uptake of Cu(II), then we would expect that Fe(III) would inhibit Cu(II) uptake. Therefore, we examined the effect of excess Fe(III) on uptake of 1 μM Cu from Cu(II)–albumin. As shown in Fig. 7, there was marked inhibition in the

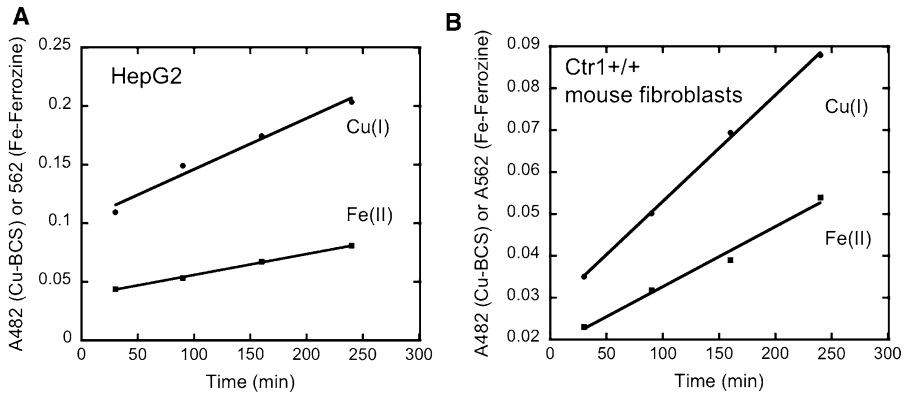

Fig. 4 External copper and iron reductase activity of human hepatic cells and mouse embryonic fibroblasts. Accumulation of Cu(I)–BCS complex (*upper curves*) and Fe(II)–ferrozine complex (*lower curves*) in the medium of HepG2 cells (**a**) and mouse embryonic fibroblasts (**b**) (Ctr1+/+). The *y* axis shows the increases in absorbance of the complexes when substrates were provided as 50 μM Cu(II) or Fe(III)-NTA. Absorbance has been corrected for cell number, values being A/mg cell protein

presence 25 μM Fe(III)-NTA. This strongly supports the concept that the reductase is mediating uptake of Cu(II) from albumin, and that Cu(II) from albumin must be reduced to Cu(I) in order to enter the cell through the unknown transporter.

Potential involvement of endocytosis

Although the saturation kinetic results for copper uptake from albumin were most consistent with uptake occurring via facilitated diffusion through a cell surface transporter, like CTR1, we considered the possibility that endocytosis of the Cu–albumin complex might be occurring instead, and so examined the effects of endocytosis inhibitors. As shown in Fig. 8, neither FSBA (which prevents the first step in endocytosis; Olusanya et al. 2001) nor nocodazole (which depolymerizes microtubules; Turner and Tartakoff 1989) inhibited uptake.

Discussion

We have further examined the relative importance of the only major copper-specific cell uptake transporter so far identified (CTR1) for uptake of this important metal ion by mammalian cells. Our studies differ from those of most other investigators because we presented the metal ion in the forms in which it is presented in vivo, namely attached to the proteins that carry it in the blood plasma. The two proteins examined here (albumin and transcuprein) are the main constituents of the exchangeable copper pool of that fluid. We examined uptake rates using cultured cells and concentrations of Cu(II)–protein complexes that are in the physiological range, which is 2–5 μM Cu (Linder 2002, 2010; Moriya et al. 2008). In the three cell types examined (hepatic, mammary epithelial and fibroblasts), uptake rates of 1 μM Cu(II) bound to the pure proteins were in the same range as those for ionic copper bound to histidine, the form most commonly used by other investigators. This indicates that although tightly bound to the plasma proteins, the copper was readily available for uptake. As concerns CTR1, knockdown or knockout of CTR1 made little difference. Uptake rates were (at most) reduced by 20%. In the case of the human cell lines, we were not able to obtain reliable immunoblotting data to verify that CTR1 protein had been depleted. However, the same results were obtained with the fibroblasts in which Ctr1 was clearly not expressed. Minimal inhibitory effects of Ag(I) corroborated these findings, indicating quite clearly that at least in the cell models examined here, CTR1 is not the major plasma membrane uptake system for copper.

Our results are in agreement with the conclusions of our previous findings for HepG2 and PMC42 cells that an excess of silver ions had little effect on Cu(II) uptake from human albumin and transcuprein (a2M) (Moriya et al. 2008). Our findings are also in agreement with the previous report of Lee et al. (2002b), using the same mouse embryonic fibroblasts, that there still is uptake of copper in the absence of Ctr1 expression (in that case most probably presented

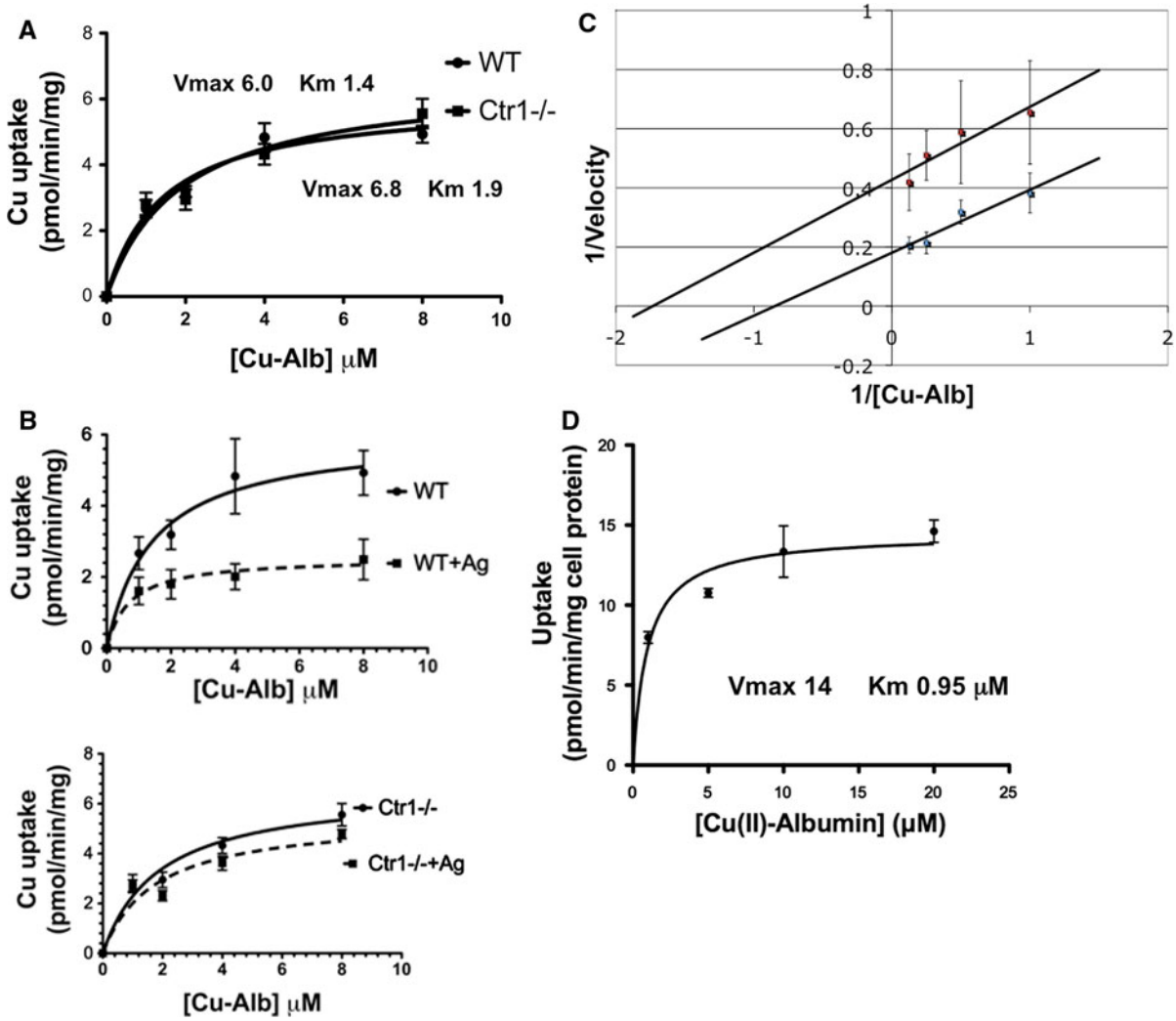

Fig. 5 Kinetics of Cu(II) uptake from human and mouse albumin by the mouse embryonic fibroblasts and effect of Ag(I). (**a**) Example of comparative results obtained with human Cu(II)–albumin for Ctr1 wild type and null cells. Vmax values (in pmol/mi/mg cell protein) and Kms (μM) for the wild type and null cells, are indicated. (**b**) Kinetics in the presence of 5 μM Ag(I) from the same experiments. Vmax values changed from 6.0 and 6.8 to 2.5 and 5.5 pmol/min/mg cell protein, Kms remaining about the same, going from 1.4 and 1.9 to 1.7 and 0.7 μM with Ag(I). (**c**) Double reciprocal plots of the ±Ag(I) data for Ctr1+/+ cells in (**b**), *Upper curve* +Ag, *lower curve* −Ag which suggested uncompetitive kinetics. (**d**) Representative example of kinetics of Cu(II) uptake from mouse albumin by Ctr1−/− mouse embryonic fibroblasts. Vmax (pmol/min/mg cell protein) and Km (μM) are indicated

as ^{64}Cu-labeled $CuCl_2$); and they showed that donation of 2 μM Cu(II) by bovine serum albumin gave uptake rates tenfold lower than from the histidine complex. In our studies with Cu(II)–albumin, rates of uptake (which were always initial rates) were generally almost the same in the Ctr1−/− and Ctr1+/+ fibroblasts in a given set of parallel experiments, although the null cells grew more slowly. This suggests that even in the fibroblasts that do express Ctr1, most of the transporter is not on the plasma membrane, although other explanations are possible.

In the Ctr1 +/+ and null fibroblasts, providing copper as the Cu(II)–albumin complex resulted in kinetics that indicated the presence of a single saturable uptake system, which had Kms of 1–2 μM, e.g. right within the physiological range. Notably, both the maximum velocities of uptake and affinity constants (Kms) were better when homologous mouse

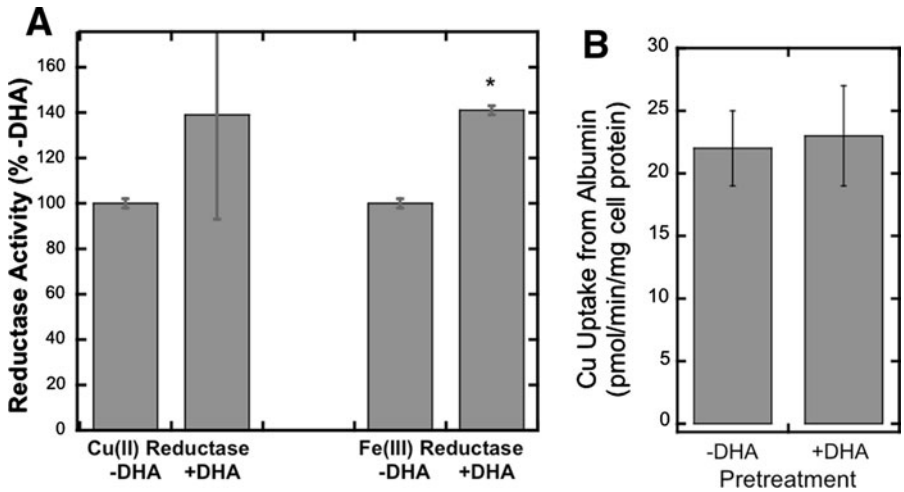

Fig. 6 Effect of dehydroascorbate (DHA) pretreatment of HepG2 and Ctr1+/+ cells on rates of copper and iron reductase activity and Cu-uptake. (**a**) Effect of 4 h DHA pretreatment on reductase activity, as percent of untreated controls in the same experiment (mean ± SD, $N = 3$). Actual activities of untreated cells averaged 230 and 26 μmol/min/mg cell protein for Cu(II) and Fe(III) reductase activity respectively in HepG2 cells, and 120 and 4 μmol/min/mg cell protein for Cu(II) and Fe(III) reductase activity respectively in Ctr1+/+ mouse embryonic fibroblasts. (**b**) Effect of DHA pretreatment of Ctr1+/+ cells on rates of Cu(II) uptake from mouse albumin (1 μM Cu) over 1 h. Values are (means ± SD, $N = 3$). *$P < 0.01$ for difference from controls

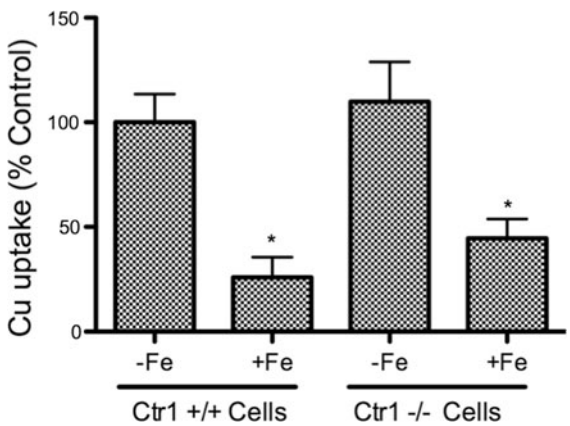

Fig. 7 Inhibition of uptake of Cu(II) from albumin by excess Fe(III). Uptake of copper from human albumin (1 μM Cu) by wild type and Ctr1-null mouse embryonic fibroblasts was measured in the absence (−Fe) and presence (+Fe) of 25 μM Fe(III)-NTA. Values are means ± SD, for $N = 6$. *$P < 0.001$ for difference from −Fe controls

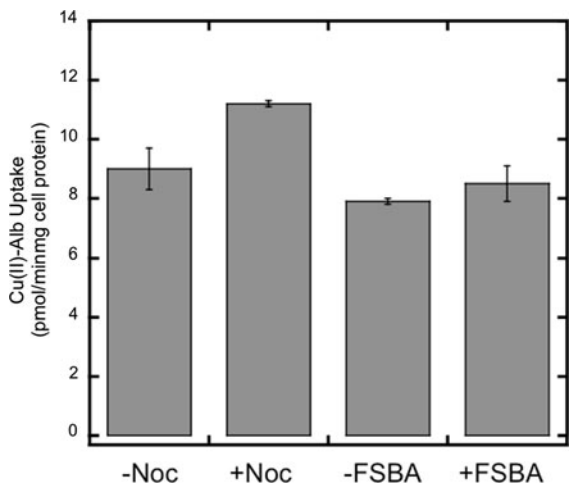

Fig. 8 Lack of effects of endocytosis inhibitors on uptake of Cu(II) from human albumin. Rates of copper uptake by Ctr1+/+ fibroblasts were measured over 15 min after 1.5 h pretreatment with 5 μM nocodazole or 100 μM FSBA (see "Materials and methods" section). Values are means ± SD, for $N = 3$. *$P < 0.01$ for difference from vehicle treated controls

albumin rather than human albumin was used to deliver the trace element. For the human albumin complex, Vmax values were 6–7 pmol/min/mg cell protein compared to 14–22 for mouse albumin, while Kms were 2 versus 1 μM, respectively. When studied in the same sets of cells, kinetic parameters for Cu(II)–histidine were close to those of Cu(II)–human albumin.

Of particular note was our finding that reduction of the copper in the histidine complex always greatly increased the rates of uptake. This was studied mainly in the Ctr1−/− cells in order to better characterize the

unknown uptake system. Although there was considerable variation in the results obtained with these cells at two different times in the period of a year (Fig. 3a vs b, c), maximal rates were always at least 12-fold higher for Cu(I) versus Cu(II). (However, Kms were also much higher.) This implies either that there are two separate uptake systems for Cu(II) and Cu(I), when presented in ionic form, or that the rate of Cu(II) uptake is limited by the rate at which it can be reduced by a cell surface cupric reductase. Since we found that cupric reductase activity was present, it seems more likely that the latter interpretation is correct. Our measurements of reductase activity gave values in the range of 120 pmol/min/mg cell protein for the Ctr1+/+ fibroblasts, when substrate [Cu(II)] concentrations were 50 μM. Maximal uptake rates for Cu(II) ranged from 6 to 40 pmol/min/mg cell protein. Since we do not have kinetic information on the reductase, including its affinities for Cu(II) provided as the histidine or albumin complexes, we can only say that it is entirely within reason that reduction could be the rate limiting step for uptake of Cu(II) from these carriers.

Further evidence that reduction is mediating uptake of copper from albumin comes from our findings that an excess of Fe(III) markedly inhibited the process. In both Ctr1+/+ and null fibroblasts, a 25:1 molar ratio of Fe(III) to Cu(II)—on human albumin resulted in 60–75% less uptake. As far as we have been able to determine from the literature, all the known mammalian cupric reductases are also ferric reductases (Knopfel and Solioz 2002; Knutson 2007; Ohgami et al. 2006). We thus reasoned that Fe(III) would compete with Cu(II) for the reductase electrons, inhibiting production of Cu(I) that could enter through the unknown Cu(I) transporter, and our findings are consistent with this hypothesis. If that is the case, the physiologically relevant scenario for delivery of copper to cells from the plasma protein, albumin (and probably also from a2M), would be that the Cu(II)-protein complex binds to the cell surface reductase in the vicinity of CTR1 or the unknown Cu(I) transporter (or binds to both the reductase and transporter), which results in reduction and transfer of the resulting Cu(I) to the uptake system. Albumin from the same species as the cells is more effective in Cu(II) delivery (higher maximum rates and lower Km). The nature of the reductase involved has not yet been determined; but based on what is known about organ cell expression (Knopfel and Solioz 2002; Knutson 2007; Ohgami et al. 2006; Vargas et al. 2003) and the fact that pretreatment with dehydroascorbate did not markedly enhance reductase activities (Wyman et al. 2008), one of the Steaps is most likely to be involved.

As concerns the unknown transporter in the mouse fibroblasts, our evidence indicates that uptake of Cu(II) from albumin is sensitive to Ag(I). Thus, either the activity of the transporter or the reductase (or both) can be inhibited by this ion which has chemical similarities with Cu(I). In the case of CTR1, it has been assumed that Ag(I) is competing with Cu(I) for sites in the transporter, since Ag(I) markedly inhibits Cu(I) uptake (Lee et al. 2002a, b). However, it appears that actual kinetic analyses of the Ag(I) effect have not been carried out, and so the nature of the inhibitory effect when studying Cu(I) uptake in the presence of Ag(I) remains unclear. In the case reported here, we found an inhibitory effect of Ag(I) on uptake of Cu(II) from albumin in the Ctr1 null fibroblasts. However, the kinetics indicated this effect was not competitive, implying no direct interaction between the metal ions at the uptake sites. [The metal ions would, however, compete for electrons and inhibit each other's reduction, consistent with the inhibition of Cu(II) uptake by excess Fe(III).]

It seems unlikely that the unknown transporter is CTR2, for reasons already provided in the Introduction, although that needs to be verified. It also seems unlikely that uptake of Cu(II) from albumin is by an independent system for albumin involving endocytosis, although this also needs to be further examined. Uptake was by a saturable system, and there was linear accumulation of copper within the cells over time, which together would not seem to fit with endocytosis. In addition, we found that two different inhibitors of endocytosis failed to prevent copper uptake.

Acknowledgments We gratefully acknowledge receipt of mouse embryonic fibroblasts that do and do not express Ctr1 from Dennis Thiele at Duke University. Supported in part by Public Health Service Grant No. RO1 HD46949 for the research, and IR24CA86307 to the Mallinkrodt Institute of Radiology (MIR) at Washington University, St. Louis, subsidizing ^{64}Cu production. Ramin Farhad, Eric Russo, and Kyoung Jin Lee were supported by an Howard Hughes Medical Institute grant for the CSUF-HHMI Undergraduate Research Scholars Program. No conflicts of interest leading to financial or other gain are connected with the research reported here.

References

Abada P, Howell SB (2010) Regulation of cisplatin cytotoxicity by Cu influx transporters. Metal-Based Drugs 1–9 (article ID 317581)

Arredondo M, Munoz P, Mura CV, Nunez MT (2003) DMT1, a physiologically relevant Cu+1 transporter of intestinal cells. Am J Physiol (Cell Physiol) 284:C1525–C1530

Bertinato J, Swist E, Plouffe LJ et al (2008) Ctr2 is partially localized to the plasma membrane and stimulates copper uptake in COS-7 cells. Biochem J 409:731–740

Blair BG, Larson CA, Adams PL et al (2011) Copper transporter 2 regulates endocytosis and controls tumor growth and sensitivity to cisplatin in vivo. Mol Pharmacol 79:157–166

Breslow E (1964) Comparison of cupric ion-binding sites in myoglobin derivatives and serum albumin. J Biol Chem 239:3252–3259

Cabrera A, Alonzo E, Chu Y-L et al (2008) Copper binding components of blood plasma and organs, and their responses to influx of large doses of ^{65}Cu, in the mouse. Biometals 21:525–543

Campbell CH, Brown RH, Linder MC (1981) Circulating ceruloplasmin is an important source of copper for normal and malignant cells. Bioch Biophys Acta 678:27–38

Festa RA, Thiele DJ (2011) Copper: an essential metal in biology. Curr Biol 21:R877–R883

Gercken B, Barnes RM (1991) Determination of lead and other trace element species in blood by size exclusion chromatography and inductively coupled plasma/mass spectrometry. Anal Chem 63:283–287

Gray LA, Kidane TZ, Nguyen A et al (2009) Multiple copper proteins and ferro-oxidases in mouse and human plasma. Biochem J 419:237–245

Knopfel M, Solioz M (2002) Characterization of a cytochrome b558 ferric/cupric reductase from rabbit duodenal brush border membranes. Biochem Biophys Res Comm 291: 220–225

Knutson MD (2007) Steap proteins: implications for iron and copper metabolism. Nutr Rev 65:335–340

Kuo Y-M, Zhou B, Cosco D et al (2001) The copper transporter CTR1 provides an essential function in mammalian embryonic development. Proc Natl Acad Sci USA 98:6836–6841

Lau S, Sarkar B (1971) Ternary coordination complex between human serum albumin, copper(II) and L-histidine. J Biol Chem 246:5938–5943

Lee SH, Lancey R, Montaser A et al (1993) Ceruloplasmin and copper transport during the latter part of gestation in the rat. Proc Soc Exp Biol Med 203:428–439

Lee J, Prohaska JR, Thiele DJ (2001) Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. Proc Natl Acad Sci USA 98:6842–6847

Lee J, Pena MMO, Nose Y et al (2002a) Biochemical characterization of the human copper transporter Ctr1. J Biol Chem 277:4380–4387

Lee J, Petris MJ, Thiele DJ (2002b) Characterization of mouse embryonic cells deficient in the Ctr1 high affinity copper transporter: identification of a CTR1-independent copper transport system. J Biol Chem 277:40253–40259

Linder MC (1991) Biochemistry of copper. Plenum Publishing, New York

Linder MC (2002) Biochemistry and molecular biology of copper in mammals. In: Massoro EJ (ed) Handbook of copper pharmacology and toxicology. Humana Press, Totowa

Linder MC (2010) Nutritional biochemistry of copper, with emphasis on the perinatal period. In: Avigliano L, Rossi L (eds) Biochemical aspects of human nutrition. Research Signpost, Trivandrum

Linder MC, Zerounian NR, Moriya M et al (2003) Iron and copper homeostasis and intestinal absorption using the $CaCO_2$ cell model. Biometals 16:145–160

Liu NM, Lo LSL, Askary SH et al (2007) Transcuprein is a macroglobulin regulated by copper and iron availability. J Nutr Biochem 18:597–608

Lutsenko S (2010) Human copper homeostasis: a network of interconnected pathways. Curr Opin Chem Biol 14:211–217

Masuoka J, Hegenauer J, Van Dyke BR et al (1993) Intrinsic stoichiometric equilibrium constants for the binding of zinc(II) and copper(II) to the high affinity site of serum albumin. J Biol Chem 268:21533–21537

Mestek O, Kominkova J, Koplik R, Zima T, Miskusova M, Stern P (2002) Speciation of Cu, Se, Zn and Fe in blood serum of hemodialysed patients. Sb Lek 103:23–27

Moriya M, Ho Y-H, Grana A et al (2008) Copper is taken up efficiently from albumin and alpha-2-macroglobulin by cultured human cells by more than one mechanism. Am J Physiol (Cell Physiol) 295:708–721

Musci G, Fraterrigo T, Calabrese L et al (1999) On the lability and functional significance of the type 1 copper pool in ceruloplasmin. J Biol Inorg Chem 4:441–446

Ohgami RS, Campagne DR, McDonald A et al (2006) The Steap proteins are metalloreductases. Blood 108:1388–1394

Olusanya O, Andrews PD, Swedlow JR et al (2001) Phosphorylation of threonine 156 of the mu2 subunit of the AP2 complex is essential for endocytosis in vitro and in vivo 11(11):896–900

Owen CA Jr (1971) Metabolism of copper 67 by the copper deficient rat. Am J Physiol 221:1722–1727

Rees EM, Thiele DJ (2007) Identification of a vacuole-associated metalloreductase and its role in Ctr2-mediated intracellular copper mobilization. J Biol Chem 282:21629–21638

Scott KC, Turnlund JR (1994) Compartmental model of copper metabolism in adult men. J Nutr Biochem 5:342–350

Shum SCK, Houk RS (1993) Elemental speciation by anion exchange and size exclusion chromatography with detection by inductively coupled plasma mass spectrometry with direct injection nebulization. Anal Chem 65:2972–2976

Turner JR, Tartakoff AM (1989) The response of the golgi complex to microtubule alterations: The roles of metabolic energy and membrane traffic in golgi complex organization. J Cell Biol 109:2081–2088

Turnlund JR, Keyes WR, Peiffer GL et al (1998) Copper absorption, excretion, and retention by young men consuming low dietary copper determined by using the stable isotope ^{65}Cu. Am J Clin Nutr 67:1219–1225

van den Berghe PV, Folmer DE, Malingre HE et al (2007) Human copper transporter 2 is localized in late endosomes and lysosomes and facilitates cellular copper uptake. Biochem J 407:49–59

Vargas JD, Herpers B, McKie AT et al (2003) Stromal cell-derived receptor 2 and cytochrome b561 are functional ferric reductases. Biochim Biophys Acta 1651:116–123

Weiss KC, Linder MC (1985) Copper transport in rats, involving a new plasma protein. Am J Physiol Endocrinol Metab 249:E77–E88

Wirth PL, Linder MC (1985) Distribution of copper among components of human serum. J Natl Cancer Inst 75:277–284

Wyman S, Simpson RJ, McKie AT et al (2008) Dcytb (Cybrd1) functions as both a ferric and a cupric reductase in vitro. FEBS Lett 582:1901–1906

Zhou B, Gitschier J (1997) hCTR1: a human gene for copper uptake identified by complementation in yeast. Proc Natl Acad Sci USA 94:7481–7486