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Mouse divalent metal transporter 1 is a copper transporter in HEK293 cells

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Abstract Divalent Metal Transporter 1 (DMT1) is an apical Fe transporter in the duodenum and is involved in endosomal Fe export. Four protein isoforms have been described for DMT1, two from mRNA with an iron responsive element (IRE) and two from mRNA without it. The sets of two begin in exon 1A or 2. We have characterized copper transport using mouse 2/−IRE DMT1 during regulated ectopic expression. HEK293 cells carrying a TetR:Hyg element were stably transfected with pDEST31 containing a 2/−IRE construct. $^{64}Cu^{1+}$ incorporation in doxycycline treated cells exhibited 18.6 and 30.0-fold increases in Cu content, respectively when were exposed to 10 and 100 μM of extracellular Cu. Cu content was ~4-fold above that of parent cells or cells carrying just the vector. ^{64}Cu uptake in transfected cells pre-incubated with 5 μM of Cu-His revealed a Vmax and Km of 11.98 ± 0.52 pmol mg protein^{-1} min^{-1} and 2.03 ± 0.03 μM, respectively. Doxycycline-stimulated Cu uptake was linear with time. The rates of apical Cu uptake decreased and transepithelial transport increased when intracellular Cu increased. The optimal pH for Cu transport was 6.5; uptake of Cu was temperature dependent. Silver does not inhibit Cu uptake in cells carrying the vector. In conclusion, Cu uptake in HEK293 cells that overexpressed the 2/−IRE isoform of DMT1 transporter supports our earlier contention that DMT1 transports Cu as Cu^{1+}.

Keywords Copper · DMT1 · Transport · HEK293 cells · Tetracycline regulation

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Introduction

Copper is both essential and potentially toxic for plants, animals and humans. Animal and human studies have shown that copper is involved in the function of several enzymes (Linder 1991; Turlund 1995). Its redox properties involve copper as a cofactor for proteins involved in essential processes as neural development (dopamine β hydroxylase), photosynthesis and respiration (cytochrome *c* oxidase), free radical management (superoxide dismutase) and iron oxidation (ceruloplasmin and hephaestin). An excess of copper, however, produces oxidative damage and cell death. Copper catalyzes, through the Fenton and Haber–Weiss reactions, production of hydrogen peroxide and hydroxyl free radicals (Okada 1996).

Body copper levels are the result of a balance between copper absorption and copper secretion.

Although important advances have been done in the understanding of copper secretion and excretion, the mechanisms that govern intestinal copper absorption remain largely a mystery (reviewed by Linder and Hazegh-Azam 1996). In particular, the molecular basis for copper absorption is poorly known. Intestinal copper uptake from foods initiates with the passage through the plasma membrane of copper from the intestinal lumen into the cytosol of the intestinal epithelial cell. Given its polar nature, this membranous passage of copper requires a system of facilitated transport. A divalent metal membrane transporter 1 (DMT1) was identified by expression cloning in *Xenopus* oocytes (Gunshin et al. 1997). Electrophysiological studies revealed that DMT1 is an electrogenic metal:proton cotransporter. Besides iron and copper, DMT1 transports the divalent forms of Zn, Cd, Mn and Co. A missense mutation, G185R, generates DMT1 deficient in membrane iron transport (Fleming et al. 1997, 1998). DMT1 mRNA expression increased several-fold in the duodenum of iron deficient animal, an indication that DMT1 is regulated by intracellular iron levels. Tissue localization revealed a wide range of tissue distribution, with preferential expression in kidney and duodenum. There are at least four isoforms of DMT1. They share 543 amino acid residues but vary at the C-terminus and N-terminus (reviewed by Garrick et al. 2003a). The former region can have an extra 18 amino acid residues encoded by the Iron Responsive Element (IRE) containing species of mRNA or an extra 25 amino acid residues encoded by the mRNA that lacks an IRE; thus we refer to these isoforms as +IRE or −IRE, respectively. The latter region can have an N-terminal extension of ~30 amino acid residues due to a proximal AUG when exon 1A is present in the mRNA; but when the mRNA species has exon 1B at its 5′ end, the first AUG is in exon 2. We refer to this variation as 1A or 2 so the four forms are 1A/+IRE, 1A/−IRE, 2/+IRE and 2/−IRE. It is clear that DMT1 participates actively in gastrointestinal uptake and endosomal mobilization of metals (Garrick et al. 2003a, b; Han and Wessling-Resnick 2002), however, it is unclear if DMT1 has a role in metal toxicity. For the present study, we worked with a cell line over-expressing the mouse 2/−IRE isoform from a strong, tetracycline-regulated promoter and used it to address whether DMT1 transported copper (Cu^{1+}) ions. Proof that DMT1 is a Cu transporter requires that one show that decreasing DMT1 by specific means lowers Cu transport and increasing DMT1 expression specifically raises Cu uptake. Previously, we provided evidence that antisense oligonucleotides to DMT1 diminished Cu transport (Arredondo et al. 2003). In the present study, we find that a regulated increment in DMT1 expression produces greater Cu uptake.

Experimental procedures

Reagents

Dulbecco's modified Eagle's medium (DMEM), hygromycin, geneticin and Lipofectamine2000 were purchased from GIBCO Life Technologies (Carlsbad, CA). Tet System Approved Fetal Bovine Serum (FBS) was from Clontech (Palo Alto, CA). Doxycycline was from Sigma (St. Louis, MO). ^{64}Cu was from Comisión Chilena de Energía Nuclear (Santiago, Chile).

Cell line and plasmid construction

The HEK293 cell line carrying the mouse 2/−IRE form of DMT1 and control cell lines were prepared and initially characterized as described by Garrick et al. (2006). Cells were cultured in a CO_2 incubator at 37 °C with 5 % CO_2 in DMEM supplemented with tetracycline-free FBS in the presence of 200 μg/mL of hygromycin, 400 μg/ml of geneticin and up to 5 μM doxycycline. Doxycycline increased expression of the 2/−IRE DMT1 isoform. There was not a significative change in the uptake of Cu in cells with empty vector treated with doxycycline compared to the regular Caco-2 cells.

Cu treatments

HEK293 cells, a model of kidney cells (ATCC # CRL-1573), were cultured as described for Hep-G2 cells (Arredondo et al. 2004) with minor changes. Briefly, cells were incubated at 37 °C in a 5 % CO_2 atmosphere and grown in plastic cell culture flasks (Nalge Nunc Int. Corp. IL, USA) containing DMEM and 10 % (v/v) tetracycline-free FBS supplemented with doxycycline where relevant. The medium was changed every three days. Cu concentration in the standard culture medium was ~0.40 μM. Cu was supplemented to the culture medium, as Cu-His complex (1:10 ratio) in the

presence of ascorbic acid (1:10 = Cu:AA) or citric acid (1:10 = Cu:CA) to present Cu^{1+} or Cu^{2+}, respectively. For ^{64}Cu uptake, cells were plated in 12-well plates at 0.5×10^6 cells/well or were seeded and grown for 12 days in 0.33-cm^2 polycarbonate cell culture inserts (Corning-Costar). Cell viability assessed by trypan blue exclusion was typically 90–95 %. For all experiments, variables were tested in triplicate samples and analyses were repeated at least twice.

Quantification of intracellular Cu concentration

HEK293 cells (control cells; empty vector cells and 2/−IRE cells) were grown for 1 week as above, supplemented with Cu-His (1, 5, 10, 20, 50 and 100 μM) in the presence of AA or CA. The day of the experiment, cells were washed three fold with cold phosphate-buffered saline (PBS)-EDTA, centrifuge and re-suspended in 50 μL PBS. 10 μL were separated for protein determination. Cellular suspension was digested with concentrate ultrapure nitric acid (1:1) overnight at 60 °C. Cu content was determined by an atomic absorption spectrometer equipped with graphite furnace (SIMAA 6100, Perkin Elmer, Shelton, CT). MR-CCHEN-002 (*Venus antiqua*) and DOlt-2 (*Dogfish liver*) preparations were used as reference materials to validate the mineral analyses. Basal copper in the culture media and with FBS was 0.85 and 2.4 μM, respectively.

Uptake and transepithelial Cu flux studies

Uptake and transepithelial experiments were carried out as reported (Tong and McArdle 1995; Tapia et al. 1996; Arredondo et al. 2001). To determine the effect of pre-loading HEK293 cells with different copper concentration over the rates of apical Cu uptake, control and 2/−IRE cells were incubated with (a) 0.5, 5 and 50 μM or (b) 1, 5, 10 and 50 μM, for 1 week as a Cu-His complex. Cells were trypsinized and grown for 10–12 days in 0.33-cm^2 polycarbonate cell culture inserts (Transwell, Corning-Costar). As a control, cells with empty vector were pre-loading with 5.0 μM of Cu. Inserts were used when they attained stable transepithelial electric resistance (TEER) values between 200 and 220 ohm cm^2. Inserts with less than 200 ohm cm^2 were discarded. Briefly, the cells were washed twice with PBS, pH 7.0, 50 μM $CaCl_2$ and then incubated for different period of time (0–60 min) with MOPS buffer (50 mM MOPS-Na, 94 mM NaCl, 7.4 mM KCl, 0.74 $MgCl_2$, 1.5 mM $CaCl_2$ and 5 mM glucose, pH 6.5) containing (a) different ^{64}Cu concentration (0.5–20 μM) or (b) 10 μM ^{64}Cu. ^{64}Cu uptake was stopped by washing the cells four times with PBS containing 1 mM EDTA at 4 °C. ^{64}Cu radioactivities in cells, apical and basolateral media were measured immediately in a dual-channel Packard beta counter. Measurements obtained from cells at each time were used to determine the amount of copper taken up by the cells. Those obtained from the basolateral media were used to determine the amount of copper transported from the apical to the basolateral side of the membrane. Data were expressed as the rates (pmol ^{64}Cu $insert^{-1}$ min^{-1}), estimated from the slopes of the kinetic data. Uptake and transport were linear over the first 90 min of incubation.

Temperature and pH dependence and competition studies

HEK293 cells were grown for 1 week in 12-well plates. Cells were incubated with 10 μM ^{64}Cu in MOPS buffer for 60 min at different temperatures (4, 20, 25 and 37 °C). The uptake was stopped washing the cells with PBS-EDTA. For pH-dependence studies, cells were incubated as above with 10 μM ^{64}Cu as a Cu:His:AA complex in MOPS buffer (pH 6.5–7.5) or MES buffer (50 mM MES-Na, 94 mM NaCl, 7.4 mM KCl, 0.74 $MgCl_2$, 1.5 mM $CaCl_2$ and 5 mM glucose, pH 5.5–6.5) and then the uptake stopped with PBS-EDTA. For competition uptake studies, HEK293 cells were grown as before. The day of the experiment, the cells were incubated with increased concentrations (0.1–500 μM) of Ag and 1 μM of ^{64}Cu, in the presence of ascorbic acid. Uptake was stopped with PBS-EDTA. Radioactivity was counted in the cellular extracts and expressed as pmol of ^{64}Cu uptake * mg $protein^{-1}$.

Statistical analysis

Variables were tested in triplicates and the experiments were repeated at least twice. Variability among experiments was <20 %. One-way ANOVA was used to test differences in means, and the Bonferroni post hoc *t* test was used for comparisons (GraphPad InStat

software). Differences were considered significant if $P < 0.05$.

Results

Intracellular Cu concentration in HEK293 cells overexpressing 2/−IRE DMT1 isoform

Cells over-expressing the 2/−IRE form of DMT1 (·) incubated with Cu^{1+} had higher levels of intracellular Cu at by at least three to four fold ($P < 0.01$) than the empty vector (+) and original cell controls (*) independent of the external copper concentration and species during growth. As expected, intracellular Cu concentration in HEK293 cells over-expressing 2/−IRE DMT1 still increased when Cu increased in the media. Intracellular Cu content increased from 1.23 ± 0.18 to 36.84 ± 2.87 nmol Cu mg protein^{-1} when the copper in the media increased from 1 to 100 μM ($P < 0.001$). An increment of Cu in the media of 100 times produced a ~30.0-fold increase in the Cu content. Above 20 μM of external Cu, the increment in internal Cu was just 1.6-fold (22.85 ± 2.86–36.84 ± 2.87 nmol Cu mg protein^{-1}, $P < 0.05$), consistent with a saturable process. Cells that were incubated in the presence of citric acid had lower intracellular copper concentration compared with cell incubated in the presence of ascorbic acid (Fig. 1; $P < 0.001$). These results suggest that DMT1 preferentially will transport Cu^{1+} instead of Cu^{2+} when both are available. Trypan blue and MTT viability analysis showed no significant differences under the different Cu exposure conditions. Probably the efflux of Cu from these cells became important in this external concentration range to protect them from the intracellular Cu excess (Qian et al. 1996; Harris 2001). The lower intracellular Cu content of wild type cells or cells transfected with the empty vector was not significantly different between the two control lines. Having established that increased mouse 2/−IRE DMT1 expressions causes HEK293 cells to accumulate much more Cu^{1+}, we will focus on the properties of DMT1 as a Cu^{1+} transporter because the likelihood that Cu^{2+} was first reduced would complicate each of the analysis. This possibility is considered further in the discussion. To do that, we performed experiments to learn more about the effect of varying internal Cu levels on uptake, apical uptake compared to transepithelial transport and the properties of DMT1 as a Cu transporter.

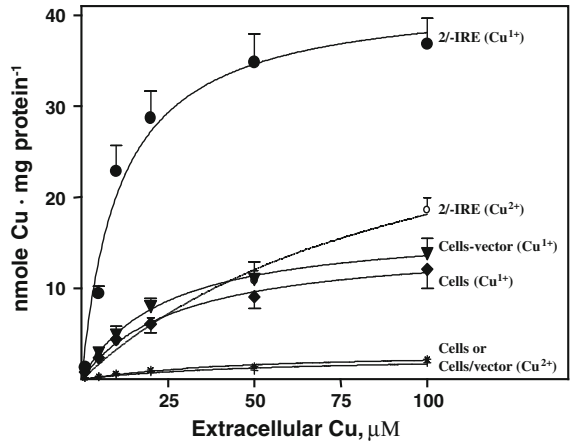

Fig. 1 Intracellular Cu content in HEK293-DMT1 cells incubated with different Cu concentrations. HEK293 cells were incubated with the indicated Cu:His complex concentrations in the presence of ascorbic acid or citric acid concentrations for 1 week. The intracellular Cu content was measured by EAA with graphite furnace (plotted as mean ± SD, $n = 5$ different experiments). *Black circle* 2/−IRE cells (Cu^{1+}), *white circle* 2/−IRE cells (Cu^{2+}), *black lower triangle* empty vector (Cu^{1+}), *black diamond* Caco-2 cells (Cu^{1*}), *asterisk* Caco-2 cells (Cu^{2+}), *plus* empty vector (Cu^{2+})

Kinetics of ^{64}Cu uptake in cells pre-incubated with copper

We next studied how the intracellular Cu content affected the uptake of apical Cu transport in these cells. For that, HEK293 cells over-expressing 2/−IRE form of DMT1 were pre-incubated with three different Cu extracellular Cu concentrations (0.5, 5 and 50 μM) as a Cu-His-AA complex for 12 days. The day of the experiment the cells were incubated with ^{64}Cu concentrations (0.5–20 μM; Fig. 2). We were able to get the cell lines to form tight junctions in transwell plates so we could see the role of 2/−IRE DMT1 and learn the effect of different intracellular Cu content in apical and transepithelial Cu transport. Although there appeared be a trend for less uptake when cells were pre-exposed to more Cu, there was no significant difference in uptake among cells over-expressing 2/−IRE. They were, however, different ($P < 0.05$) from the control cells. A double-reciprocal analysis of uptake data (from Fig. 2) showed that cells grown in 0.5, 5 and 50 μM Cu had apparent *K*m's (in μM: 2.03 ± 0.3; 2.03 ± 0.2 and 2.2 ± 0.5, respectively) lower than cells with empty vector grown in 5 μM Cu (5.5 ± 1.1 μM). The Vmax's (in pmol ^{64}Cu mg protein^{-1} min^{-1}: 13.1 ± 0.6;

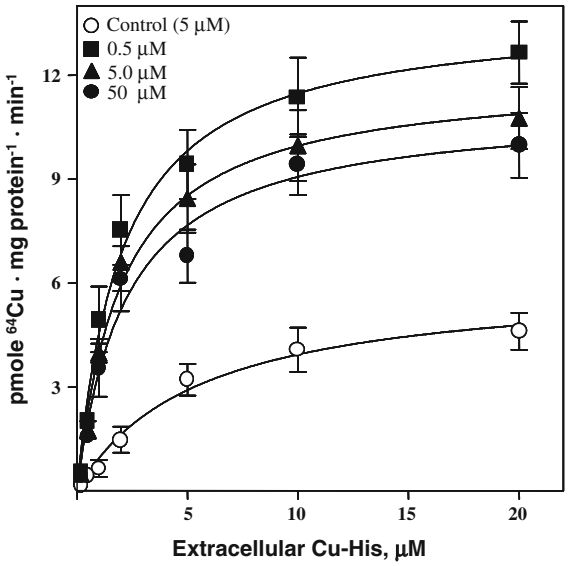

Fig. 2 Apical Cu uptake in HEK293-DMT1 cells exposed to different extracellular Cu concentrations. HEK293 cells over-expressing 2/−IRE form of DMT1 were pre-incubated for 12 days with different Cu concentrations (0.5; 5 and 50 μM) as a Cu:His:AA complex. The day of the experiment, cells were incubated with increasing concentrations of ^{64}Cu. Uptake was stopped with PBS-EDTA after 30 min incubations. Radioactivity was counted in cellular extract (plotted as mean ± SD, $n = 3$ independent samples). *Black square* 2/−IRE cells (0.5 μM Cu^{1+}), *black upper triangle* 2/−IRE cells (5 μM Cu^{1+}), *black circle* 2/−IRE cells (50 μM Cu^{1+}), *white circle* control cells (empty vector) (2.5 μM Cu^{1+})

12.0 ± 0.5 and 11.1 ± 0.7, respectively) were ~2-fold higher than cells with empty vector (6.1 ± 0.5). Higher fluxes indicated that half or more of total Cu uptake in cells expressing mouse 2/−IRE DMT1 is due to the ectopically expressed transporter; while the difference in *K*m's suggests that endogenous Cu uptake in HEK293 cells relies at least in part on another lower affinity Cu transporter (although it remains possible that endogenous human DMT1 has a weaker affinity for Cu if one relies only on these data).

Rate of apical uptake and transepithelial transport of Cu in HEK293 cells exposed to different extracellular Cu concentration

HEK293 cells (control and 2/−IRE cells) were grown in 1, 5, 10 or 50 μM of extracellular Cu for 2 weeks in bicameral chambers and then incubated with 10 μM of ^{64}Cu (Fig. 3). Cu uptake was higher in cells exposed to lower extracellular Cu and thus having lower intracellular Cu content, and lower in cells exposed to higher extracellular Cu and thus having higher intracellular content (in pmol ^{64}Cu mg protein^{-1}: 4.59 ± 0.31 at 1 μM extracellular Cu compared to 2.46 ± 0.14 at 50 μM Cu, $P < 0.05$). The rates of apical ^{64}Cu uptake (Fig. 3, inset) were high at low intracellular Cu concentrations, and reach a plateau over 10 nmol of Cu mg protein^{-1} (in pmol ^{64}Cu mg protein^{-1} min^{-1}: 0.094 ± 0.004 at 1.2 nmol Cu mg protein^{-1} to 0.047 ± 0.002 at 33.8 nmol Cu mg protein^{-1}). All the rates of Cu uptake were higher in cells over-expressing 2/−IRE DMT1 than control cells. However, both types of cells showed otherwise similar behavior relative to extracellular or intracellular Cu or time.

Transepithelial Cu flux was low in cells with low Cu concentrations and high in cells with high intracellular Cu (Fig. 4) (in pmol ^{64}Cu mg protein^{-1}: 0.59 ± 0.04 at 1 μM Cu compared to 1.21 ± 0.10 at 50 μM, $P < 0.01$). The rates of transepithelial Cu flux (Fig. 4, inset) increased when the intracellular Cu increased (in pmol ^{64}Cu mg protein^{-1} min^{-1}: 0.0099 ± 0.0004 at 1.2 nmol Cu mg protein^{-1} to 0.0234 ± 0.0009 at 33.8 nmol Cu mg protein^{-1}). 2/−IRE cells showed higher rates of transepithelial Cu flux than cells that only express endogenous Cu transporter(s), although the two types of cells otherwise exhibited similar responses to extracellular or intracellular Cu or time.

Effect of varying temperature and pH on Cu uptake in HEK293 cells

The temperature dependence of inducible Cu uptake was studied in HEK293 cells (Fig. 5). As expected, the transfected cells exhibited higher uptake and Cu uptake was affected by incubation temperature. The uptake decreased from 5.36 ± 0.42 nmol mg protein^{-1} at 37 °C to 2.16 ± 0.18 at 4 °C ($P < 0.01$). Controls or transfected HEK293 cells showed a similar pattern of uptake at different temperatures although the latter had higher levels.

DMT1 transporter activity is associated with proton flux; in that context we studied the effect of varying the incubation pH on Cu uptake in HEK293 cells. HEK293 cells over-expressing 2/−IRE DMT1 exhibited pH dependent uptake with an optimum at pH 6.5. There was a significant difference in the uptake between 6.5 and 5.0, 5.5 and 7.5 pH ($P < 0.01$); however, the difference between 6.5 and 7.0 was not significant (Fig. 6).

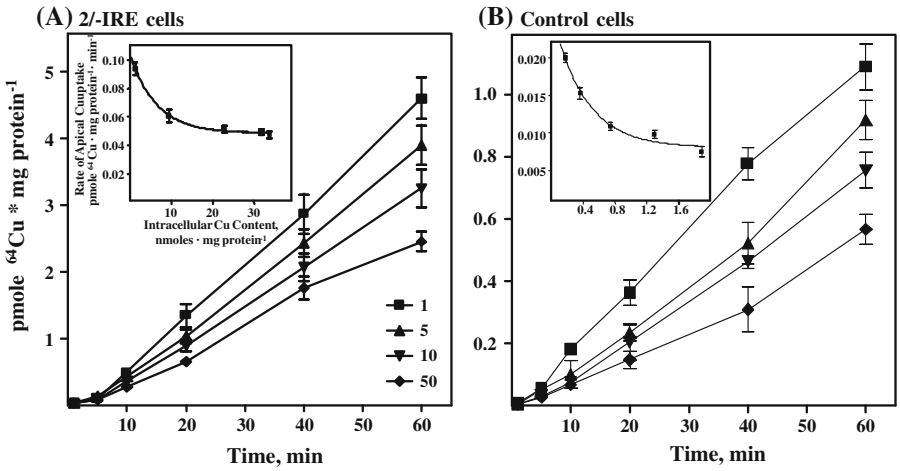

Fig. 3 Kinetics of apical Cu uptake in HEK293 cells exposed to different extracellular Cu concentrations. HEK293 cells were incubated for 10–12 days with different Cu concentrations as a Cu:His:AA complex and then were incubated with 10 μM ^{64}Cu for different period of time (0–60 min). These analyses represent **a** 2/−IRE cells, **b** control HEK293 cells. The uptake was stopped with PBS-EDTA. The radioactivity was counted in cellular extracts. The insets relate the rates of apical Cu uptake to the intracellular Cu (plotted as mean ± SD, $n = 3$ independent experiments)

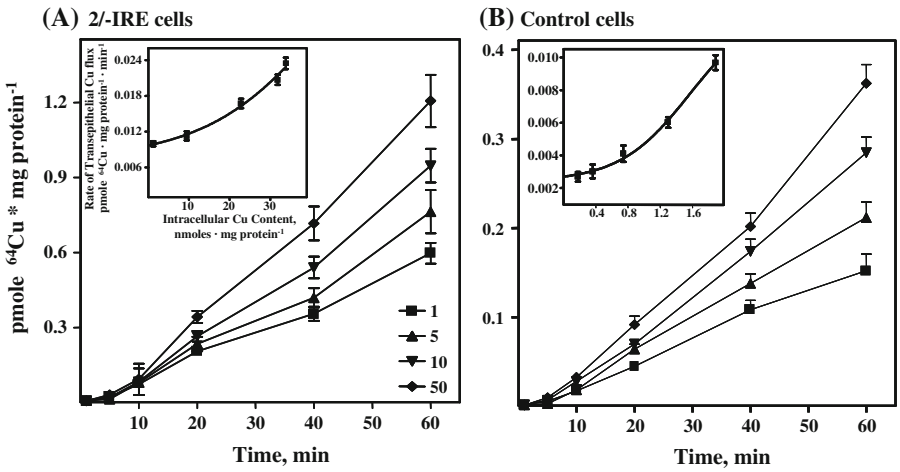

Fig. 4 Kinetics of transepithelial Cu flux in HEK293 cells exposed to different extracellular Cu concentrations. HEK293 cells were incubated for 10–12 days with different Cu:His:AA concentrations. The cells were incubated with 10 μM ^{64}Cu for different period of time (0–60 min). Transepithelial Cu flux was stopped with PBS-EDTA. The radioactivity was counted in cellular extract. These analyses represent **a** 2/−IRE cells, **b** control HEK293 cells. The insets relate the rates of transepithelial Cu flux to the intracellular Cu (plotted as mean ± SD, $n = 3$ independent experiments)

Effect of increasing extracellular concentrations of Ag over Cu uptake

To study the specificity of Cu uptake by DMT1, HEK293 cells (carrying the vector and control) were incubated with increasing Ag extracellular concentrations. Independently of the extracellular Ag concentrations (Fig. 7), ^{64}Cu uptake was not affected in all the range of Ag studied.

Discussion

In the present study, we set out to characterize Cu uptake in HEK293 cells that were stably transfected with 2/−IRE DMT1. After establishing that there was more uptake than in control cells, we studied the state of the Cu ions, in the effect of different external Cu concentrations, apical uptake compared to

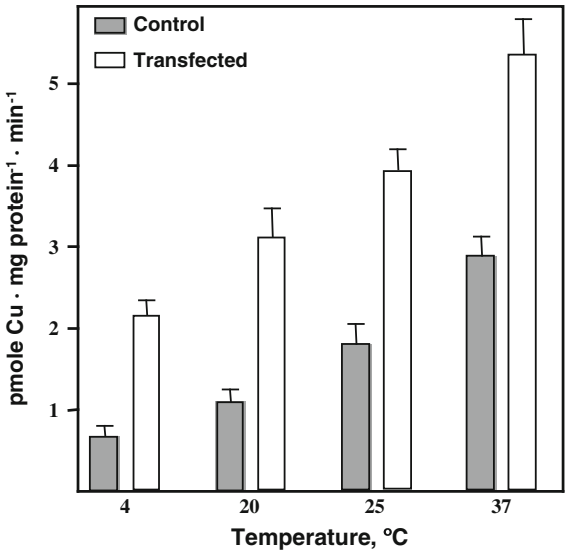

Fig. 5 Effect of temperature on Cu uptake. HEK293 cells were grown as before. ^{64}Cu uptake was measured at different temperatures (4, 20, 25 and 37 °C) for 60 min. ^{64}Cu uptake was stopped with PBS-EDTA. The radioactivity was counted in cellular extract (plotted as mean ± SD, $n = 4$ independent experiments)

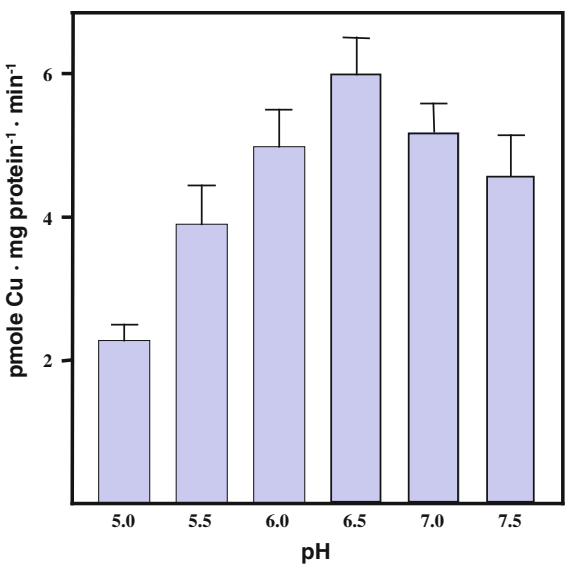

Fig. 6 Effect of pH on Cu uptake. HEK293 cells were grown as before. ^{64}Cu uptake was measured at different pH in the transport buffer (5.0–7.5) for 60 min. ^{64}Cu flux was stopped with PBS-EDTA. The radioactivity was counted in cellular extract (plotted as mean ± SD, $n = 4$ independent experiments)

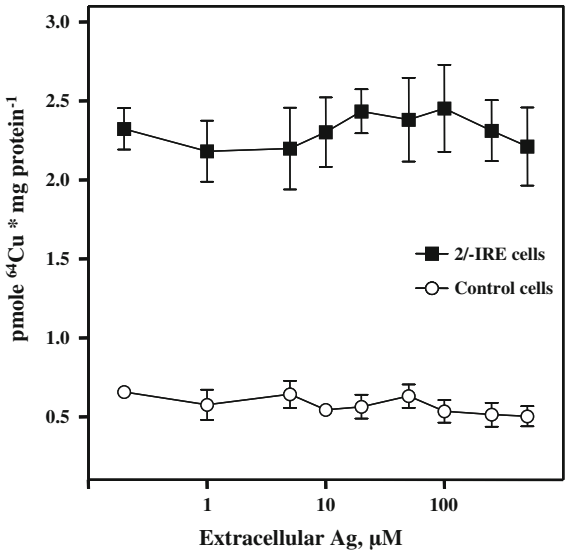

Fig. 7 Competition uptake studies between Cu and Ag. HEK293 cells were grown as before. The day of the experiment, the cells were incubated with increased concentrations (0.1–500 µM) of Ag and 5 µM of ^{64}Cu, in the presence of ascorbic acid. Uptake was stopped with PBS-EDTA. Radioactivity was counted in the cellular extracts and expressed as pmol of ^{64}Cu uptake * mg protein^{-1}. ($n = 4$ independent experiments)

transepithelial transport and the temperature and pH dependence and competition with Ag.

The properties of DMT1 as a Cu transporter are similar to those of it as a transporter of Mn and Fe (Garrick et al. 2006). What is most critical is that transport activity is higher in the transfected cells overexpressing DMT1 than in the control cells. The degree of stimulation is less, however, for Cu. This difference could depend on differences in assay conditions in this submission for Cu. For examples, the two papers relied on different media, time spans, metal ion concentration ranges and as also discussed below, and the optimal pH for Cu was higher than for Mn or Fe. This difference could reflect behavior of Cu^{1+} as substrate compared to Mn^{2+} and Fe^{2+} as the forms transported for these two metals. Additional experiments could help to understand these differences, but it now clear that over-expressing DMT1 increases Cu transport.

In previous work, we had demonstrated that DMT1 is the main iron transporter and is a physiologically relevant in Cu^{1+} transporter in an intestinal model, Caco-2 cells (Arredondo et al. 2003). Our data

illustrated clearly that intestinal absorption of copper and iron are intertwined. We continued studying their interrelationship in HepG2 cells (Arredondo et al. 2004) showing that loading with Cu lowered DMT1 levels and increased the apparent Km for Cu uptake. These observations motivated our current study because DMT1 levels would be responsive to doxycycline treatment rather than Fe or Cu levels. The results presented here suggested that DMT1 transport Cu^{2+} and Cu^{1+}, however if DMT1 is also a Cu^{2+} transporter, it favors Cu^{1+} binding by nearly ten fold (Fig. 1). Nevertheless, we prefer an alternative explanation: that a cupric reductase is present in HEK293 cells. This argument receives support from the observation (Arredondo et al. 2003) that there was less Cu^{2+} than Cu^{1+} transport in Caco-2 cells. Garrick, Singleton, Vargas and Garrick (personal communication) have similar data comparing Fe^{3+} and Fe^{2+} in these HEK293 cells overexpressing DMT1 and their controls. These data point to the presence of a ferri-reductase. The issue of reductase acting on these metal ions merits additional studies, but such experiments are beyond the scope of this paper. As a control of Cu uptake, we studied the Ag–Cu uptake competition. Results by Lee et al. (2002) showed that Cu uptake by CTR1 was inhibited by Ag. Our results showed that in HEK293 cells carrying the vector the Cu uptake was not inhibited by Ag, suggesting that Cu uptake by CTR1 was minimal and mainly by 2/−IRE DMT1 isoform in HEK293 cell carrying the vector.

In HEK293 cells over-expressing 2/−IRE DMT1, external Cu has less effect on Cu uptake but there is still some depression. Possibly this result is due to an effect on DMT1 itself. We observed decreasing uptake of Cu when intracellular Cu increased, an observation that parallels our results in HepG2 cells, supporting a role for DMT1 in renal handling of Cu (Arredondo et al. 2004). Others (Ghio A and Dailey LA, personal communication from A Ghio to M Garrick, cited with permission); have also grown these lines of HEK293 cells in transwells and seen polarization and tight junctions. The HEK293 cells might therefore be representative of kidney epithelium, but it remains to be determined to what extent and for what part of the kidney.

If DMT1 has such a role, then changes in DMT1 expression would be expected to affect the rate of renal Cu uptake or excretion. In any case there are potential similarities between how the cells take up and increase their storage of copper in HEK293 cells, Caco-2 cells and HepG2 cells. Cells with low intracellular copper acquire more copper rapidly initially, and then reached a plateau as extracellular copper exceeds 5 μM. The uptake was linear with time in all the conditions and within the range of times studied. The *K*m values reported here are very similar to the obtained in other cell lines under similar conditions, especially with HepG2 cells (Table 1). The higher value in Vmax in HEK293 cells is probably due to the over-expression of the DMT1 (compared to control HEK293 cells or HepG2 cells). This result suggests that probably that endogenous DMT1 may participate in Cu re-absorption in the kidney.

In duodenum and placenta, as in the kidney, low-iron diets induce an increase in DMT1 expression. Renal DMT1 expression is strongly modulated in response to altered dietary iron intake and these responses are associated with changes in urinary iron excretion rate. These findings suggest that changes in DMT1 expression influence urinary iron excretion and potentially provide the body with one means of conserving or excreting iron. Therefore, the kidney plays an important role in iron homeostasis and that 98 % of the iron filtered at the glomerulus is reabsorbed (Cannone-Hergaux and Gros 2002). DMT1 is not expressed in the basolateral membranes of the nephron, indicating that this protein is not involved in basolateral iron movement (Wareing et al. 2000). Therefore, expression of DMT1 in the kidney responds in the same way as the duodenum and placenta, but the opposite to the liver, to altered dietary iron intake. DMT1 could also be involved in the uptake of Cd, a toxic metal, in kidney epithelial cells (Ferguson et al. 2001; Olivi et al. 2001).

Table 1 Kinetic parameters of Cu transport in three different cells lines

Cells lines	Cu_{ext} (μM)	Km	Vmax
HepG2	0.4	0.70	6.9
	10	1.48	7.1
	100	1.33	5.3
Caco-2	10	0.35	102.0
Hek293 2/−IRE	0.5	2.03	13.8
	5	2.03	12.0
	50	2.23	11.1
Hek293 wt	5	5.47	6.1

Km in (μM) Vmax in pmol Cu mg $protein^{-1}$ min^{-1}

We showed here that the optimal activity of the transporter was at 37 °C and pH 6.5. The temperature dependence agrees with Garrick et al. (2006) as well as experiments in *X. laevis* oocytes and in transfected CHO cells expressing DMT1 at the plasma membrane. The same studies have shown that DMT1-mediated transport of divalent cations is strictly pH-dependent, but it is most active at acidic pH of 5.5–6.0. It is unclear why the optimal pH is somewhat higher in the current study. It could be specifically related to Cu as a substrate or it could be due to the contributions of other Cu transporters to the results, given that we see less stimulation of DMT1 expression for Cu uptake than Garrick et al. (2006) see for Mn or Fe uptake. Therefore, the acidic pH of the filtrate present in the proximal tubules is compatible with the known requirements for divalent cations transport by DMT1 (Lee et al. 2002).

In summary, we have shown that the 2/−IRE isoform of DMT1 transporter in HEK293 cells functions in uptake of Cu^{1+}. Further experiments should be made in order to clarify the differences or similarities between the DMT1 isoforms, their role in metals transport and the extent to which DMT1 (in any isoform) relies on reductases for presentation of substrates like Fe^{3+} or Cu^{2+}.

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