

ATP-driven copper transport across the intestinal brush border membrane

Martin Knöpfel^{a,1}, Craig Smith^b, Marc Solioz^{a,*}

^a Department of Clinical Pharmacology, University of Berne, 3010 Berne, Switzerland

^b School of Biological Sciences, University of Manchester, UK

Received 3 March 2005

Available online 16 March 2005

Abstract

The divalent metal ion transporter DMT1 is localized in the brush border membrane (BBM) of the upper small intestine and has been shown to be able to transport Mn^{2+} , Fe^{2+} , Co^{2+} , Ni^{2+} , and Cu^{2+} . Belgrade rats have a glycine-to-arginine (G185R) mutation in DMT1, which affects its function. We investigated copper transport with BBM vesicles of Belgrade rats loaded with calcein, which exhibits fluorescence quenching by various metal ions. Transport of copper was disrupted in unenergized BBM vesicle of *b/b* Belgrade rats, as had been described for iron transport, while *+/b* vesicles exhibited normal transport by DMT1. When either *b/b* or *+/b* vesicles were loaded with ATP and magnesium, similar high-affinity accumulation of copper was observed in both types of vesicles. Thus, brush border membranes possess an ATP-driven, high-affinity copper transport system which could serve as the primary route for copper uptake by the intestine.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Brush border membrane; Copper transport; Belgrade rat; Calcein fluorescence; Intestine; Duodenum; ATPase

Copper and iron are essential trace elements through their function as cofactors in numerous biochemical reactions, but knowledge of their homeostasis by eukaryotic cells is still fragmentary (see [1–8] for recent reviews). Recently, a divalent metal ion transporter, DMT1, has been identified by expression cloning in *Xenopus laevis* oocytes [9]. DMT1 is the homolog of mouse Nramp2, a member of the 'natural resistance-associated macrophage protein' family [10–12], and is also called DCT1. Its official designation is Slc11A2 (OMIM database, <http://www3.ncbi.nlm.nih.gov/omim>); we here use the term DMT1, which is commonly used in the literature. DMT1 features 12 putative membrane

spanning helices and occurs in four splice variants: a central domain of 543 common amino acids can have a 31 amino acid N-terminal extension and a diverging C-terminus of either 18 or 25 amino acids (see [13] for review). The functional significance of the four isoforms remains to be demonstrated. DMT1 is strongly expressed in the proximal duodenum and the kidneys, but can be found in all tissues. In enterocytes, DMT1 is mainly localized in the brush border, but is also present in basolateral and internal membranes, and is upregulated by iron deficiency [13]. This transporter thus appears to be the key mediator of intestinal iron absorption, but has also a function in the release of endosomal iron, originating from transferrin-mediated iron acquisition.

In transport studies with erythroid cells, brush border membrane (BBM) vesicles or *Xenopus* oocytes, the transport specificity of DMT1 was broad, including Fe^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Ni^{2+} , and Pb^{2+}

* Corresponding author. Fax: +41 31 632 4997.

E-mail addresses: tracechem@tiscali.ch (M. Knöpfel), marc.solioz@ikp.unibe.ch (M. Solioz).

¹ Present address: TraceChem, Belpstrasse 41, 3007 Berne, Switzerland.

[9,14–16]. Belgrade rats as well as anemic *mk/mk* mice harbor the same glycine-to-arginine (G185R) mutation in DMT1 and it has been shown that this abolishes the transport of Mn^{2+} (and presumably other divalent metal ions) into BBM and basolateral membrane vesicles [17]. Belgrade rats are anemic, but exhibit normal copper status [18–20]. This would suggest that other intestinal copper transporters are at work. At least two other copper transporters are known to be expressed in enterocytes: Ctr1 and ATP7A.

Ctr1 can be detected in all cell types tested and exhibits plasma membrane as well as intracellular localization. This transporter has been shown to catalyze the transport of Cu(I) across the plasma membrane with nanomolar affinity, but Ctr1 also has a role in copper transport in intracellular compartments [21]. The transport of biologically relevant transition metal ions other than Cu(I) has not been reported for Ctr1. Whether Ctr1 has a role in intestinal copper absorption remains unclear. The second known copper transporter in enterocytes, ATP7A, is a copper ATPase. It serves in the delivery of copper to the Golgi for copper incorporation into cuproenzymes such as ceruloplasmin, but also in the secretion of copper from cells [6,22]. ATP7A is expressed in most tissues, but is absent in the liver, where the related enzyme ATP7B fulfills this function. Under normal copper load, ATP7A is mainly localized in the *trans*-Golgi network, but excess copper induces trafficking of ATP7A to the plasma membrane for the secretion of copper. Like Ctr1, ATP7A appears to only transport Cu(I) and no other biometals [23].

In spite of the advance in the understanding of copper homeostasis, it is still unclear which transporter is responsible for intestinal copper uptake. We here report the properties of a novel, ATP-driven copper transport activity in BBM vesicles. This copper transport did not appear to be catalyzed by DMT1, Ctr1 or ATP7A. Transport activity exhibited a sub-micromolar affinity for copper and may thus have a primary role in intestinal copper absorption.

Materials and methods

Materials. All chemicals were of analytical grade and were obtained from Fluka (Buchs, Switzerland). Protein molecular weight standards were purchased from Bio-Rad.

Preparation of brush border membrane vesicles. Rat small intestines were excised from decapitated rats, copiously rinsed with ice-cold saline, cut open longitudinally, and frozen at -80°C . The first 20 cm of proximal rat small intestine from two rats was used to prepare BBM vesicles by the method of Kessler et al. [24], but using a 2:1 rescaled $MgCl_2$ precipitation instead of a $CaCl_2$ precipitation to avoid the activation of proteases [25]. The resulting BBM vesicles were stored frozen at -80°C until use, but no longer than four weeks.

Transport measurements. For measuring metal ion transport by unenergized BBM vesicles, vesicles were loaded with $33\ \mu\text{M}$ calcein as

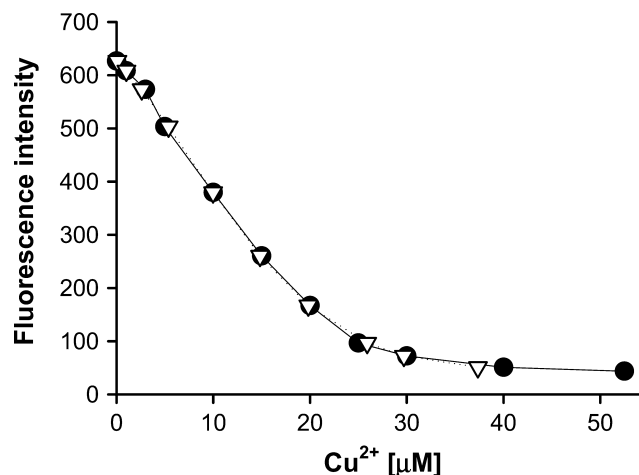


Fig. 1. Quenching of calcein fluorescence by copper. A $33\ \mu\text{M}$ calcein solution in Na–Hepes buffer, pH 7.4, was quenched with increasing concentrations of Cu^{2+} at 25°C . The experimental data (●) were fitted with a Gaussian function (▽) as described previously for nickel [15].

described earlier [15]. Briefly, BBM vesicles were homogenized in the presence of $33\ \mu\text{M}$ calcein with a Potter–Elvehjem homogenizer, followed by gel filtration through a Sepharose 4B column to remove extravesicular calcein. To measure ATP-driven transport, BBM vesicles were simultaneously loaded with $33\ \mu\text{M}$ calcein, $10\ \text{mM}$ ATP, and $10\ \text{mM}$ $MgCl_2$ by the same method. Transport was measured by measuring calcein fluorescence quenching as described [15]. Briefly, $160\ \mu\text{l}$ of BBM vesicles at a protein concentration of 0.1 – $0.2\ \text{mg/ml}$ in $50\ \text{mM}$ Na–Hepes, pH 7.4, $0.1\ \text{M}$ NaCl were equilibrated to 25°C in a 4-by-4 mm microcuvette. Fluorescence quenching was measured on a Perkin-Elmer LS 50 B spectrofluorometer, using excitation and emission wavelengths of 490 and 515 nm, respectively. Changes in metal ion concentration were estimated on the basis of relative changes in fluorescence, based on the calibration curve shown in Fig. 1. There was no interference by light scattering as verified by solubilizing the vesicles with detergent in the course of an experiment.

Miscellaneous methods. Protein concentrations were determined by the method of Bradford [26].

Results

It has been shown that DMT1 plays a major role in the intestinal uptake of Fe(II) and may also play a role in the uptake of other divalent ions, including copper [13,15,27,28]. Belgrade rats as well as *mk/mk* anemic mice, which possess the same glycine-to-alanine mutation in DMT1, have a defect in the uptake of iron from the lumen of the gut, leading to iron deficiency in many tissues [13]. Belgrade rats have elevated plasma iron, but the iron cannot get into tissues because of the lack of functional DMT1 in endocytic vesicles, where this transporter is required for the release of iron acquired by the transferrin-mediated pathway. However, Belgrade rats do not appear to be compromised in their copper status. This could either be due to the low requirement for copper compared to iron or to the presence of another copper uptake route. This prompted us to investigate

copper transport by BBM vesicles of Belgrade rats, which are homozygous (*b/b*) for a G185R missense mutation in DMT1. Heterozygous (*+/b*) rats, which are phenotypically not anomalous, were used as a control. For unexplained reasons, BBM vesicles of the heterozygotes exhibited more robust and reproducible transport than those from wild-type rats (Martin Knöpfel, personal observation).

Transport into isolated BBM vesicles was monitored by loading them with the fluorescent dye calcein, which is quenched by divalent metal ions. Fig. 1 shows the calibration curve of the quenching of calcein by copper. The decrease in fluorescence intensity with increasing concentrations of Cu^{2+} could be fitted by a Gaussian function and was used to quantify all experimental curves. Corresponding calibration curves were derived for the other metal ions used in this study (not shown).

Fig. 2 shows divalent metal ion transport by unenergized calcein-loaded BBM vesicles of heterozygous Belgrade rats (*+/b*). Fluorescence quenching of the calcein in the vesicle lumen indicates uptake of metal ions. The quenching by different metal ions proceeded at varying rates, reflecting different transport affinities. The greatest uptake rate was observed for Co^{2+} , which was rapidly transported into vesicles already at $1 \mu\text{M}$ Co^{2+} in the extravesicular space. In contrast, Ni^{2+} exhibited a much lower rate of uptake. Cu^{2+} at $1 \mu\text{M}$ gave a bi-phasic signal, not indicating net transport (artifactual signals were often observed with copper, but their cause remained unknown), while $50 \mu\text{M}$ Cu^{2+} induced rapid copper uptake by the BBM vesicles. These transport characteristics appear typical for DMT1-catalyzed uptake, as described previously [15].

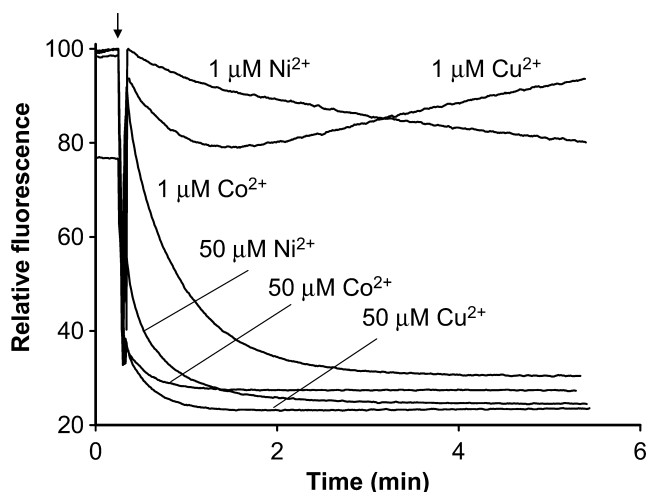


Fig. 2. Transport of Cu^{2+} , Ni^{2+} , and Co^{2+} into unenergized (*+/b*) BBM vesicles. Transport was measured by monitoring the change in fluorescence of 0.18 mg/ml calcein loaded (*+/b*) BBM vesicles. Transport was initiated by the addition of 1 or $50 \mu\text{M}$ metal ions (arrow). Other details of the experiment were as outlined under Materials and methods.

There is conflicting information as to whether Cu(I) or Cu(II) is being transported by DMT1 [13,15,27]. Also, it is not clear if Ctr1 or ATP7A has a role in intestinal copper absorption. Ctr1 and ATP7A apparently catalyze high affinity uptake of Cu(I) [7]. To test for the presence of high affinity uptake of Cu(I) by the BBM vesicle preparation, we conducted transport experiments under reducing conditions. Fig. 3 shows uptake of copper, nickel, and cobalt by unenergized BBM vesicles of (*+/b*) heterozygous rats in the presence of 2 mM ascorbate as a reducing agent. Each metal was added at $1 \mu\text{M}$ (arrow) and at $50 \mu\text{M}$ (arrowhead) to qualitatively demonstrate the different affinities. At $1 \mu\text{M}$, no net uptake of Cu(I) could be observed; there was, however, an artifactual increase in fluorescence. Adding $50 \mu\text{M}$ of copper led to copper accumulation inside the vesicles. Since these experiments were conducted in the presence of ascorbate, the added Cu(II) was reduced to Cu(I). It remains unclear whether the uptake of copper at $50 \mu\text{M}$ was catalyzed by DMT1 or by another transport system. However, it appears unlikely to be catalyzed by Ctr1; this transporter has been reported to have an affinity for Cu(I) of a few micromolar and transport should thus be observed at $1 \mu\text{M}$ Cu(I) [29]. ATP7A on the other hand would require ATP to catalyze copper transport [23]. Ni^{2+} at $1 \mu\text{M}$ was accumulated at a low rate, while $50 \mu\text{M}$ Ni^{2+} led to rapid transport into the vesicle lumen. As already seen in Fig. 2, Co^{2+} was accumulated at the highest rate by the vesicles, exhibiting rapid quenching already at $1 \mu\text{M}$ and almost instantaneous uptake to equilibrium at $50 \mu\text{M}$. So in essence, there was no positive effect of ascorbic acid on copper ion uptake by (*+/b*) vesicles. When the same transport experi-

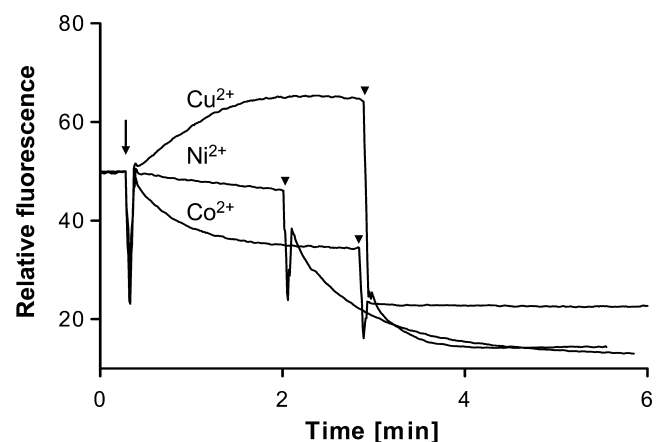


Fig. 3. Transport of Cu^{2+} , Ni^{2+} , and Co^{2+} into unenergized (*+/b*) BBM vesicles in the presence of ascorbate. Transport was measured by monitoring the change in fluorescence of 0.091 mg/ml calcein loaded (*+/b*) BBM vesicles in the presence of 2 mM ascorbate. Transport was initiated by the addition of $1 \mu\text{M}$ metal ion (arrow), followed by a second addition of $50 \mu\text{M}$ metal ion (arrowheads). Other details of the experiment were as outlined under Materials and methods.

ments were conducted with BBM vesicles from Belgrade rats (*b/b*), no transport activity was observed (not shown). These results confirm the transport by DMT1 and its absence/non-function in Belgrade rats, which has been described previously [2,13,15,17].

Average copper in US drinking water is 0.066 mg/L, corresponding to approximately 1 μM [30]. The copper content of most food is around 1 mg/kg, but only a fraction of this copper (e.g., 18% in cow milk) is bioavailable [31]. So available copper in the diet will be in the low micromolar range. It appears unlikely that the observed affinity of DMT1 for copper suffices to accomplish proficient copper uptake from the diet. This led us to probe the existence of an ATP-driven high-affinity transport processes. A procedure was devised to load BBM vesicles with ATP and magnesium. These ATP-loaded, or ‘energized,’ vesicles exhibited high-affinity transport of metal ions not observed in unenergized vesicles. Interestingly, ATP-driven high-affinity transport was essentially identical in Belgrade heterozygous (*+/b*) or homozygous (*b/b*) vesicles; only results for (*b/b*) vesicles are shown here.

Fig. 4 shows ATP-driven transport of Cu^{2+} , Ni^{2+} , and Co^{2+} into energized, homozygous (*b/b*) BBM vesicles. Under these experimental conditions, the uptake of cobalt could readily be observed at a metal ion concentration of only 0.2 μM . In contrast to unenergized vesicles, ATP-loaded vesicles accumulated copper and nickel when present at low micromolar concentrations. Thus, vesicles from Belgrade rats exhibited ATP-driven, high-affinity transport of copper, not detectable in unenergized vesicles. The vesicle preparation used here has been well characterized and is enriched for alkaline

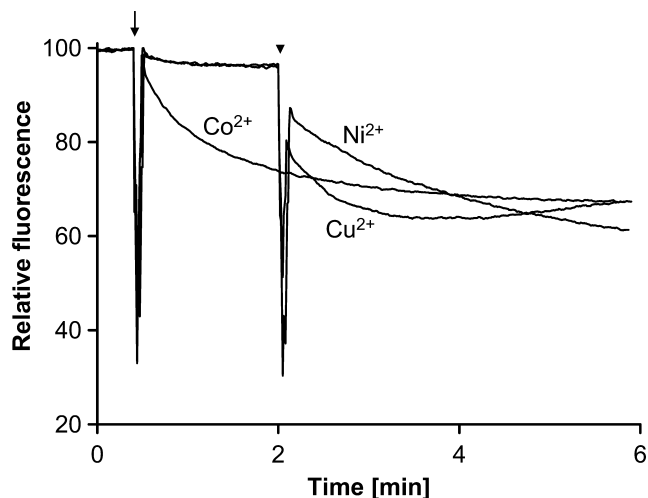


Fig. 4. Transport of Cu^{2+} , Ni^{2+} , and Co^{2+} into energized (*b/b*) BBM vesicles. Transport was measured by monitoring the change in fluorescence of 0.18 mg/ml calcein and ATP loaded (*b/b*) BBM vesicles. Transport was initiated by the addition of 0.2 μM metal ion (arrow), followed by a second addition of 2 μM metal ion (arrowhead). Other details of the experiment were as outlined under Materials and methods.

phosphatase activity, a marker for BBM, by 15 ± 5 -fold [17,25]. It can clearly not be ruled out that membranes other than BBM contribute to transport in this preparation, but if only a minor, contaminating fraction of vesicles would be involved in transport, fluorescence would only be quenched in these and the overall extent of fluorescence quenching would only reach a few percent. However, we observed fluorescence quenching to the extent of 50–80%, indicating that the majority of vesicles participate in the transport reaction and thus contain the ATP-driven transport system. Most likely, the same system catalyzes the transport of Cu^{2+} , Co^{2+} , and Ni^{2+} , as shown in Fig. 4. The divalent and diverse nature of the transported ions argues against an involvement of Ctr1 or ATP7A, which appears to be specific for Cu(I). DMT1 on the other hand is inactivated by the G185R mutation in the Belgrade rat [17].

Kinetic parameters of the ATP-driven copper transport were assessed by determining initial rates of uptake for a range of low copper concentrations under optimized conditions (Fig. 5). Plotting the data as a Lineweaver–Burk plot revealed a bi-phasic behavior (Fig. 6). The data in the range of 0.2–1 μM copper could be fit by linear regression and resulted in a v_{max} for ATP-driven copper transport of 22 nmol/min/mg of membrane protein and a K_m for copper of 0.3 μM ($R = 0.998$). At higher copper concentrations, there was an increase in initial rate and a decrease in affinity for copper.

It has previously been shown that DMT1 transports metal ions in symport with protons, thus generating an electrochemical proton gradient. The ATP-driven ion transport described here was tested for the coupling to an electrochemical proton gradient, e.g., an ATPase could serve in the ejection of protons, which in turn

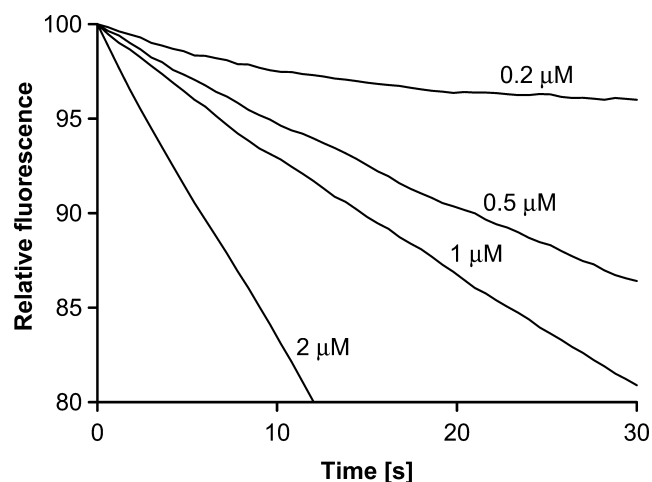


Fig. 5. Initial rates of quenching by copper in energized (*b/b*) BBM vesicles. ATP-loaded vesicles were used at a protein concentration of 0.098 mg. Other details of the measurements were as outlined under Materials and methods. The copper concentrations used are indicated in the figure.

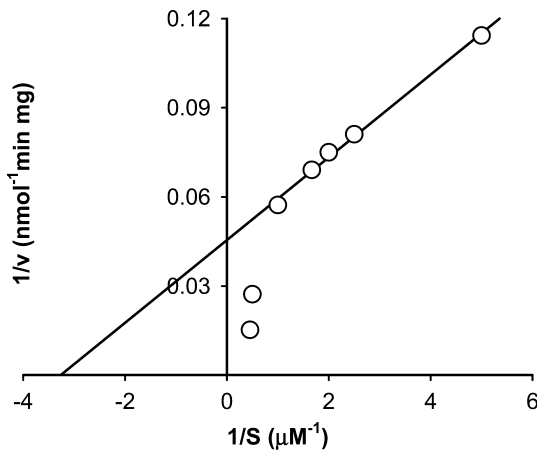


Fig. 6. Lineweaver–Burk plot for copper transport. The initial rate of ATP-driven copper uptake by (*b/b*) BBM vesicles was determined as a function of the copper concentration. Measurements were conducted as described in Fig. 5.

could provide the driving force for metal accumulation. Such coupling would be sensitive to uncoupling by ionophores. This possibility was tested with ionophores. The electroneutral sodium-proton exchanger monensin had no significant effect on ATP-driven copper accumulation by BBM vesicles (Fig. 7). The electrogenic protonophore (uncoupler) carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), on the other hand, stimulated copper accumulation twofold. This would suggest that copper transport is electrogenic, and CCCP stimulated transport by relieving the electrochemical gradient. However, such a conclusion requires further verification. More importantly, if copper transport were coupled to another system via protons, CCCP would

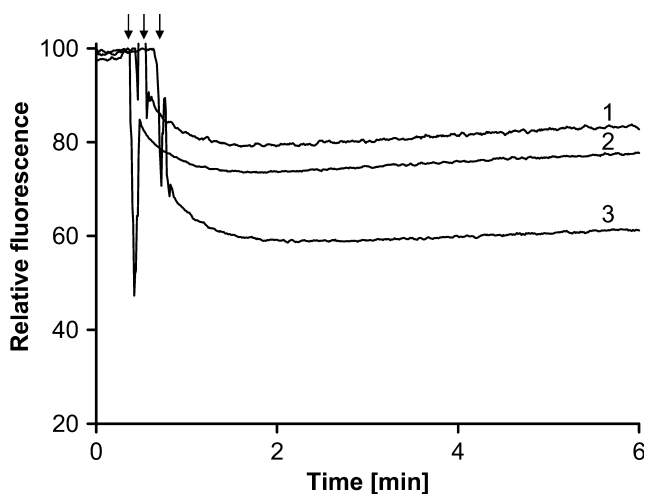


Fig. 7. Effect of ionophores on ATP-driven copper transport by (*b/b*) BBM vesicles. Vesicles at 0.093 mg/ml were preincubated for 5 min at 25 °C with ionophores as indicated. Transport was then initiated by the addition of 2 μM copper (arrows) and transport was measured as described in Fig. 4. Trace 1, control without addition; trace 2, 2.8 μg/ml monensin; and trace 3, 2.8 μg/ml CCCP.

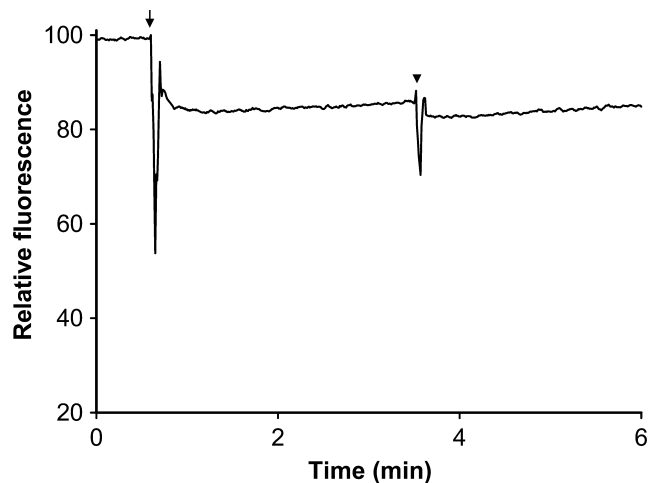


Fig. 8. Transport of Cu^{2+} by (*b/b*) BBM vesicles loaded with AMPPCP. Transport was measured by monitoring the change in fluorescence of 0.092 mg/ml calcein-loaded (*b/b*) BBM vesicles, also loaded with 10 mM MgCl_2 and 10 mM of the ATP analog AMPPCP (β - γ - CH_2 -ATP). Transport was initiated by the addition of 1.25 μM Cu(II) (arrow), followed by a second addition of 1.25 μM Cu(II) (arrowhead). Other details of the experiment were as outlined under Materials and methods.

have abolished transport. Other ions like K^+ or Ca^{2+} were absent during the measurements and thus could not be involved in the coupling of copper transport to a secondary ion gradient. The results of these control experiments are in line with the operation of a primary, ATP-driven copper transporter in BBM vesicles.

To rule out an indirect effect of the ATP or the magnesium in the vesicle lumen, *b/b* vesicles were loaded with 10 mM of the non-hydrolyzable ATP-analog β , γ -methylene adenosine triphosphate (AMPPCP) instead of ATP (Fig. 8). No copper transport was observed under these conditions. There was some artifactual fluorescence quenching upon the first copper addition, but no quenching with the second copper addition, arguing against a ‘copper-sink’ function of the nucleotide triphosphates in the vesicle lumen. Taken together, our results suggest that brush border membranes possess an ATP-driven high affinity transport system for copper and other divalent metal ions. This system may represent the primary system for uptake of these metals from the diet.

Discussion

We here show for the first time an ATP-driven transport mechanism for copper, and probably other divalent metal ions, in brush border membranes of rat intestines. The affinity for copper of this system was 0.3 μM, which appears to be sufficiently high to accomplish copper extraction from food in the lumen of the intestine. Detection of the ATP-driven transport activity

depended on an experimental protocol whereby BBM vesicles were loaded with both, the fluorescent indicator calcein to measure intravesicular metal ion concentrations, and Mg-ATP as an energy source. ATP-driven copper uptake was essentially the same in vesicles derived from normal rats and in vesicles derived from homozygous Belgrade rats. These possess a G185R mutation which inactivates DMT1, the principal iron uptake system of the brush border membrane [17,20]. DMT1 has also been suggested to function in the uptake of copper and other divalent metal ions, including Zn^{2+} , Cd^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} , and Pb^{2+} [9,15]. Conceivably, the ATP-driven copper uptake system described here is the principal copper uptake mechanism under copper limiting conditions, while DMT1 could serve in copper uptake under conditions of copper excess.

In some studies demonstrating copper transport by DMT1, high copper concentrations were employed (up to 80 μ M; e.g. [9,15]), which would have masked a high-affinity, low capacity copper transporter activity, like the one described here. But a number of observations do suggest the existence of one or several copper transport systems other than DMT1 in the brush border membrane. First, Belgrade rats, which are systemically iron deficient due to mutation of DMT1, are not copper deficient, and thus must be able to acquire copper by a different route. In several studies involving sub-micromolar copper concentrations, it was concluded that copper uptake by duodenal cells was not, or not exclusively, catalyzed by DMT1. Arredondo et al., using Cu(II)-histidine as a substrate, observed high-affinity copper flux from apical to cellular, and from apical to basolateral domains, by intestinal Caco-2 cells growing in polarized monolayers. The estimated affinity for this transport was in the sub-micromolar range and thus probably not mediated by DMT1 [28]. In similar studies, it was found that depletion of cellular copper levels markedly increased uptake and overall transport of copper by Caco-2 cells [32]. No changes in DMT1 mRNA levels by copper could be observed [33], arguing for a different uptake route. In these studies, 10–1000 μ M ascorbate, which reduces Cu^{2+} to Cu^{+} , failed to significantly alter copper transport [32], suggesting that Cu^{2+} was being transported.

At variance with other studies, Arredondo et al. concluded that DMT1 is a physiologically relevant Cu^{+} transporter. This was based on the observation that apical copper uptake from 5 μ M ^{64}Cu -histidine in the presence of ascorbate was 47% inhibited by a DMT1 antisense oligonucleotide [27]. However, Fe^{2+} transport was inhibited by 80% under these conditions, suggesting that a substantial part of the copper transport was not mediated by DMT1. Conceivably, this additional copper transport was catalyzed by the ATP-driven copper transporter described here. Even in the presence of ascorbate, sufficient copper could disproportionate to Cu^{2+} and be transported in the presence of a high affin-

ity Cu^{2+} binding site. Indeed, the mode of intestinal absorption of copper may be similar to that of zinc, where several transporters appear to be at work, depending on the region of the intestine and the available zinc concentrations [34].

It had previously been shown that DMT1 transports metal ions in symport with protons. In principle, loading vesicles with ATP could activate a proton pumping ATPase. The electrochemical proton gradient thus generated could then couple ATP hydrolysis to copper-proton symport by DMT1. Such coupling would be abolished by protonophores and would only be possible in vesicles derived from wild-type rats or heterozygous Belgrade rats with functional DMT1. However, ATP-driven copper transport was essentially the same in wild-type, homo- and heterozygous Belgrade rats, and the protonophore CCCP stimulated, rather than inhibited, copper accumulation. The stimulation of ATP-driven copper uptake by an electrogenic protonophore suggests that the transport mechanism under observation is electrogenic, most likely transporting net charge (Cu^{2+}) to the vesicle lumen. However, this conclusion requires additional experimentation.

At physiological pH and in the presence of oxygen, iron and copper will be present in the oxidized iron(III) and copper(II) forms. Since DMT1 transports Fe(II), the iron will first have to be reduced [9,15,35]. Thus, corresponding reductases and oxidases working in concert with the transport proteins are required on either side of the membrane. A reductase, duodenal cytochrome *b* (Dcytb), that appears to be involved in iron and copper reduction in mice, has recently been described [36–38]. Dcytb is a b_{558} cytochrome with an apparent molecular weight of 33 kDa and shares 45–50% sequence similarity to the cytochrome b_{561} family of membrane reductases. Dcytb was suggested to act as dehydroascorbate reductase which provides luminal ascorbate for the reduction of iron and copper for absorption [36]. Such a reducing function could aid intestinal absorption of Cu(I), such as by Ctr1, but not absorption of Cu(II) [21]. In the purified membrane preparation employed here, we would not expect the presence of significant amounts of a reductant (e.g., ascorbate, NADH, and NADPH) which could fuel Dcytb or a similar reductase to catalyze the reduction of metal ions.

Two well-characterized copper transporters, Ctr1 and ATP7A, are also present in enterocytes. However, the properties of these transporters are quite different and make it highly unlikely that they are involved in the ATP-driven transport described here. First, Ctr1 has been shown to be independent of ATP [21], while the copper transport described here requires ATP. Second, Ctr1 apparently transports Cu(I), thus requiring reduction of the added Cu(II) to Cu(I) with ascorbate or a similar reductant, and has not been observed to transport other transition metal ions; the ATP-driven copper

transport is not specific for copper and also accepts cobalt and nickel, and possibly other divalent metal ions. The same line of arguments also applies to the Menkes copper ATPase, ATP7A. This ATP-driven copper pump transports Cu(I), not Cu(II), and is not known to transport other transition metal ions with the exception of Ag(I), which is a Cu(I) mimetic without a biological function. Also, ATP7A pumps copper in a direction opposite to the direction of transporter described here, namely from the cytoplasm into the Golgi-network, or from the cytoplasm to the extracellular space [22,23]. ATP7A-containing vesicles which could accumulate copper would have to have the ATP binding site on the vesicle outside. Since ATP has been removed from the extravascular space in our experiments, ATP7A in such vesicles would not “see” ATP and be inactive. Finally, if ATP-driven copper transport were due a minor contamination by an uncharacterized vesicle population, it would only result in marginal fluorescence quenching, maximally by the fraction of calcein present in such contaminating vesicles. However, we observed fluorescence quenching to the extent of 50–80%, indicating that the majority of vesicles participate in the ATP-driven transport reaction. Taken together, our results suggest the presence of a novel, ATP-driven high-affinity copper transporter in BBM vesicles. Its molecular identification will likely help to resolve some of the open issues regarding the mechanism of intestinal copper absorption.

Acknowledgments

We are grateful to Michael Garrick for his valuable help with Belgrade rats and many helpful suggestions. This work was supported by Grant 31-68075.02 from the Swiss National Foundation (M.S.) and a grant from the International Copper Association (M.S.).

References

- [1] J.R. Prohaska, A.A. Gybina, Intracellular copper transport in mammals, *J. Nutr.* 134 (2004) 1003–1006.
- [2] M.D. Garrick, M.T. Nunez, M. Olivares, E.D. Harris, Parallels and contrasts between iron and copper metabolism, *Biomaterials* 16 (2003) 1–8.
- [3] J.D. Gitlin, Wilson disease, *Gastroenterology* 125 (2003) 1868–1877.
- [4] E.D. Harris, Basic and clinical aspects of copper, *Crit. Rev. Clin. Lab. Sci.* 40 (2003) 547–586.
- [5] E. Luk, L.T. Jensen, V.C. Culotta, The many highways for intracellular trafficking of metals, *J. Biol. Inorg. Chem.* 8 (2003) 803–809.
- [6] J.F. Mercer, R.M. Llanos, Molecular and cellular aspects of copper transport in developing mammals, *J. Nutr.* 133 (2003) 1481S–1484S.
- [7] D.J. Thiele, Integrating trace element metabolism from the cell to the whole organism, *J. Nutr.* 133 (2003) 1579S–1580S.
- [8] N.C. Andrews, Metal transporters and disease, *Curr. Opin. Chem. Biol.* 6 (2002) 181–186.
- [9] H. Gunshin, B. Mackenzie, U.V. Berger, Y. Gunshin, M.F. Romero, W.F. Boron, S. Nussberger, J.L. Gollan, M.A. Hediger, Cloning and characterization of a mammalian proton-coupled metal-ion transporter, *Nature* 388 (1997) 482–488.
- [10] F. Canonne-Hergaux, S. Gruenheid, P. Ponka, P. Gros, Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron, *Blood* 93 (1999) 4406–4417.
- [11] S.M. Vidal, D. Malo, K. Vogan, E. Skamene, P. Gros, Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg, *Cell* 73 (1993) 469–485.
- [12] S. Gruenheid, M. Cellier, S. Vidal, P. Gros, Identification and characterization of a second mouse Nramp gene, *Genomics* 25 (1995) 514–525.
- [13] M.D. Garrick, K.G. Dolan, C. Horbinski, A.J. Ghio, D. Higgins, M. Porubcin, E.G. Moore, L.N. Hainsworth, J.N. Umbreit, M.E. Conrad, L. Feng, A. Lis, J.A. Roth, S. Singleton, L.M. Garrick, DMT1: a mammalian transporter for multiple metals, *Biomaterials* 16 (2003) 41–54.
- [14] D.L. Savigni, E.H. Morgan, Transport mechanisms for iron and other transition metals in rat and rabbit erythroid cells, *J. Physiol.* 508 (1998) 837–850.
- [15] M. Knöpfel, G. Schulthess, F. Funk, H. Hauser, Characterization of an integral protein of the brush border membrane mediating the transport of divalent metal ions, *Biophys. J.* 79 (2000) 874–884.
- [16] A. Sacher, A. Cohen, N. Nelson, Properties of the mammalian and yeast metal-ion transporters DCT1 and Smf1p expressed in *Xenopus laevis* oocytes, *J. Exp. Biol.* 204 (2001) 1053–1061.
- [17] M. Knöpfel, L. Zhao, M.D. Garrick, Transport of divalent transition-metal ions is lost in small-intestinal tissue of *b/b* Belgrade rats, *Biochemistry* 44 (2005) 3454–3465.
- [18] F. Canonne-Hergaux, A.S. Zhang, P. Ponka, P. Gros, Characterization of the iron transporter DMT1 (NRAMP2/DCT1) in red blood cells of normal and anemic mk/mk mice, *Blood* 98 (2001) 3823–3830.
- [19] M.D. Fleming, C.C. Trenor III, M.A. Su, D. Foernzler, D.R. Beier, W.F. Dietrich, N.C. Andrews, Microcytic anaemia mice have a mutation in *Nramp2*, a candidate iron transporter gene, *Nat. Genet.* 16 (1997) 383–386.
- [20] M.D. Fleming, M.A. Romano, M.A. Su, L.M. Garrick, M.D. Garrick, N.C. Andrews, *Nramp2* is mutated in the anemic Belgrade (b) rat: evidence of a role for *Nramp2* in endosomal iron transport, *Proc. Natl. Acad. Sci. USA* 95 (1998) 1148–1153.
- [21] J. Lee, M.M. Pena, Y. Nose, D.J. Thiele, Biochemical characterization of the human copper transporter Ctr1, *J. Biol. Chem.* 277 (2002) 4380–4387.
- [22] I. Voskoboinik, J. Camakaris, Menkes copper-translocating P-type ATPase (ATP7A): biochemical and cell biology properties, and role in Menkes disease, *J. Bioenerg. Biomembr.* 34 (2002) 363–371.
- [23] I. Voskoboinik, H. Brooks, S. Smith, P. Shen, J. Camakaris, ATP-dependent copper transport by the Menkes protein in membrane vesicles isolated from cultured Chinese hamster ovary cells, *FEBS Lett.* 435 (1998) 178–182.
- [24] M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Muller, G. Semenza, A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems, *Biochim. Biophys. Acta* 506 (1978) 136–154.
- [25] H. Hauser, K. Howell, R.M. Dawson, D.E. Bowyer, Rabbit small intestinal brush border membrane preparation and lipid composition, *Biochim. Biophys. Acta* 602 (1980) 567–577.

- [26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [27] M. Arredondo, P. Munoz, C.V. Mura, M.T. Nunez, DMT1, a physiologically relevant apical Cu^{1+} transporter of intestinal cells, *Am. J. Physiol. Cell. Physiol.* 284 (2003) C1525–C1530.
- [28] M. Arredondo, R. Uauy, M. Gonzalez, Regulation of copper uptake and transport in intestinal cell monolayers by acute and chronic copper exposure, *Biochim. Biophys. Acta* 1474 (2000) 169–176.
- [29] J.F. Eisses, J.H. Kaplan, Molecular characterization of hCTR1, the human copper uptake protein, *J. Biol. Chem.* 277 (2002) 29162–29171.
- [30] WHO, Guidelines for Drinking-Water Quality. Addendum to Volume 2, second ed., World Health Organization, Geneva, 1998.
- [31] L. Landner, L. Lindström, Copper in Society and in the Environment, Swedish Environmental Research Group (MFG), Västerås, 1999.
- [32] N.R. Zerounian, C. Redekosky, R. Malpe, M.C. Linder, Regulation of copper absorption by copper availability in the Caco-2 cell intestinal model, *Am. J. Physiol. Gastrointest. Liver Physiol.* 284 (2003) G739–G747.
- [33] M.C. Linder, N.R. Zerounian, M. Moriya, R. Malpe, Iron and copper homeostasis and intestinal absorption using the Caco2 cell model, *Biometals* 16 (2003) 145–160.
- [34] J. Condomina, T. Zornoza-Sabina, L. Granero, A. Polache, Kinetics of zinc transport in vitro in rat small intestine and colon: interaction with copper, *Eur. J. Pharm. Sci.* 16 (2002) 289.
- [35] M.E. Conrad, J.N. Umbreit, E.G. Moore, L.N. Hainsworth, M. Porubcin, M.J. Simovich, M.T. Nakada, K. Dolan, M.D. Garrick, Separate pathways for cellular uptake of ferric and ferrous iron, *Am. J. Physiol. Gastrointest. Liver Physiol.* 279 (2000) G767–G774.
- [36] M. Knöpfel, M. Solioz, Characterization of a cytochrome b_{558} ferric/cupric reductase from rabbit duodenal brush border membranes, *Biochem. Biophys. Res. Commun.* 291 (2002) 220–225.
- [37] A.T. McKie, P. Marciani, A. Rolfs, K. Brennan, K. Wehr, D. Barrow, S. Miret, A. Bomford, T.J. Peters, F. Farzaneh, M.A. Hediger, M.W. Hentze, R.J. Simpson, A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation, *Mol. Cell* 5 (2000) 299–309.
- [38] A.T. McKie, D. Barrow, G.O. Latunde-Dada, A. Rolfs, G. Sager, E. Mudaly, M. Mudaly, C. Richardson, D. Barlow, A. Bomford, T.J. Peters, K.B. Raja, S. Shirali, M.A. Hediger, F. Farzaneh, R.J. Simpson, An iron-regulated ferric reductase associated with the absorption of dietary iron, *Science* 291 (2001) 1755–1759.