Effects of copper on the expression of metal transporters in human intestinal Caco-2 cells

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Abstract Copper is an essential dietary trace metal, however the mechanisms involved in intestinal copper uptake are unclear. Two putative copper transporters are expressed in Caco-2 cells, the divalent metal transporter (DMT1) and copper transporter (Ctr1). Our data demonstrate that copper could compete with iron for uptake via DMT1 and that DMT1 protein and mRNA expression were decreased following exposure (24 h) to high copper. Expression of Ctr1, which acts as a copper transporter in transfected cell lines, was unaffected by copper treatment. Interestingly, exposure to copper increased iron efflux from Caco-2 cells and up regulated IREG1 (iron-regulated mRNA) expression. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: DMT1; IREG1; Ctr1; Copper; Caco-2 cell

1. Introduction

Copper is an essential trace metal required for a number of key physiological and biochemical functions in the body. Copper can exist in either the reduced (Cu^{+}) or oxidised (Cu^{2+}) state and can therefore act as an important co-factor in a number of fundamental redox reactions. However, due to this powerful redox activity, excess copper can lead to the production of the highly damaging hydroxyl radical. Nonetheless, copper-requiring proteins are widespread and are involved in a number of biological processes including oxidative phosphorylation (cytochrome *c* oxidase), cellular antioxidant status (superoxide dismutase) and oxidation of iron (ceruloplasmin). To maintain these essential processes, dietary copper intake must match endogenous copper losses (mainly via the bile) of 0.6–1.6 mg copper per day. Accordingly, copper is readily absorbed from the diet (up to 60% of the daily intake), mainly in the duodenum and early jejunum [1,2], and rapidly dispersed to the copper-requiring proteins. The precise mechanisms controlling copper uptake are unclear, but evidence suggests that they can be regulated at the intestinal level in line with the body's copper requirements, i.e. decreased absorption is observed following exposure to high copper levels, whilst absorption is increased by low copper intake [3].

Two high affinity copper transporters (Ctr1 and Ctr3) have been identified in the yeast *S. cerevisiae* [4,5], which have a K_m in the low micromolar range. Studies using complementation strategies with yeast Ctr mutants have identified candidate mammalian homologues of these proteins [6,7]. Human Ctr1 can functionally complement yeast lacking Ctr1 and Ctr3 [6], suggesting that the protein may function as a copper transporter in mammalian cell systems. Recently, mammalian Ctr1 has been shown to be ubiquitously expressed with the highest levels being found in the liver and kidney [6,7]. In support of a major role for Ctr1 in mammalian copper homeostasis, studies using Ctr1 knockout mice have shown that the homozygotes died in utero with severe developmental defects at the time of death, while heterozygotes have severely restricted tissue copper levels [8,9]. In addition, studies with Hek293 cells transfected with human Ctr1 have shown that the protein is expressed at the plasma membrane and acts as a copper transporter [10].

The divalent metal transporter (DMT1), a member of the Nramp (natural resistance-associated macrophage proteins) superfamily of proteins (reviewed in [11]), may represent an alternative copper uptake pathway since expression of DMT1 in *Xenopus* oocytes results in transport of several divalent metals including Fe^{2+}, Cd^{2+}, Cu^{2+} and Mn^{2+} [12]. The purpose of our current work was to study the expression of the putative copper transporters Ctr1 and DMT1 in the Caco-2 TC7 cell model of human intestinal epithelial cells and to investigate the effects of copper-supplemented medium on metal transporter mRNA.

2. Materials and methods

2.1. Materials

Radiochemicals and materials for Western blotting were supplied by Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK. Cell culture medium, heat-inactivated foetal bovine serum and plasticware were purchased from Life Technologies (Paisley, UK) unless stated. All other chemicals were of the highest grade available and bought from Sigma-Aldrich (Poole, Dorset, UK) or Merck (Poole, Dorset, UK).

2.2. Cell culture

Caco-2 TC7 cells were maintained in 25 cm^2 plastic flasks and cultured in a 90% air/10% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium, supplemented with 20% heat-inactivated foetal bovine serum. All experiments were carried out on cells between passage numbers 32 and 42. For experiments, cells were seeded at a density of 1×10^4 cells/cm^2 onto Transwell inserts (Costar UK, Buckinghamshire, UK) to measure iron transport, or 75 cm^2

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Abbreviations: DMT1, divalent metal transporter; IREG1, iron-regulated mRNA; IRE, iron responsive element; UTR, untranslated region; RT-PCR, reverse transcription-polymerase chain reaction; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid

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tissue culture flasks for isolation of plasma membrane protein and total RNA, and used 21 days later. Cells were incubated in the presence or absence of 100 μM Cu^{2+} for the final 24 h of the culture period to determine the effects of increased copper on metal transporter expression.

2.3. Transepithelial iron flux across Caco-2 TC7 cell monolayers

The measurement of iron uptake by Caco-2 TC7 cells has been described previously [13]. The integrity of cell monolayers was determined for each well before all uptake experiments by measuring transepithelial resistance (TER, measured as Ω.cm^2), using a Millicell electrical resistance system (Millipore UK Ltd, Watford, UK). Cell monolayers with a TER of less than 200 Ω.cm^2 were excluded from experiments. Transepithelial pH gradients were produced using HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid)-buffered salt solution (HBSS, pH 7.5; 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, 0.2% bovine serum albumin) in the basolateral chamber, with either HBSS or MBSS (pH 5.5 – substituting 2-(*N*-morpholino)-ethanesulphonic acid for HEPES) added to the apical chamber. Uptake was initiated by the addition of 1 or 10 μM $^{55}Fe^{2+}$ complexed with 1 mM ascorbic acid (prepared freshly prior to the start of each experiment) to the apical chamber and terminated after 60 min. Cells were washed three times in ice cold transport buffer containing a 10-fold excess of iron, solubilised overnight in 200 mM NaOH, and finally subjected to scintillation counting to determine cell uptake. Aliquots of the basolateral buffer were taken to measure transport across the Caco-2 TC7 epithelium. Parallel experiments in which [^{14}C]mannitol was substituted for ^{55}Fe were performed to distinguish the passive transport component.

2.4. Western blot analysis

Following removal of culture medium, cell monolayers were washed twice in phosphate buffer and harvested using a cell scraper. Total plasma membranes (apical and basolateral pooled) were prepared as described previously [14] and used for Western blotting.

Cell membranes (20 μg) were solubilised in sample buffer and subjected to 10% SDS–PAGE. The proteins were transferred onto nitrocellulose (Hybond ECL – Amersham Pharmacia Biotech, Buckinghamshire, UK) and blocked overnight in phosphate buffer containing 0.05% Tween 20 and 1% fat free milk. The nitrocellulose was incubated for 2 h at room temperature with either a polyclonal antibody (1:250 dilution) raised in rabbit against a synthetic peptide corresponding to amino acids 310–330 of the human DMT1 sequence, common to both isoforms, or commercially available antibodies to iron-regulated mRNA (IREG1) (1:500 dilution, Alpha Diagnostics International, TX, USA) or villin (1:250 dilution, Santa Cruz Biotechnology Inc, CA, USA). Following removal of the primary antibody, a secondary anti-IgG antibody (horseradish peroxidase-labelled) was used, and cross reactivity visualised using ECL Plus and Hyperfilm ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Band densities were semi-quantified by densitometric analysis using Scion Image software (Scion Corporation, MD, USA).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from Caco-2 TC7 cells using Trizol reagent (Life Technologies, Paisley, UK) and stored at −70°C in 75% ethanol until required. RT-PCR was performed in a single step reaction, using Ready-to-go RT-PCR Beads (Amersham Pharmacia Biotech) on total RNA samples (1 μg per tube) using the following primer sequences: β-actin 5′-CCAAGGCCAACCGCGAGAAGAT-GAC-3′ (forward), 5′-AGGGTACATGGTGGTGCCGCCAGAC-3′ (reverse); DMT1 (+IRE) 5′-AGTGGTTTATGTCCGGGACC-3′ (forward), 5′-TTTAACGTAGCCACGGGTGG-3′ (reverse); DMT1 (non-IRE) 5′-TCTAGATGACTGACAGCC-3′ (forward), 5′-CCAA-GACACAAGCCCATA-3′ (reverse); IREG1 5′-ATTGCTGCTAGA-ATCGGTCT-3′ (forward), 5′-AGACTGAAATCAATACGAGC-3′ (reverse); Ctr1 5′-TAAGATTCGGAGAGAGAGGTGC-3′ (forward), 5′-AGGCTCTCTCGGGCTATCTT-3′ (reverse).

The cDNA transcript was produced by incubation at 42°C for 30 min. PCR (28 cycles) was in a Hybaid Omn-E thermal cycler (Hybaid Ltd, Middlesex, UK). PCR products were stained with ethidium bromide on a 2% agarose gel and visualised using Fluor-S MultiImager (Bio-Rad Laboratories Ltd, Hertfordshire, UK), and bands analysed using Scion Image analysis software. Transporter mRNA was normalised to β-actin expression.

2.6. Data analysis

Data are presented as the mean ± S.E.M. Statistical analysis was carried out using SPSS statistics package, and utilised one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test, or Student's unpaired *t*-test where appropriate. Differences were considered significant at $P < 0.05$.

3. Results and discussion

As copper is a redox active metal and can cause cytotoxicity and membrane damage, the integrity of all cell monolayers was determined immediately prior to commencing transport experiments by measuring TER. In our studies there was no significant difference between the TER of control cells (256 ± 41 Ω.cm^2) or copper-treated cells (231 ± 26 Ω.cm^2). All wells with a TER of less than 200 Ω.cm^2 were discarded from the experimental protocol. There was no difference in paracellular permeability, estimated by [^{14}C]mannitol transfer from the apical medium to the basolateral chamber, between control and copper-treated cells (data not shown) indicating that the monolayer remained intact throughout our studies. This is in contrast with previous studies showing that high copper can decrease Caco-2 cell monolayer integrity [15]. These differences could well reflect our use of a more differentiated (in terms of a number of small intestinal markers) Caco-2 cell subclone [16].

DMT1 expressed in *Xenopus* oocytes is capable of transporting Fe^{2+}, Cd^{2+}, Cu^{2+} and Mn^{2+} with equal affinity via an electrogenic, voltage- and proton-dependent mechanism [12]. However, more recent work has shown that DMT1 is predominantly an iron transporter with lower affinity for other metals [13,17–19]. In our current study, we have demonstrated that addition of a 100-fold excess of copper could reduce iron uptake by 50% ($P < 0.05$), suggesting that copper has significant affinity for this transport system (Fig. 1). The interaction between the biology of copper and iron is well recognised, particularly in the transport of iron across the intestinal barrier. A recent study in which Caco-2 cells were supplemented with copper (1 μM) for the final 7–8 days prior to experimentation demonstrated enhanced apical iron uptake and DMT1 expression [20]. Interestingly, over the 24 h time

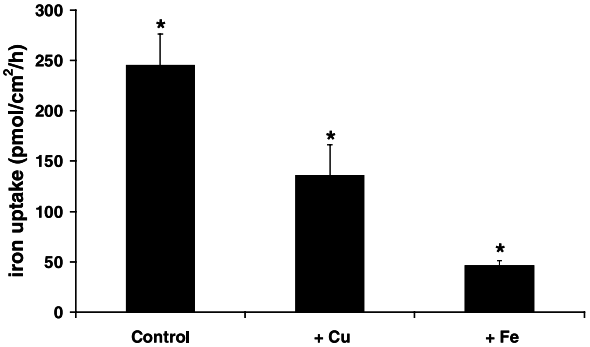

Fig. 1. Competition between copper and iron for uptake at the apical membrane of Caco-2 TC7 cells. Uptake of 1 μM $^{55}Fe^{2+}$ was significantly inhibited in the presence of 100 μM unlabelled iron ($P < 0.001$) or unlabelled copper ($P < 0.05$) compared with control (ANOVA and Scheffe's post hoc test). Data are means ± S.E.M. of four observations. * indicates that all experimental groups are significantly different from each other ($P < 0.05$).

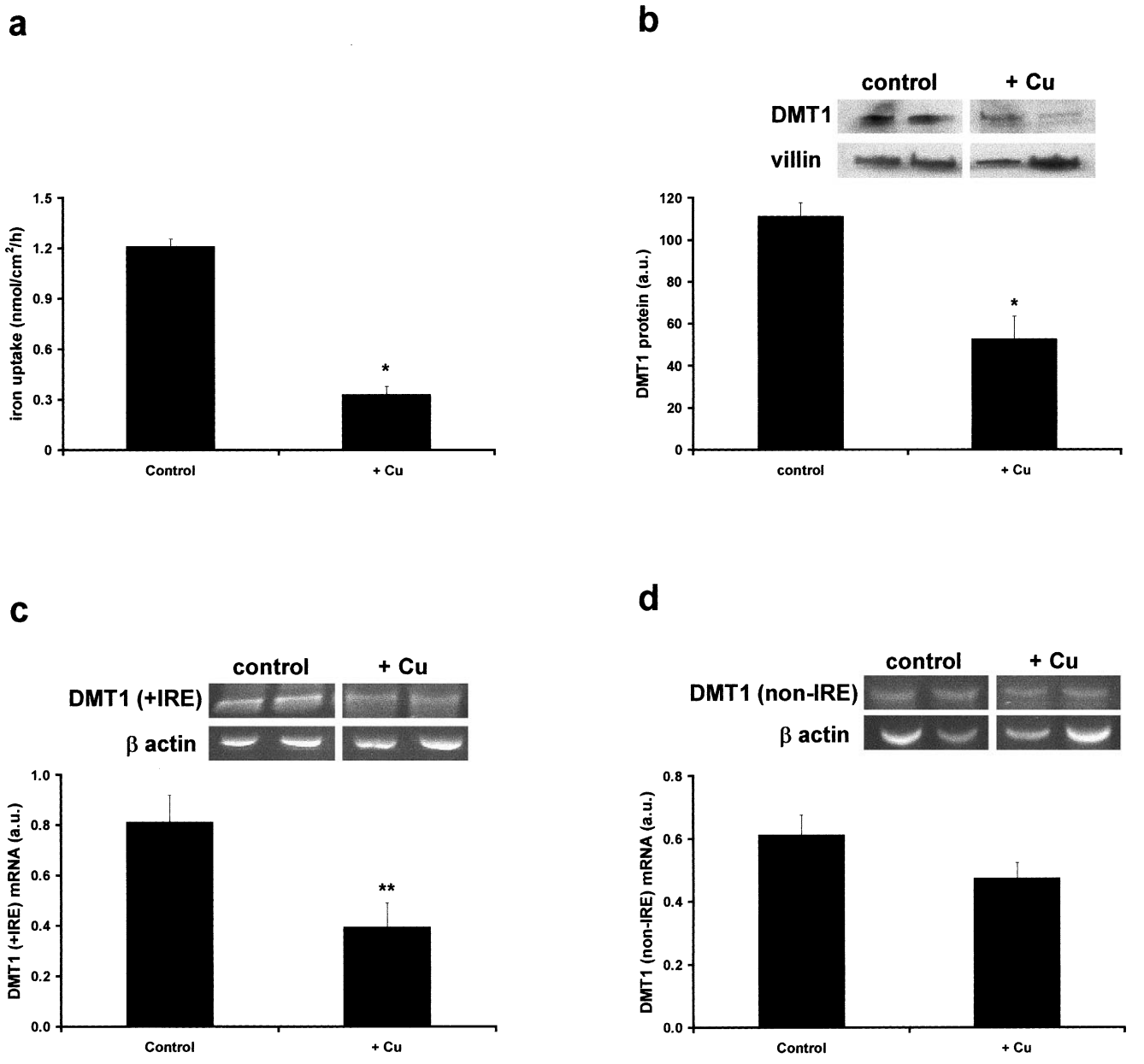

Fig. 2. Effect of copper on DMT1 function and expression. Caco-2 TC7 cells were incubated in the presence of 100 μM copper for the final 24 h of the culture period. pH-dependent Fe^{2+} uptake across the apical membrane (a) and DMT1 protein levels (b) (detected on Western blots as a 66 kDa band) in plasma membranes of Caco-2 TC7 cells were significantly decreased by high copper (*$P < 0.01$, Student's unpaired *t*-test). DMT1 (+IRE) mRNA levels were significantly decreased by exposure to high copper for 24 h (**$P < 0.05$, Student's unpaired *t*-test) (c), whereas DMT1 (non-IRE) mRNA levels were unaffected by copper treatment (d). Data are mean ± S.E.M. of four to six separate experiments. Two representative bands from control and copper-treated cells are shown above the data bars for DMT1 and for villin (b) and β-actin (c and d) in the same cell samples to indicate equal loading of protein and RT-PCR products onto gels. Copper treatment did not affect villin or β-actin densities compared with control cells.

period we found no significant effect of 1 μM copper on any of the variables measured in our study (data not shown).

To determine the effect of copper loading on the function and expression of the apical membrane resident transporter, DMT1, Caco-2 TC7 cells were incubated for the final 24 h of the culture period in the presence of 100 μM $CuCl_2$. Following this manoeuvre, pH-dependent iron uptake was significantly decreased ($P < 0.01$) compared with control cells (Fig. 2a). Taken together, the uptake data (Figs. 1 and 2a) are consistent with the hypothesis that DMT1 is not only an iron transporter but may also act as a copper transport pathway [12]. Ongoing studies in our laboratory are investigating copper transport kinetics via DMT1. The effects of copper loading on DMT1 protein and mRNA expression were also determined. There are two functional splice variants of DMT1, one contains a single iron responsive element (IRE) in the 3′ untranslated region (UTR), which can bind iron regulatory proteins (IRPs) in vitro [21,22], whereas the second isoform lacks this 3′ IRE. In agreement with our uptake data, DMT1 protein expression (our antibody recognises both DMT1 isoforms) was decreased following 24 h copper treatment (Fig. 2b). Interestingly, exposure to copper significantly

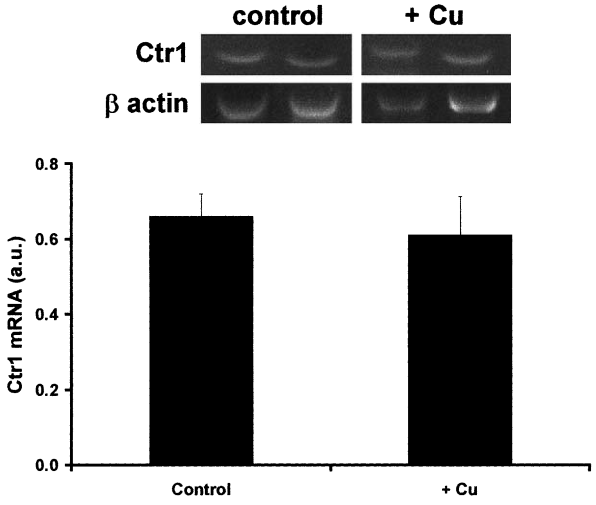

Fig. 3. Effects of copper on Ctr1 mRNA expression. mRNA expression of the putative copper transporter Ctr1 in Caco-2 TC7 cells was unaltered following exposure to high copper for the final 24 h of the culture period. Data are mean ± S.E.M. of six observations in each group. β-Actin bands show equal loading of RT-PCR products from control and copper-treated cells. Band density was not different in the two groups of cells (data not shown).

reduced DMT1 (+IRE) mRNA (Fig. 2c) but had no effect on DMT1 (non-IRE) mRNA (Fig. 2d). Intestinal copper uptake is known to be down regulated following exposure to high dietary copper levels [3]. The copper-mediated decrease in DMT1 expression gives further credence to a role for the transporter in copper absorption. Previous work in our laboratory has shown that DMT1 mRNA expression is regulated by other divalent metals including iron [23,24] and zinc [19]. In agreement with the current copper data, exposure to iron decreases DMT1 (+IRE) mRNA but does not change in the non-IRE isoform [24]. Taken together, these data suggest that the molecular information required for the regulation of DMT1 by copper and iron resides in the 3′ UTR since both DMT1 isoforms share the same 5′ promoter [25]. Support for this hypothesis is provided by recent data in human hepatoma cells demonstrating that copper decreases IRP1/IRE binding, possibly by replacing the labile fourth position iron in the iron–sulphur cluster of IRP1, and down regulates transferrin receptor mRNA [26].

Ctr1 represents an alternative copper uptake pathway in Caco-2 TC7 cells. Ctr1 mRNA was expressed in this cell

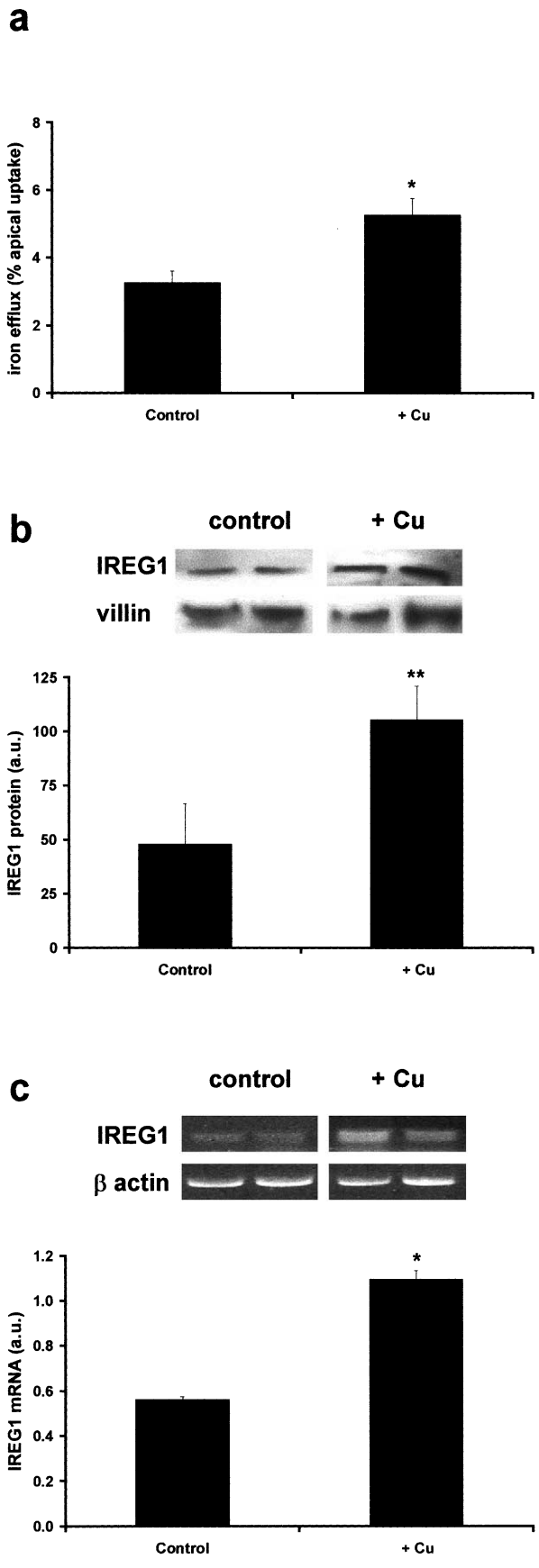

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Fig. 4. Effects of copper on iron transport across Caco-2 TC7 cell monolayers and IREG1 expression. Iron transport, expressed as a percentage of apical uptake, was significantly higher following exposure to copper (100 μM) compared with control cells (*$P < 0.02$ Student's unpaired t-test) (a). Data are means ± S.E.M. of six observations in each group. Protein (b) and mRNA (c) expression of the basolateral iron transporter, IREG1, were both significantly increased by exposure to copper, compared with control (**$P < 0.05$; *$P < 0.01$ Student's unpaired t-test). Data are presented as means ± S.E.M. of four to six separate determinations in each group. Two representative bands from control and copper-treated cells run on the same gel are shown above the data bars for each IREG1 and for villin (b) and β-actin (c) in the same cell samples to show equal loading of protein and RT-PCR products onto gels. Copper treatment did not affect villin or β-actin densities compared with control cells.

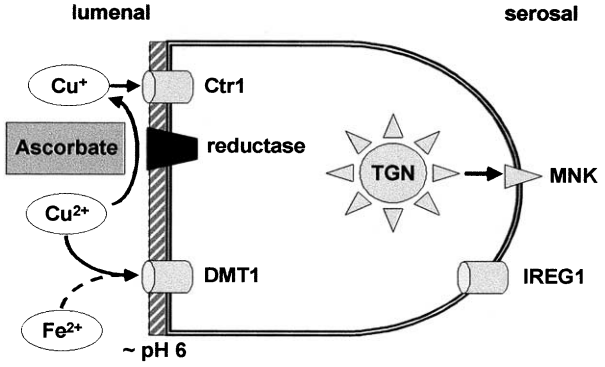

Fig. 5. Working model for the absorption of dietary copper by intestinal enterocytes. Copper, present in the diet as Cu^{2+}, could be absorbed across the apical membrane of enterocytes as either Cu^{2+} via DMT1 (i.e. utilising a common pathway with iron) or reduced (by endogenous reductases and dietary components such as ascorbate) to Cu^{+} prior to uptake by Ctr1. Both of these transport processes are coupled to the proton gradient that exists across the enterocyte apical membrane. Absorbed copper is immediately bound by intracellular chaperones and directed to the *trans*-Golgi network (TGN) and is ultimately exported from the cell via the Menke's ATPase (MNK). Iron, also absorbed via DMT1, is released across the basolateral membrane of enterocytes via its own specific transporter, IREG1.

line but, unlike DMT1, mRNA levels were not influenced by incubation with copper-loaded medium (Fig. 3). These findings are consistent with previous studies with rats, which despite being fed a high copper diet showed no regulation of Ctr1 expression in either the liver or the intestine [7]. However, Ctr1 does function as a copper transporter and is expressed at the plasma membrane of a transfected human cell line [10]. It is possible that transport function and protein expression could be regulated by copper at the post-translational level, similar to the situation that occurs in yeast [4]. Clearly, despite the evidence that Ctr1 can function as a transporter, further studies are necessary to determine the role of Ctr1 in intestinal copper homeostasis.

Previous studies have shown that copper deficiency results in tissue retention of iron [27,28] and in many cases leads to a microcytic hypochromic anaemia similar to that seen in iron deficiency [28], which can be normalised by administration of copper but not iron [29]. Hence, there seems to be a reciprocal arrangement between cellular copper and iron status. To test the hypothesis that high copper levels should increase iron efflux from Caco-2 TC7 cells, we measured accumulation of ^{55}Fe in the basolateral medium. Following exposure to copper, iron efflux (when normalised to the level of uptake across the apical membrane) increased by approximately 40% (Fig. 4a). A key component in the cellular release of iron is the basolateral iron transporter IREG1 [30] – also known as ferroportin 1 and MTP1 [31,32]. Intriguingly, and in agreement with our efflux data, exposure to high levels of copper increased both IREG1 protein and mRNA expression in Caco-2 TC7 cells (Fig. 4b,c). Previous studies have shown that IREG1 mRNA is increased in response to changes in body iron status [30,31] and following exposure to high levels of zinc [19]. The mechanisms involved in the regulation of IREG1 by metal ion status are unclear but may be related to regulatory elements in the IREG1 promoter.

In summary, two possible copper transporters DMT1 and Ctr1 are expressed in the Caco-2 TC7 cell model of human intestinal epithelial cells (Fig. 5). The relative contribution of these proteins to intestinal copper homeostasis at this time is unclear. Whilst evidence points towards DMT1 acting as a transporter of divalent metals [12,13], Ctr1 appears to transport monovalent ions including Cu (I) and Ag (I) [10]. Since copper is largely present as Cu (II) in the diet, it would first have to be reduced to Cu (I) to be transported via Ctr1. There are numerous mechanisms potentially capable of reducing copper including endogenous plasma membrane reductases [33,34] as well as dietary components such as ascorbate. However, previous work has suggested that reduction of dietary copper into its cuprous state (for example, by ascorbate) inhibits absorption [35]. Clearly there are several issues relating to copper bioavailability that remain to be resolved. Current work in our laboratory is attempting to delineate between these two potential copper transporting mechanisms and should determine the relative role of each in maintaining intestinal copper homeostasis.

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