Rapid regulation of divalent metal transporter (DMT1) protein but not mRNA expression by non-haem iron in human intestinal Caco-2 cells

Paul Sharp[a,*], Sarah Tandy[b], Sachie Yamaji[c], Jason Tennant[a], Mark Williams[b], Surjit Kaila Singh Srai[c,1]

[a] *Centre for Nutrition and Food Safety, School of Biomedical and Life Sciences, University of Surrey, Guildford GU2 7XH, UK*
[b] *School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK*
[c] *Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School, London NW3 2PF, UK*

Received 14 September 2001; revised 20 November 2001; accepted 21 November 2001

First published online 4 December 2001

Edited by Veli-Pekka Lehto

Abstract A divalent metal transporter, DMT1, located on the apical membrane of intestinal enterocytes is the major pathway for the absorption of dietary non-haem iron. Using human intestinal Caco-2 TC7 cells, we have shown that iron uptake and DMT1 protein in the plasma membrane were significantly decreased by exposure to high iron for 24 h, in a concentration-dependent manner, whereas whole cell DMT1 protein abundance was unaltered. This suggests that part of the response to high iron involved redistribution of DMT1 between the cytosol and cell membrane. These events preceded changes in DMT1 mRNA, which was only decreased following 72 h exposure to high iron.

Key words: Divalent metal transporter; Iron transport; Caco-2 cell

1. Introduction

Iron is an essential dietary trace metal due to its role in numerous biochemical processes in the body. However, excess iron is extremely toxic to cells and organs and, coupled with the fact that mammals do not posses a defined iron excretory pathway, this means that the intestinal enterocytes play a key role in the control of iron homeostasis. Iron enters the body from the diet across the apical membrane of duodenal enterocytes via the divalent metal transporter, DMT1, previously DCT1/Nramp2 [1,2]. Previous work has shown that this transporter is pH-dependent and ferrous iron-specific [1,3].

The duodenal crypt–villus axis represents a differentiation pathway and is furnished with a heterogeneous population of cells. DMT1 expression along the crypt–villus length is also heterogeneous with the absorptive cells, in the upper villus region, expressing the highest transporter levels [4–6]. Despite the obvious importance of DMT1 in maintaining body iron homeostasis, relatively little is known concerning the regulation of this transport pathway in intestinal cells by dietary iron. Data from animals fed on an iron-restricted diet have shown that iron transport [7] and DMT1 expression [1] are increased. In addition, a very recent study also suggests that DMT1 is up-regulated in human patients with iron deficiency and hereditary haemochromatosis [4]. However, it is unclear whether these changes in iron transporter expression are due to pre-programming in the crypts in response to the prevailing levels of the body iron stores, or whether they occur during the journey along the crypt–villus axis as a result of changes in local (i.e. dietary) iron levels (reviewed in [8]). In our current study, using human intestinal Caco-2 TC7 cells, we have addressed this issue by investigating the effect of 'dietary' non-haem iron on DMT1 expression and iron transport function during cellular differentiation.

2. Materials and methods

2.1. Cell culture

Stock cultures of Caco-2 TC7 cells (obtained from Drs Monique Rousset and Edith Brot-Laroche, INSERM U505, Paris, France) 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 30–40. 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 tissue culture flasks for isolation of plasma membrane protein and total RNA. Experiments were carried out on either day 7, 10, 14 or 21 following seeding. In some experiments, cells were incubated with 10–100 μM Fe^{3+} for the final 24 or 72 h of the culture period to determine the effects of increased iron on DMT1 function and expression.

2.2. Iron uptake by Caco-2 TC7 cell monolayers

The measurement of iron uptake by Caco-2 TC7 cells has been described previously [3]. Briefly, transepithelial pH gradients were produced using *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) 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 3–100 μM Fe^{2+} complexed with 1 mM ascorbic acid (prepared freshly prior to the start of each experiment) and 37 kBq/ml^{55} $FeCl_3$ to the apical chamber and terminated after 60 min. Cells were washed three times in ice

*Corresponding author. Fax: (44)-1483-576978.
E-mail addresses: p.sharp@surrey.ac.uk (P. Sharp), ksrai@rfc.ucl.ac.uk (S.K. Singh Srai).

[1] Also corresponding author. Fax: (44)-20-77949645.

Abbreviations: DMT1, divalent metal transporter; IRP, iron regulatory protein; IRE, iron responsive element; UTR, untranslated region; RT-PCR, reverse transcriptase polymerase chain reaction; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; TfR, transferrin receptor

0014-5793/02/$22.00
PII: S0014-5793(01)03225-2

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.

2.3. 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 [10] and used for Western blotting.

Cell membranes (20 μg) were solubilised in Laemmli buffer [11] and subjected to 7.5% 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, which recognises both DMT1 splice variants, or commercially available antibodies to transferrin receptor (TfR; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and villin (Santa Cruz). 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). Villin levels were used to demonstrate equal protein loading onto gels.

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from Caco-2 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: GAPDH 5′-GCCATCAATGACCCCTTCAT-3′ (forward) and 5′-GAGGGGGCAGAGATGATGAC-3′ (reverse); DMT1 5′-GGTGTTGTGCTGGGATGTTA-3′ (forward) and 5′-AGTACATATTGATGGAACAG-3′ (reverse). DMT1 primers recognised both splice variants. The cDNA transcript was produced by incubation at 42°C for 30 min. PCR was performed by 28 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 1 min, followed by a final single extension at 72°C for 10 min in a PTC-100 thermal cycler (MJ Research, NV, USA). PCR products were stained with ethidium bromide on a 2.5% agarose gel and visualised using Fluor-S MultiImager (Bio-Rad Laboratories Ltd., Hertfordshire, UK), and bands were analysed using MultiAnalyst (Bio-Rad) image analysis software. DMT1 mRNA levels were normalised to GAPDH expression.

2.5. Data analysis

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

2.6. Materials

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

3. Results and discussion

The enterocytes that line the duodenum are produced as a result of stem cell division and proliferation in the crypts of Lieberkühn, and migrate onto the villus where they differentiate, taking on their absorptive function in the upper villus regions before finally undergoing cell death at the villus tip. To study cellular events in defined populations of cells undergoing differentiation we have employed the Caco-2 TC7 cell model, using undifferentiated proliferative cells (day 7) and cells in the initial (day 10), intermediate (day 14) and terminal (day 21) stages of differentiation. The advantage of the cell culture approach is that we can measure changes in iron transporter mRNA and protein expression as well as associated regulation of transport function in defined populations of cells during the differentiation process. However, in interpreting these data, it is important to remember that Caco-2 cells are derived from an adenocarcinoma of the colon and as such are not identical to normal duodenal enterocytes. Characterisation of the Caco-2 TC7 subclone during the transition from a proliferative to a fully differentiated phenotype has been performed extensively by others [9,12], and in our hands is associated with an increase in the expression of the brush border membrane structural protein villin (Fig. 1a) and a decrease in the proliferative cell marker TfR (Fig. 1b).

DMT1 mRNA (normalised to GAPDH expression) was expressed in Caco-2 TC7 cells at all stages of differentiation,

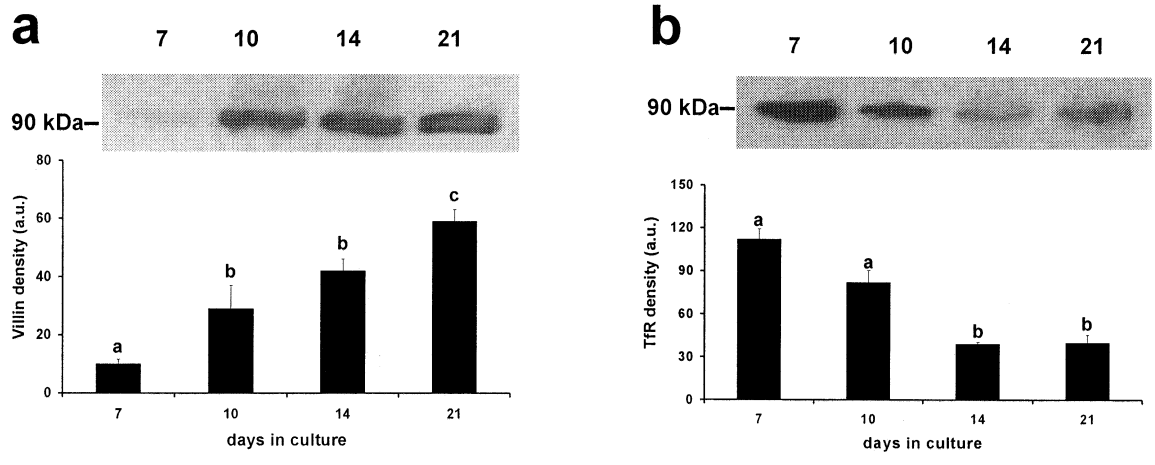

Fig. 1. Differentiation of intestinal epithelial cells. Development of the brush border membrane during differentiation is associated with an increase in the expression of the structural protein, villin (a), but a decrease in the expression of the proliferative cell marker, TfR (b). Data are the means ± S.E.M. of three separate experiments. Different letters above data bars indicate that these groups are statistically different from each other ($P < 0.05$).

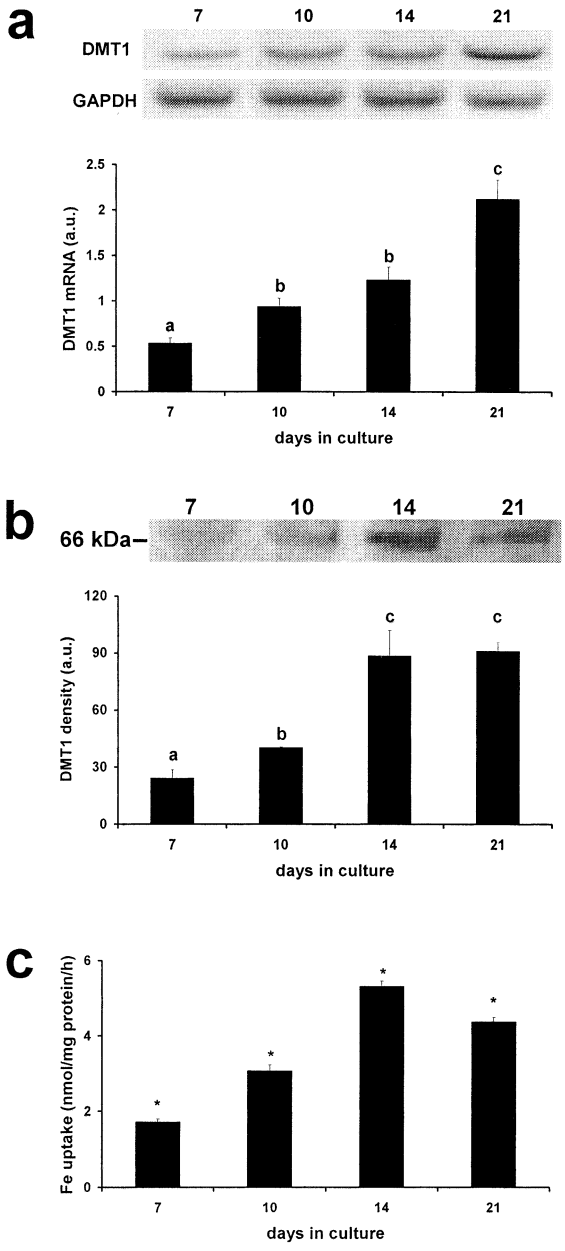

Fig. 2. Differentiation-dependent regulation of DMT1 expression and function in Caco-2 TC7 cells. DMT1 mRNA levels in arbitrary units (normalised to GAPDH expression) increased during differentiation (a). This pattern of expression was also seen in relation to DMT1 protein levels (b) and pH-dependent iron uptake (c) that were highest in fully differentiated cells. Data are mean ± S.E.M. of three to six experiments. Different letters above data bars in (a) and (b) indicate that these groups are statistically different from each other ($P < 0.05$). In (c), $^*P < 0.05$ indicates that all groups are statistically different from each other.

reaching maximal levels at day 21 (one-way ANOVA and Scheffe's post-hoc test, $P < 0.05$; Fig. 2a). While a similar increase in DMT1 mRNA with time has been noted previously in post-confluent Caco-2 cells [13], our study also found DMT1 mRNA in pre-confluent undifferentiated proliferating cells (day 7). This finding is consistent with the key requirement for DMT1 in cellular accumulation of iron from transferrin via the endosomal pathway [5,14–17]. In accordance with the observed expression of DMT1 mRNA, both DMT1 protein (Fig. 2b) and pH-dependent iron transport (Fig. 2c) showed lowest activity in the proliferating cells (day 7) and highest activity in the differentiated cells (days 14 and 21). Despite the low abundance of DMT1 in proliferative Caco-2 TC7 cells, expression was associated with measurable pH-dependent iron uptake. Interestingly, in rat duodenal crypt cells, we have also observed membrane expression of DMT1 protein as well as evidence for iron uptake in this region [18].

To investigate further the relationship between extracellular (i.e. dietary) non-haem iron and the functional expression of DMT1, we exposed Caco-2 TC7 cells at various stages of differentiation to high iron (100 μM) for the final 24 h of the culture period. DMT1 protein levels in the plasma membrane were significantly decreased at days 10, 14 and 21 (though not at day 7; Fig. 3a) in the iron-treated cells compared with controls (Student's t-test, $P < 0.05$). In addition, pH-dependent iron uptake across the apical membrane was significantly reduced at all stages of differentiation by exposure to high iron (Fig. 3b). By analogy with the duodenum, these data suggest that all cells along the crypt–villus differentiation axis could have the capacity to respond to changes in the local dietary environment, and this may be important in optimising dietary iron absorption. Interestingly, incubating cells with 100 μM iron for 24 h did not significantly alter DMT1 mRNA levels at any stage during the differentiation process (Fig. 3c). These findings are in agreement with a previous study in mouse fibroblasts where following 20 h exposure to ferric ammonium citrate iron uptake and DMT1 mRNA expression were unaltered [19]. There are two functional splice variants of DMT1, one contains a single iron responsive element (IRE) in the 3′-untranslated region (UTR) [1,19], which can bind iron regulatory proteins (IRP) in vitro [19], whereas the second isoform lacks this 3′-IRE. Our own data (not shown) and work by other groups [5,20,21] suggest that the IRE containing isoform predominates in intestinal tissue. By analogy with the TfR, which contains five IREs in the 3′-UTR, it has been suggested that exposure to high iron should reduce IRP/IRE binding and thereby decrease DMT1 mRNA stability. However, it is important to note that for the TfR, IRP interaction with at least three IREs is essential for the regulation of mRNA stability [22] and consequently, DMT1 mRNA may be less sensitive to changes in iron status than TfR and could take longer to respond to alterations in the cellular environment. In support of this hypothesis, following 72 h exposure to high iron, DMT1 mRNA levels in fully differentiated (i.e. 21 day) Caco-2 TC7 cells were reduced by 50% (Fig. 3d). In addition to cellular iron levels, it is clear than other factors can regulate DMT1 expression, including other dietary metals [23], changes in cellular nitric oxide levels [19] and the presence of inflammatory cytokines [24]. These events may occur as a result of the presence of a number of response motifs in the 5′-regulatory region of DMT1, highlighting the importance of this region in controlling transporter expression.

Further investigation revealed that both iron uptake (Fig. 4a) and DMT1 protein expression (Fig. 4b) were decreased by 24 h exposure to iron in a dose-dependent manner. Both parameters were significantly lower than the respective controls at iron concentrations greater than 20 μM. Importantly, this means the DMT1 expression and transport function can be

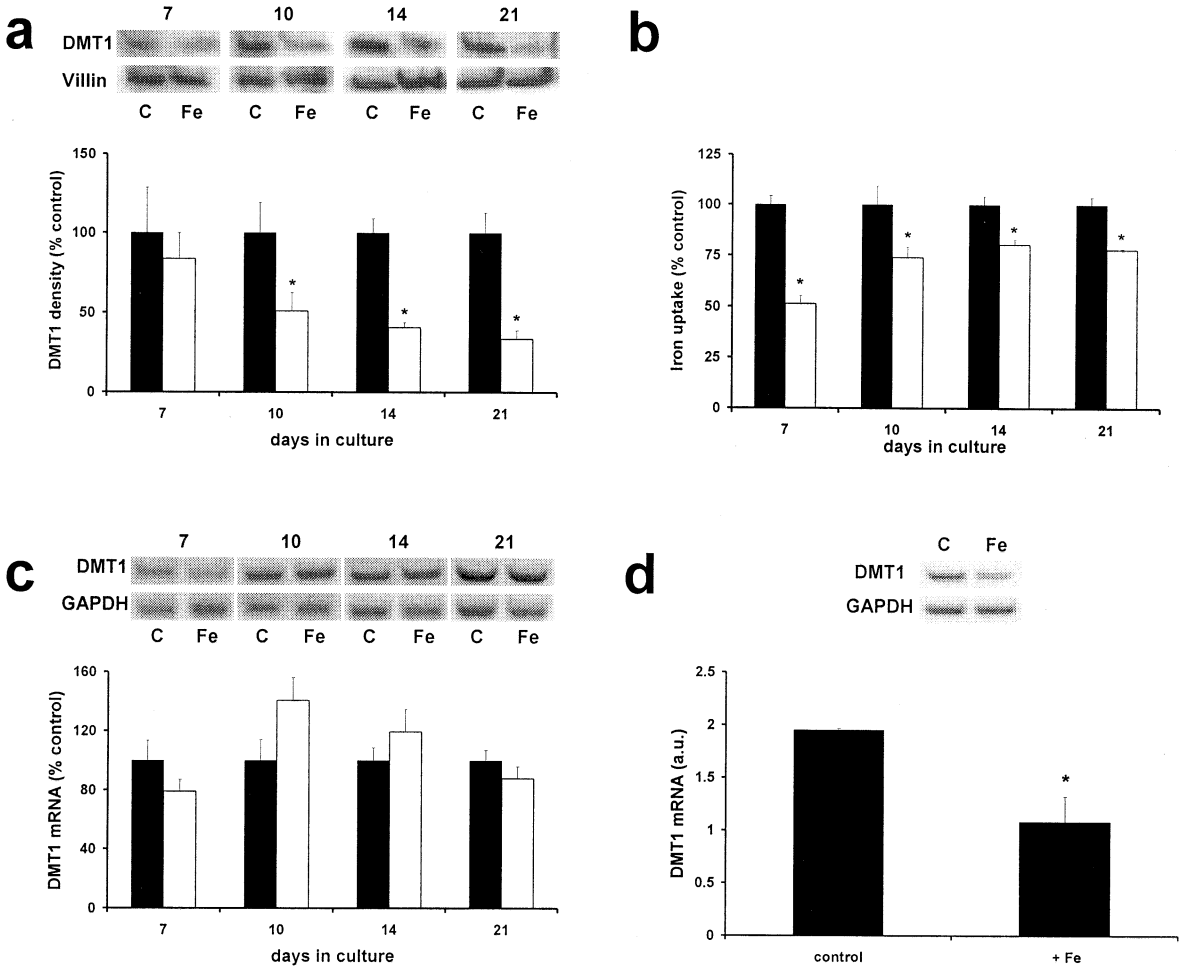

Fig. 3. Effect of exposure to high iron on DMT1 function and expression. Caco-2 TC7 cells were incubated in the presence of 100 μM iron for the final 24 h of the culture period. DMT1 protein levels in plasma membranes (a) and pH-dependent Fe^{2+} uptake across the apical membrane (b) of Caco-2 TC7 cells were significantly decreased by high iron. DMT1 mRNA levels were unaffected by exposure to high iron for 24 h (c), but were significantly decreased following 72 h iron treatment (d). Data are mean ± S.E.M. of three to six experiments. $*P < 0.05$ (Student's unpaired *t*-test). Villin (a) and GAPDH (c and d) show equal loading of protein and RT-PCR products, respectively, from control and iron-treated cells.

regulated by physiologically relevant levels of iron found in the diet. It is interesting to note that the magnitude of the decrease in iron uptake was much less than the decrease in DMT1 protein in the cell membrane following iron treatment. This may indicate that, in addition to decreasing DMT1 protein expression per se, exposure to iron could activate other cellular mechanisms that alter the intrinsic activity of the transporters remaining in the cell membrane. It is also possible that membrane transporters other than DMT1 contribute to non-haem iron uptake. Our recent work has postulated that a common uptake pathway for iron and zinc, distinct from DMT1, exists in the apical membrane of Caco-2 cells [23]. In addition, a Fe^{3+}-specific transporter has been identified in K562 erythroleukaemia cells [25].

The large decrease in membrane expression of DMT1 protein, without parallel changes in mRNA suggests that these events are post-translational and their nature remains to be fully determined. Interestingly, we have found that although membrane levels of DMT1 protein are reduced following high iron, whole cell levels of the protein remain unchanged (Fig. 5a,b), suggesting that one regulatory mechanism might involve re-distribution of transporters between the cytosol and the plasma membrane. In support of this hypothesis, changes in the cellular localisation of DMT1 have been noted in duodenal enterocytes from control and iron-loaded animals [6] and this may involve rapid internalisation of DMT1 into cytosolic vesicles [26], following ingestion of dietary iron.

In conclusion, iron uptake by Caco-2 TC7 cell monolayers and plasma membrane DMT1 protein levels are down-regulated rapidly (within 24 h) following exposure to high non-haem iron levels at the apical membrane. These iron-dependent events occur at all stages of differentiation and intriguingly cannot be accounted for by changes in mRNA expression. We are currently investigating the nature of these regulatory mechanisms.

Acknowledgements: This study was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) Grant number 90/D13400. S.R.T. was funded by a BBSRC Research Committee Studentship.

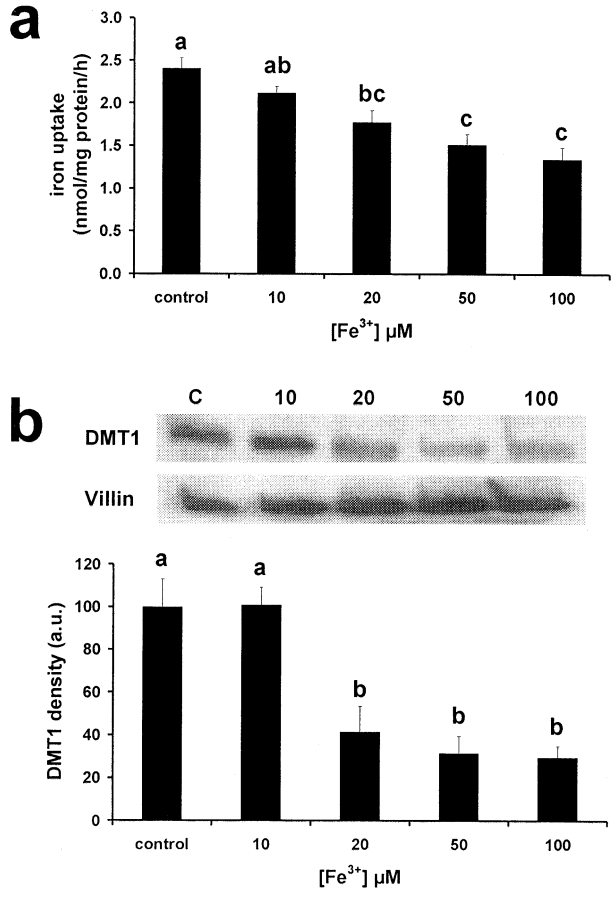

Fig. 4. DMT1 protein and iron transport function are regulated by apical iron levels in a dose-dependent manner. Caco-2 TC7 cells were cultured in the absence or presence of 10–100 μM iron for the final 24 h of the culture period. Fe^{2+} (10 μM) uptake at apical pH 5.5 (a) and DMT1 protein expression (b) were decreased in a reciprocal relationship to increasing iron levels. Data are means ± S.E.M. of four experiments. Different letters above data bars indicate that these groups are statistically different ($P < 0.05$).

References

[1] Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L. and Hediger, M.A. (1997) Nature 388, 482–487.
[2] Fleming, M.D., Trenor, C.C., Su, M.A., Foernzler, D., Beier, D.R., Dietrich, W.F. and Andrews, N.C. (1997) Nat. Genet. 16, 383–386.
[3] Tandy, S., Williams, M., Leggett, A., Lopez-Jimenez, M., Dedes, M., Ramesh, B., Srai, S.K. and Sharp, P. (2000) J. Biol. Chem. 275, 1023–1029.
[4] Zoller, H., Koch, R.O., Theurl, I., Obrist, P., Pietrangelo, A., Montosi, G., Haile, D.J., Vogel, W. and Weiss, G. (2001) Gastroenterology 120, 1412–1419.
[5] Canonne-Hergaux, F., Greunheid, S., Ponka, P. and Gros, P. (1999) Blood 93, 4406–4417.
[6] Trinder, D., Oates, P.S., Thomas, C., Sadleir, J. and Morgan, E.H. (2000) Gut 46, 270–276.
[7] Smith, M.W., Debnam, E.S., Dashwood, M.R. and Srai, S.K. (2000) Pflug. Arch. 439, 449–454.
[8] Roy, C.N. and Enns, C.A. (2001) Blood 96, 4020–4027.
[9] Mahraoui, L., Rodolosse, A., Barbat, A., Dussaulx, E., Zweibaum, A., Rousset, M. and Brot-Laroche, E. (1994) Biochem. J. 298, 629–633.
[10] Mahraoui, L., Rousset, M., Dussaulx, E., Darmoul, D., Zweibaum, A. and Brot-Laroche, E. (1992) Am. J. Physiol. 263, G312–G318.
[11] Laemmli, U.K. (1970) Nature 277, 680–685.
[12] Chantret, I., Rodolosse, A., Barbat, A., Dussaulx, E., Brot-Laroche, E., Zweibaum, A. and Rousset, M. (1994) J. Cell Sci. 107, 213–225.
[13] Han, O., Fleet, J.C. and Wood, R.J. (1999) J. Nutr. 129, 98–104.
[14] Fleming, M.D., Romano, M.A., Su, M.A., Garrick, L.M., Garrick, M.D. and Andrews, N.C. (1998) Proc. Natl. Acad. Sci. USA 95, 1148–1153.
[15] Su, M.A., Trenor III, C.C., Fleming, J.C., Fleming, M.D. and Andrews, N.C. (1998) Blood 92, 2157–2163.
[16] Gruenheid, S., Canonne-Hergaux, F., Gauthier, S., Hackam, D.J., Grinstein, S. and Gros, P. (1999) J. Exp. Med. 189, 831–841.
[17] Tabuchi, M., Yoshimori, T., Yamaguchi, K., Yoshida, T. and Kishi, F. (2000) J. Biol. Chem. 275, 22220–22228.
[18] Debnam, E.S., Smith, M.W., Simpson, R.J., Churchill, L. and Srai, S.K.S. (1999) J. Physiol. 520P, 26P.
[19] Wardrop, S.L. and Richardson, D.R. (1999) Eur. J. Biochem. 263, 41–49.

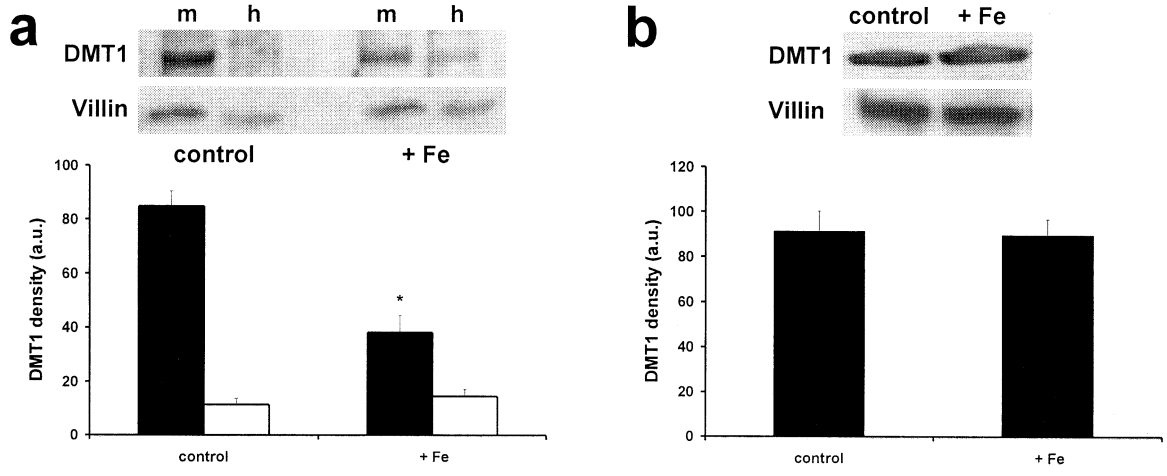

Fig. 5. Cellular distribution of DMT1 protein following exposure to high iron. Western blotting (20 mg protein per lane) for DMT1 and villin in Caco-2 TC7 cell membranes (m) and whole cell homogenates (h) revealed a prominent 66 kDa band (a). Quantification of DMT1 band density shows that there was a significant reduction in transporter levels in membrane fractions (filled bars) from iron-treated cells, whereas there was no significant difference in whole cell DMT1 levels (open bars). Data are means ± S.E.M. of three to five experiments. *$P < 0.05$. Representative Western blots using higher loading (50 μg) of whole cell protein confirms that there was no difference in DMT1 or villin expression following exposure to high iron (b). Data bars are mean ± S.E.M. of four separate experiments.

[20] Lee, P.L., Gelbart, T., West, C., Halloran, C. and Beutler, E. (1998) Blood Cells Mol. Dis. 24, 199–215.
[21] Cannone-Hergaux, F., Fleming, M.D., Levy, J.E., Gauthier, S., Picard, V., Andrews, N.C. and Gros, P. (2000) Blood 96, 3964–3970.
[22] Casey, J.L., Koeller, D.M., Ramin, V.C., Klausner, R.D. and Harford, J.B. (1989) EMBO J. 8, 3693–3699.
[23] Yamaji, S., Tennant, J., Tandy, S., Williams, M., Srai, S.K.S. and Sharp, P. (2001) FEBS Lett. 507, 137–141.
[24] Wardrop, S.L. and Richardson, D.R. (2000) Eur. J. Biochem. 267, 6586–6593.
[25] Conrad, M.E., Umbreit, J.N., Moore, E.G., Hainsworth, L.N., Porubcin, M., Simovich, M.J., Nakada, M.T., Dolan, K. and Garrick, M.D. (2000) Am. J. Physiol. 279, G767–G774.
[26] Yeh, K.-Y., Yeh, M., Watkins, J.A., Rodriguez-Paris, J. and Glass, J. (2000) Am. J. Physiol. 279, G1070–G1079.