Nramp2 Expression Is Associated with pH-dependent Iron Uptake across the Apical Membrane of Human Intestinal Caco-2 Cells*

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The absorption of dietary non-heme iron by intestinal enterocytes is crucial to the maintenance of body iron homeostasis. This process must be tightly regulated since there are no distinct mechanisms for the excretion of excess iron from the body. An insight into the cellular mechanisms has recently been provided by expression cloning of a divalent cation transporter (DCT1) from rat duodenum and positional cloning of its human homologue, Nramp2. Here we demonstrate that Nramp2 is expressed in the apical membrane of the human intestinal epithelial cell line, Caco-2 TC7, and is associated with functional iron transport in these cells with a substrate preference for iron over other divalent cations. Iron transport occurs by a proton-dependent mechanism, exhibiting a concurrent intracellular acidification. Taken together, these data suggest that the expression of the Nramp2 transporter in human enterocytes may play an important role in intestinal iron absorption.

Iron is a trace element that is essential for life, since it plays a critical role in many biochemical and physiological mechanisms. As a consequence, nature has developed an array of elaborate processes for the absorption, storage, and transport of iron within the body, and a homeostatic balance between these mechanisms is essential for good health.

The vast majority of dietary non-heme iron is absorbed in the duodenum, where it is most soluble due to the acidic environment. Bioavailable iron is always in the ferrous (Fe²⁺) state, but most ingested iron is in the ferric (Fe³⁺) form. Reduction of Fe³⁺ to Fe²⁺ can be promoted by the action of a putative ferric reductase (1) and reducing components in the diet, such as ascorbate (2). The regulation of iron absorption from the diet by the small intestine is of crucial importance in determining body iron status, and consequently, a great deal of research interest has focused on the cellular mechanisms involved in iron accumulation. This has culminated in the expression cloning of an elaborate process for the absorption, storage, and transport of iron within the body, and a homeostatic balance between these mechanisms is essential for good health.

Intracellular iron transport that has been mapped to the gene encoding the protein Nramp2. Taken together these data suggest to us that Nramp2 should be expressed on human enterocyte plasma membranes and function as an iron transporter. Our present study has tested this hypothesis, and accordingly, we present evidence that human enterocytes absorb iron across their apical membrane in a pH-dependent fashion. Using cells loaded with the fluorescent dye BCECF, we show that there is a concurrent intracellular acidification induced by iron in the bathing medium. With an antibody generated againstNramp2, we demonstrate the expression of a 66-kDa apical membrane-resident protein in Caco-2 TC7 cells. Finally, we present evidence that in human enterocytes the pH-dependent iron uptake associated with Nramp2 expression has a different substrate specificity profile than the rat homologue, showing selectivity for iron over other divalent metals.

** Experimental Procedures

**Cell Culture**—Caco-2 TC7 cells were obtained from Drs. Monique Roussel and Edith Brot-Laroche (INSERM U178, Villejuif). Stock cultures of cells were maintained in 25-cm² plastic flasks and cultured in a 90% air, 10% CO₂ atmosphere in Dulbecco's modified Eagle’s minimal essential medium supplemented with 20% heat-inactivated fetal bovine serum. All experiments were carried out on cells between passage numbers 30 and 35. For experiments, cells were seeded at a density of 1 × 10⁶ cells/cm² onto either glass coverslips for pH measurements or Transwell inserts (Costar) for all other experiments and used 20 days later. Caco-2 TC7 cells were fully differentiated at this time and demonstrated a small intestinal phenotype (data not shown), which has been described previously (4). To investigate the regulation of Nramp2 by iron status, in some experiments cells were grown for the last 5 days in medium supplemented with 50 μM Fe²⁺ (complexed with a 2-fold excess of nitritotriacetic acid).

**Transepithelial Iron Flux across Caco-2 TC7 Cell Monolayers**—To determine the nature of iron transport across the Caco-2 TC7 cell monolayer, cells were grown on Transwell inserts. To achieve transepithelial pH gradients, either Hepes-buffered salt solution (HBSS), pH 7.5, containing 140 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose, 10 mM Hepes, 0.2% bovine serum albumin, HBBS (pH 6.5, substituting Pipes for Hepes) or MBSS (pH 5.5, substituting MES for Hepes) were added to the apical chamber. HBBS was placed in the basolateral chamber. Uptake was initiated by the addition of 100 μM Fe³⁺, ascorbate (1:10 molar ratio) and 37 kBq/ml [⁵⁵FeCl₃] to the apical chamber and terminated after 60 min. Cells, solubilized in 200 mM NaOH, were subjected to scintillation counting to determine cell uptake. An aliquot of the basolateral medium was counted to determine transepithelial iron movement. Parallel experiments in which [¹⁴C]mannitol was substituted for ⁵⁵Fe³⁺ were performed to distinguish the passive transport component. Experiments measuring

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The abbreviations used are: BCECF, 2',7'-bis-(carboxyethyl)-5(6)carboxyfluorescein; pHₐp, apical pH; pHᵢ, basolateral pH; pHₑ, intracellular pH; pHₑ, extracellular pH; Pipes, piperazine-N,N'-(2-ethanesulfonic acid); MES, 4-morpholineethanesulfonic acid; HBBS, Hepes-buffered salt solution.
zinc uptake used a similar protocol and employed $^{65}$ZnCl$_2$ as the radioligand tracer. For inhibition studies, 1 $\mu$m $^{55}$Fe$^{2+}$ and 100 $\mu$m appropriate divalent cation were added to the apical chamber, pH 5.5, and after 1 h, incubation the cells processed as above.

**Western Blot Analysis**—After removal of culture medium, cell monolayers were washed twice in phosphate buffer, and harvested using a cell scraper. Enriched apical membranes were prepared by the MgCl$_2$ precipitation technique (8) and used for Western blotting.

Cell membranes and samples of the whole cell homogenates were solubilized in Laemmli buffer (9) and subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose (Hybond ECL, Amersham Pharmacia Biotech) 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 a polyclonal antibody (1:250 dilution) raised in rabbit against a synthetic peptide corresponding to amino acids 310–330 of the human Nramp2 sequence or with a commercially available (Chemicon, Harrow, UK) polyclonal antibody to the sodium glucose cotransporter, SGLT1 (1:2000 dilution). After removal of the primary antibody, a secondary antibody against rabbit IgG (horseradish peroxidase-labeled) was used, and cross-reactivity was visualized using enhanced chemiluminescence and Hyperfilm ECL (Amersham Pharmacia Biotech). Western blots were analyzed by scanning densitometry to determine the enrichment of the transporters in the apical membrane of the intestinal epithelial cells.

**Confocal Microscopy**—Caco-2 TC7 cells grown on semi-permeable supports were fixed for 30 min with 4% paraformaldehyde ($v/v$) in phosphate-buffered saline and permeabilized with 0.1% Triton X-100 for a further 30 min. After blocking with normal goat serum, monolayers were incubated overnight at 4 °C with Nramp2 (1:20) or SGLT1 (1:200) antibodies. A fluorescein isothiocyanate-labeled anti-rabbit secondary antibody was used to visualize cross-reactivity. Optical sections (2 $\mu$m) were obtained using a Bio-Rad DVC-250 confocal microscope through the z plane of the Caco-2 TC7 cell monolayer.

**Iron-dependent Changes in Intracellular pH**—To assess the effect of extracellular iron on intracellular pH, cells grown for 20 days on glass coverslips were loaded in HBSS containing the pH-sensitive dye BCECF-AM (5 $\mu$m) for 40 min at 37 °C. Coverslips were transferred to fresh HBSS for 20 min to allow de-esterification of the dye. The glass coverslip formed the base of an experimental chamber attached to the stage of an inverted epifluorescence microscope (Zeiss IM35, ×40 neofluor Nikon objective) that was coupled to a fluorescence imaging system (Photon Technology International) with an intensified CCD video camera (Photonic Sciences, Sussex, UK). The cells were continuously perfused with oxygenated HBSS or PBSS (37 °C) during the course of the experiment, and changes in the experimental solutions (pre-warmed to 37 °C) were administered via a two-way tap. The fluorescence ratio (495/450-nm excitation, 530 nm-emission) was measured in the biplane of the transporters in the apical membrane of the intestinal epithelial cells.

**RESULTS**

**55Fe Uptake across the Apical Membrane of Caco-2 TC7 Cells**—The time course of iron uptake by Caco-2 TC7 cells could be fitted by a 1-exponential relationship (Fig. 1A). The uptake of ferrous ascorbate (100 $\mu$m Fe$^{2+}$) across the apical membrane of cell monolayer was determined by the pH$_{6.5}$ and was significantly higher at both pH$_{6.5}$ and pH$_{5.5}$ compared with pH$_{7.5}$ (Fig. 1B). Transepithelial flux of iron from apical to basal was also increased significantly at low pH$_{6.5}$ (Fig. 1C). Further analysis of these data revealed that the vast majority of iron utilizes the transcellular rather than paracellular route and that approximately 96% of this iron is retained within the cell within the time course of the experiment. Interestingly, transfer to the basolateral chamber was not pH-dependent when expressed as a function of apical uptake (Fig. 1D). When the pH gradient across the epithelium was reversed, i.e. pH$_{7.5}$/pH$_{5.5}$, there was no pH-dependent movement of iron from the basolateral chamber into the cells (data not shown), confirming that pH-dependent transport was confined to the apical membrane.

**Nramp2 Expression by Caco-2 TC7 Cells**—Western blotting revealed a major cross-reacting band in the apical membrane of Caco-2 TC7 cells at 66 kDa, which is in agreement with the predicted molecular mass of this transporter (Fig. 2A). Nramp2 was enriched (66%) in the apical membrane fraction of Caco-2 TC7 cells compared with whole cell protein levels (Fig. 2A). Parallel blots for the apical membrane-resident transporter SGLT1 showed similar levels of enrichment (57%) in the plasma membrane fraction compared with whole cell amounts (Fig. 2B).

Confocal microscopy using Caco-2 TC7 cells grown on semi-permeable supports showed significant apical staining for both Nramp2 (Fig. 2C) and SGLT1 (Fig. 2E). Sequential sections (2 $\mu$m) through the z plane of the monolayer revealed that staining was confined to the apical section and was not present at the basolateral surface of the cells (Figs. 2, D and F).

**Nramp2 Expression Is Associated with pH-dependent Iron Uptake across the Apical Membrane of Caco-2 TC7 Cells**—The specificity of the antibody for Nramp2 was confirmed by preabsorbing the antiserum with the original immunizing peptide before blotting (Fig. 3A). Lanes 1 and 3 show the 69-kDa molecular mass marker as an internal control for development of the blots. The cross-reacting band at 66 kDa. In the antibody-treated whole cell homogenates the pH-sensitive dye BCECF-AM (5 $\mu$m) for 40 min at 37 °C. Coverslips were transferred to fresh HBSS for 20 min to allow de-esterification of the dye. The glass coverslip formed the base of an experimental chamber attached to the stage of an inverted epifluorescence microscope (Zeiss IM35, ×40 neofluor Nikon objective) that was coupled to a fluorescence imaging system (Photon Technology International) with an intensified CCD video camera (Photonic Sciences, Sussex, UK). The cells were continuously perfused with oxygenated HBSS or PBSS (37 °C) during the course of the experiment, and changes in the experimental solutions (pre-warmed to 37 °C) were administered via a two-way tap. The fluorescence ratio (495/450-nm excitation, 530 nm-emission) was measured in a group of approximately 50 cells. In our system a decrease in the fluorescence ratio corresponds to an intracellular acidification (10).

**Northern Blotting**—Total RNA (20 $\mu$g), isolated using Triazol reagent (Life Technologies, Inc.) was fractionated by electrophoresis on 1% agarose gels under denaturing conditions. RNA was transferred to nitrocellulose filters (Bio-Rad) using the baking technique (8) and used for Northern hybridization with a full-length cDNA clone labeled with $^{32}$PdCTP using a random priming kit (Amersham Pharmacia Biotech). Membranes were washed in 5 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS at 50 °C for 2 × 30 min and 0.1 × SSC, 0.1% SDS at 65 °C for 3 × 20 min before exposure to x-ray film.

**Data Analysis**—Data are presented as the mean ± S.E. Statistical analysis was carried out using SPSS statistics package employing either Student’s unpaired t test when comparing 2 data sets or a one-way analysis of variance followed by Scheffe’s post-hoc analysis when comparing multiple data sets. Differences were considered significant at $p < 0.05$.

**Materials**—Radiochemicals and materials for Western blotting were supplied by Amersham Pharmacia Biotech. Cell culture medium and plastic ware were purchased from Life Technologies unless stated. Heat-inactivated fetal bovine serum was from Sigma. All other chemicals were of the highest grade available and bought from Sigma, Merck, or Fluka.
Fig. 1. pH dependence of iron uptake by Caco-2 TC7 cells. A, iron uptake across the apical membrane of Caco-2 TC7 (pH₇.5/pH₇.75) cells followed a 1-exponential relationship, which could be fitted by the equation \( y = y_{\text{max}} (1 - \exp^{-kt}) \), where \( k \), the rate constant, is 0.04 min⁻¹. B, uptake was dependent on pH of the apical chamber shown on x axis (filled bars, left axis), whereas passive movement of mannitol (open bars, right axis) was not affected by apical pH \( \ast, p < 0.01 \), significant difference in iron uptake at pH 7.5 and 6.5 compared with pH 7.75. C, transepithelial flux of iron from apical to basal was greatest in the monolayer experiments with pH 6.5 or 5.5 compared with pH 7.75 (filled bars, left axis). Passive diffusion was not influenced by the imposition of a pH gradient (open bars, right axis). \( \ast, p < 0.01 \) significant difference in basolateral iron appearance at pH 7.5, 5.5 and 6.5 compared with pH 7.75. D, when expressed as a percentage of apical uptake, it was clear that the appearance of iron in the basolateral chamber was directly proportional to iron uptake across the apical membrane, and there was no statistical difference between the three groups. In all experiments, basolateral pH was 7.5. All data are presented as means ± S.E. of 20 observations. Statistical analysis employed analysis of variance and Scheffe’s post-hoc test.

**DISCUSSION**

Little is known about the cellular mechanisms involved in the control of intestinal non-heme iron absorption, but the results of several previous studies have shown that iron uptake across the apical membrane of enterocytes is of major importance in determining the rate of absorption across the intestinal epithelium (11–14). The requirement for the apical iron transporter Nramp2 in maintaining iron homeostasis is very evident, since a mutation in this protein is responsible for microcytic anemia in both mk/mk mice (4) and Belgrade rats (6). The regulation and mechanisms of iron uptake across this membrane are therefore of great interest.

Caco-2 cells have been used extensively as a human model to study many facets of iron homeostasis, including the expression of ferritin (15) transferrin receptor (16), and IRP-1 and IRP-2 (17). In addition, Caco-2 cells are similar to “normal” enterocytes with regard to the expression of most of their differentiation markers (18) and yield very similar data to those achieved with human absorption studies (19), making them a very pertinent human model to study the regulation of iron absorption.

Preliminary studies from our laboratory have demonstrated that iron absorption by Caco-2 TC7 cells is time- and concen-
protein (Figs. 2A and 3A), which is very close to the predicted molecular mass of this transporter (64 kDa). Immunofluorescence labeling of Caco-2 TC7 cells with the same Nramp2 antibody, viewed by confocal microscopy, confirmed that the transporter was localized to apical membrane (Fig. 2C). There was no evidence of basolateral staining (Fig. 2D). This is in good agreement with a recent report (22) that demonstrated the presence of Nramp2 at the apical surface and associated with microsomal fractions in rat enterocytes. Previous studies using transfected cells showed that this microsomal locus is the recycling endosome, suggesting a role for Nramp2 in iron ac-
cumulation from the transferrin receptor pathway (23).

Our study has established a direct link between Nramp2 expression and iron uptake in Caco-2 TC7 cells. Iron uptake was blocked by 45% by co-incubation with our Nramp2-specific antibody (Fig. 3B). This provides the first physiological evidence that Nramp2 plays a major role in iron transport across the apical membrane of human small intestinal enterocytes.

In accordance with proton-dependent iron uptake, we found that the addition of ferrous ascorbate to the apical medium resulted in intracellular acidification at both pH₇.5 and 6.5 (Fig. 4). The rate of acidification (directly proportional to the BCECF fluorescence ratio, data not shown) was approximately 3-fold greater at pH₇.5. Importantly, the relative increase in the rate of intracellular acidification is an underestimate of the increase in apical proton influx, since the intrinsic buffering capacity is greater at lower intracellular pH (data not shown). Iron-induced intracellular acidification at pH₇.5, where the pH gradient would be expected to be outwardly directed, could be explained by the presence of an acidic microclimate, which surrounds enterocytes in the unstirred layers both in vivo (24) and in vitro (25) Additionally, Caco-2 cells have a membrane potential of −43mV, which would result in an inwardly directed proton motive force in our experimental system. These data are consistent with the hypothesis that iron absorption across the apical membrane of enterocytes is proton-coupled and is dependent on the functional expression of Nramp2.

Nramp2 contains an iron response element in the 3′-untranslated region (3), which should lead to decreased mRNA stability under iron-loaded conditions. Our data are consistent with this hypothesis, demonstrating that Nramp2 is down-regulated at mRNA (Fig. 5A) and protein level (Fig. 5B) by 2P. Sharp, E. Debnam, and S. Srai, unpublished information.

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**Fig. 4.** Nramp2 expression is associated with Fe²⁺-induced intracellular acidification. In Caco-2 TC7 cells, the addition of 100 μM Fe²⁺ with 500 μM ascorbic acid (AA) to the apical perfusing buffers (pH 7.5 and 6.5, respectively) resulted in an intracellular acidification that was over and above that observed in the presence of ascorbic acid alone. Data represent BCECF fluorescence emission at 530 nm expressed as a ratio of the two excitation wavelengths (495 and 450 nm). A decrease in pHₗ is represented by a decrease in fluorescence ratio (Williams et al. (10)).

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**Fig. 5.** Nramp2 expression is regulated by cellular iron status. Caco-2 TC7 cells were grown in medium supplemented with 50 μM Fe³⁺ nitrilotriacetic acid for the final 5 days of the culture period. A, a representative Northern blot (n = 3) showing that exposure to iron-loaded conditions reduces Nramp2 mRNA levels (upper panel), whereas expression of the housekeeper gene, glyceraldehyde-3-phosphate dehydrogenase, is unaffected (lower panel). The decrease in mRNA is reflected by a 2-fold reduction in Nramp2 protein expression in apical membrane fractions from Caco-2 TC7 cells (B), and this is paralleled by decreased iron uptake (30%) across the apical membrane of cells grown on Transwell inserts (C). D, interestingly, zinc uptake was not affected by these changes in Nramp2 expression and function. Experiments were performed 3–5 times, and data are shown as means ± S.E. and analyzed by Student’s unpaired t test. *, p < 0.01.
increasing cellular iron status. These iron-dependent effects on protein and mRNA expression translate into modulation of transport function in Caco-2 TC7 cells (Fig. 5C). Interestingly, zinc uptake was unaffected by these iron-dependent changes in Nramp2 expression and function (Fig. 5D). Previous work has suggested that zinc is also a major substrate for the Nramp2 transport pathway (3), and as such its uptake should also be modified by changes in transporter expression. This is not the case, and therefore, this led us to investigate the role that Nramp2 plays in the absorption of other divalent cations.

Previous studies on the substrate specificity of the Nramp2 transporter were based on an indirect methodology, measuring inward currents evoked in the presence of metal ions (3). In light of our finding that zinc uptake by Caco-2 TC7 cells was not affected by changing the levels of expression of the transporter, we employed radioligand uptake techniques to determine precisely the divalent metal transport characteristics of Nramp2 in these cells. By assessing $^{55}$Fe$^{2+}$ uptake in competition with several divalent metals, we have demonstrated that iron is the preferred substrate for the Nramp2 pathway, and only cadmium and cobalt exhibited inhibition of iron uptake by 50% or greater (Fig. 6A).

To further address the issue of substrate specificity, we measured $^{65}$Zn uptake by Caco-2 TC7 cells. If zinc, which showed the lowest inhibition of iron transport in our competition assay (Fig. 6A), crosses the apical membrane via Nramp2, we would have expected to see pH-dependent absorption. However, unlike iron, the uptake of zinc is not pH-dependent (Fig. 6B). Moreover, previous studies using small intestinal brush border membrane vesicles showed that imposition of an inwardly directed pH gradient had an inhibitory effect on zinc transport (26).

Uptake of iron and other divalent metals, including zinc, via Nramp2 in *Xenopus* oocytes is dependent on membrane potential (3). We have shown in this study and previously (13) that depolarization of enterocytes with high K$^+$ solutions (giving a resting membrane potential of $-5$ mV, data not shown) results in a significant decrease in iron transport across the apical membrane of intestinal epithelial cells (Fig. 6C). In our experiments, the uptake of zinc is unaffected by dissipation of the membrane potential (Fig. 6C). Taken together, our findings that 1) zinc uptake is not affected by decreased expression of Nramp2 (Fig. 5D), 2) zinc does not compete with iron for uptake via this transport pathway (Fig. 6A), 3) the absorption of zinc is not pH-dependent (Fig. 6B), and 4) zinc transport is not dependent on membrane potential (Fig. 6C) strongly suggests that iron and zinc are absorbed by different and distinct transport mechanisms in this acknowledged model of human intestinal enterocytes. In agreement with this hypothesis, a number of other zinc uptake pathways are present in the apical membrane of Caco-2 cells that are not affected by metabolic inhibitors (27) or are linear and nonsaturable (28) and therefore likely to be distinct from Nramp2. Clearly, although iron is the preferred substrate for the Nramp2 pathway, further work is required to fully elucidate the relative contribution of Nramp2 to the absorption of other divalent metals by intestinal enterocytes and to determine the nature of other transport pathways that may be involved.

In conclusion, we have demonstrated that iron uptake occurs across the apical membrane of Caco-2 TC7 cells in a pH-dependent fashion, and the presence of the Nramp2 transporter in the apical membrane is associated with iron transport function. Furthermore, when iron is added to the bathing medium there is a concomitant intracellular acidification. Nramp2 expression and function are modulated as a function of the prevailing iron levels in the culture medium, and transport via this pathway shows a preference for iron over several other divalent metals. Taken together, these data suggest that, first and foremost, Nramp2 acts as an iron transporter in the apical membrane of human intestinal Caco-2 TC7 cells.

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