IEC-6 Cells Are an Appropriate Model of Intestinal Iron Absorption in Rats

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ABSTRACT  Regulation of iron absorption, which is the primary mechanism for maintaining body iron stores, occurs primarily in the proximal small intestine. Recent identification of proteins that are involved in iron absorption such as the uptake transporter-divalent metal transporter (DMT1), the basolateral transporter, IREG1, and the ferroxidase-hephaestin provide new opportunities to study this process. We evaluated the rat intestinal cell line, IEC-6, as a model of intestinal iron transport. This involved measuring the expression of DMT1 and IREG1 by Western blot analysis and confocal microscopy, and hephaestin by protein-dependent copper oxidase activity. DMT1 and IREG1 were expressed in IEC-6 cells. The uptake of 1 μmol/L ferrous iron [Fe(II)];ascorbate and its efflux also was associated with the expression of DMT1 under different levels of iron loading. The expression of DMT1 changed inversely with iron levels as did the uptake of Fe(II). However, with different levels of cellular iron, IREG1 expression remained constant, as did the release of iron from the cells, suggesting that they could be related. Ceruloplasmin and apotransferrin did not enhance the rate or extent of iron release. Copper oxidase activity, considered to indicate hephaestin activity, was detected only intracellularly. Confocal microscopy showed DMT1 and IREG1 on the cell membrane of IEC-6 cells at 4°C but at intracellular locations at 37°C, indicating that these proteins can function at the cell membrane and intracellularly. In terms of iron absorption, IEC-6 cells have a villous enterocyte phenotype and are regulated by iron stores as occurs in vivo; therefore, they represent an appropriate cell model with which to study this process. J. Nutr. 132: 680–687, 2002.

KEY WORDS: • iron uptake • efflux • DMT1 • IREG1 • hephaestin

Iron is a trace element that is required for numerous cellular functions (1). However, because it reacts with oxygen to cause oxidative damage, body iron stores must be maintained within strict limits (2). This is controlled at the level of iron absorption by duodenal enterocytes. Despite extensive study, details on the mechanism of iron absorption remain unclear. Recent identification of proteins involved in this process such as divalent metal transporter 1 (DMT1)3 (3,4), IREG1/ferropor- tin/metal transporter protein 1 (MTP1) (5–7) and hephaestin (8) provide new opportunities to clarify this mechanism.

DMT1 is expressed on the apical membrane of the enterocyte; in the presence of a proton gradient provided by gastric secretions, it cotransports ferrous iron [Fe(II)] from the membrane into the cell (3,9,10). Supporting evidence for the role of DMT1 and IREG1 in the uptake of iron is found in homozygous microcytic anemic mice and Belgrade rats in which uptake of iron is impaired due to a G185R mutation in DMT1 (4,11–13).

The recent cloning and characterization of IREG1/ferroportin/MTP1 suggests that it is the efflux carrier of the enterocyte as shown in expression systems in which IREG1 exported iron in the presence of ceruloplasmin (Cp) (5–7). However, the efflux of iron from the enterocyte probably requires the Cp homologue hephaestin (8) because its mutation as seen in sex-linked anemic mice impairs iron release (14).

Understanding how these proteins coordinate iron absorption is assisted by studies using cell lines of human origin (15,16). However, it would be advantageous to have a cell line derived from the same species used in studies of whole animals, such as the rat, so that additional mechanistic questions can be studied in conjunction with those obtained in vivo and in turn compared with those found in humans. These cells must be of intestinal origin, express the above genes, have functional uptake and efflux transporters, and be able to be transfected for the further study of genes of interest. In view of this we evaluated the nontransformed rat small intestinal epithelial cell line IEC-6 as a model for the study of nonheme iron absorption. This involved comparing the expression and cellular localization of DMT1 and IREG1 in IEC-6 cells and rat duodenum. Also, the existence of uptake and efflux iron transport processes along with changes in the expression of the above genes with variations in iron loading was investigated in IEC-6 cells.

MATERIALS AND METHODS

Cell culture. IEC-6 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and routinely main-
tainated in the presence of Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum (FCS), 100 U/ml insulin, 50 mg/ml penicillin, 50 mg/ml streptomycin and 2.5 mg/ml fungizone (Intramed, Meriden, CT). Cells were grown at 37°C in a humidified atmosphere of 5% CO2/95% air and viability was monitored using phase contrast microscopy and trypan blue staining. All experiments were done on cells between passages 17 and 30.

Variations in iron loading were performed as described below (see ferrous uptake). Total cellular iron concentration was measured by atomic absorbance spectrometry. This involved washing the cells with PBS and removing them from a 25 cm2 culture flask. The cells were centrifuged (700 x g for 5 min) and resuspended in 2.8 molar nicric acid and left at 90°C for 60 min. The solution was centrifuged (12,000 × g for 5 min) to remove denatured protein and appropriately diluted supernatant measured for Fe concentration using an atomic absorbance spectrometer (Varian, Victoria, Australia).

The results presented in this study have been normalized to the protein content of the cells. The protein content of IEC-6 cells was determined using the BCA protein assay kit (Pierce, Rockford, IL).

Animals. The use of animals in this study has been approved by the Animal Welfare Committee of the University of Western Australia. Outbred male Wistar rats (6 wk old) were obtained from the Animal Resources Center (Animal Resource Centre, Murdoch, Western Australia). Rats were fed a semipurified diet (PMI, St. Louis, MO) or a control diet either normal or supplemented with no added iron, containing 5 mM FeCl3 and 5.6 mM FeSO4 in a 1:10 molar ratio, with a 100-fold molar excess of sodium ascorbate, was added to an isotonic solution of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl plus 0.5% Triton X-100, and the protease inhibitors phenylmethylsulfonyl fluoride, leupeptin and trypsin.

Glycosylation of DMT1 in vivo. To determine whether DMT1 was glycosylated in vivo, 150 μg of the total duodenal mucosal protein was precipitated with trichloroacetic acid, washed, solubilized in buffer containing 5% N-glycosidase F (Hoffman-La Roche, Basel, Switzerland) and incubated overnight. Digestion and undigested proteins were then subjected to Western blot analysis.

Uptake of ferrous iron by IEC-6 cells. IEC-6 cells were seeded in 12-well plates at 7.5 × 104 cells/well or 10-3 before the experiment, resulting in the cells being in the exponential or stationary growth phase, respectively, at the time of study. To study the uptake of Fe(II), a solution containing 1 μmol/L iron (concentration determined from preliminary studies that saturated the uptake transport process) as 57 FeCl3 and 57 FeSO4 in a 1:10 molar ratio, with a 100-fold molar excess of sodium ascorbate, was added to an isotonic solution of minimum essential medium (MEM) containing 20 mM HEPES-Tris (pH 5.0, 5.5 and 7.4) and 10 μl borivine serum albumin. Cells were washed 3 times in buffer solution (BSB) to remove culture media and were incubated for 0-1 h in the 1 μmol/L Fe solution at 37°C in an atmosphere of 5% CO2/95% air. Cells were washed 5 times in BSS and lysed in 0.1 mol/L NaOH, 1% Triton-X100 and the radioactivity counted in a gamma counter (Packard, Meriden, CT).

Copper oxidase activity. The intracellular and extracellular copper oxidase activity of IEC-6 cells was measured by the rate of oxidation of p-phenylenediamine (18). A sodium acetate solution (200 mmol/L) containing 1.5 mg/mL p-phenylenediamine was incubated with viable cells in a 6-well plate, fresh and freshly boiled homogenates for 15-60 min at 37°C. The reaction was stopped by the addition of 1.5 mol/L sodium azide. Parallel reactions with sodium azide present throughout the incubation were carried out to account for spontaneous oxidation of p-phenylenediamine. The color change was quantified by measuring its absorbance at 530 nm. Activity is expressed in units as follows: 1 U = 1 absorbance unit change/30 min.

Western blot analysis. Protein concentrations of IEC-6 cells, glycosidase F-treated and untreated intestinal mucosal scrapings were determined; 50 μg of each preparation was heated at 100°C for 5 min in standard Laemmli buffer and separated on a 12% SDS-polyacrylamide gel. Gels were electroblotted onto Immobilon-P transfer membrane (Millipore, Bedford, MA). Similar loading and transfer of proteins to the membranes was verified by staining the blots with Ponceau red. The blots were washed, dried and then subjected to antigen detection. Primary antibodies were used as follows: 1) mouse monoclonal proliferating cell nuclear antigen (PCNA) (1:1000), affinity purified rabbit polyclonal anti-rat DMT1 (1:2000) (9). This antibody recognizes both the iron response element (IRE) and non-IRE forms of DMT1 (3,4,12); 2) a rabbit polyclonal anti-rat ferritin (Fn) antibody that recognizes both heavy and light chain (1:2000) (9). This antibody recognizes both the iron response element (IRE) and non-IRE forms of DMT1 (3,4,12); 3) rabbit polyclonal anti-rat IREG1 antibody (1:1500) (see below for verification). All polyclonal antibodies were produced in the laboratory. An anti-rabbit secondary antibody conjugated to horseradish peroxidase was used at 1:2000 for the polyclonal antibodies (Serotech, Oxford, UK) and an anti-mouse secondary antibody conjugated to horseradish peroxidase was used at 1:2000 for the monoclonal antibody (Serotech). The immune complexes were detected using the ECL chemiluminescence assay (Amer sham, Bucks, UK).

Production and validation of IREG1-1 antibody. A synthetic peptide corresponding to amino acids 247-264 of rat IREG1 was conjugated to keyhole limpet hemocyanin and then used to immunize a rabbit for production of anti-IREG1 antisera as described previously (9). Several weeks after sensitization, antisera was obtained and subjected to an ELISA using immobilized immunizing peptide as antigen to determine antibody titer.

IREG1 cDNA construction. A polymerase chain reaction (PCR) fragment representing nucleotides 269-2012 of the published rat IREG1 sequence (Accession #U76714) and encompassing the open reading frame of the gene was generated by a one-step reverse transcriptase (RT) PCR (Invitrogen) using purified total RNA from rat duodenum. Primer used for the RT step was also the 3′ primer, i.e., CACATCTTATAGGCTGACT; the 5′ primer was CTAGCATCCTGAACAAACAAG. The IREG1 cDNA was unidirectionally cloned in the sense direction into the Not1 and BamH1 sites of pcDNA3.1/V5/His-TOP eucaryotic expression vector (Invitrogen). The orientation and sequence of the cDNA clone were confirmed by sequencing.

Production of IREG1 cDNA in Cos-7 cells and duodenal tissue. ICREG1 cDNA was transfected by Lipofectamine treat-
ment (Invitrogen). One day before transfection, 3 × 10⁵ cells were seeded per 25cm² culture flask, so that on the day of transfection, the culture was 70% confluent. No antibiotics were used throughout the transfection period. Cos-7 cells were transfected with either pcDNA3.1-IREG1 or pcDNA3.1 plasmids. Lipofectamine at 5 μL/DNA was used for the transfections. The DNA/lipofectamine mixture was removed after 5 h of incubation at 37°C and the cells were incubated for 24 h in DMEM with 5% FCS before they were lysed in lysis buffer as described above. The lysate was incubated with 5 μL of anti-IREG1 antibody for 1 h at 4°C. Immunoprecipitates were collected by incubation with 100 μL of protein A-sepharose for 2 h at 4°C, washed 3 times with PBS, heated at 100°C for 5 min in standard Laemmli buffer and separated on a 12% SDS-polyacrylamide gel. Western blot analysis was carried out as above. As an additional control, the tissue sections were incubated with the IREG1 antibody in the presence or absence of immunizing peptide (see below).

**Validation of the isolation of villous mucosa from crypt mucosa.** Protein (50 μg) isolated from mid-villus, crypt cells and IEC-6 cells in log-phase were subjected to Western blot analysis using PCNA to detect cells in proliferation.

**Immunofluorescence microscopy.** IEC-6 cells were grown on glass coverslips in 35-mm dishes. Cells were seeded at 1.5 × 10⁵ cells/dish 2 d before the experiment and were in the exponential phase of cell growth for the experiment. Cells were prepared for immunofluorescence microscopy either while viable or after fixation in 12% formalin. Primary antibodies were used as follows: monoclonal anti-sucrase-isomaltase (SI) (1:100) (20), affinity purified rabbit polyclonal anti-rat DMT1 (1:300) and rabbit polyclonal anti-rat IREG1 (1:100). Rabbit preimmune serum was used as a control. Secondary antibodies were anti-rabbit Texas-red conjugated (Molecular Probes, Eugene, OR) or anti-mouse FITC-conjugated (Sternstech) diluted 1:100 with BSS. Viable and fixed cells were incubated with primary and secondary antibodies together for 1 h at 37°C or 4°C and then washed 5 times with PBS. The viable cells were then fixed. Co-incubation of both primary and secondary antibodies with viable cells enabled the detection of these proteins after movement from the cell surface if this was a temperature-dependent process.

The proximal 0.5-cm segment of the duodenum from an iron-deficient and a normal rat were frozen in liquid nitrogen–cooled isopentane. This tissue was then stored at −80°C until sectioned. At the time of sectioning, the tissue was glued to a chuck with Tissue Tek O.C.T. compound (Miles Scientific, Naperville, IL). Sections (7 μm) were then cut at −13°C and adhered to a 0.5 g/L gelatin-coated microscope slides. The sections were dried for 10 min at room temperature and fixed in 12% formalin. The tissue was then used immediately for immunofluorescent microscopy for the localization of SI, DMT1 and IREG1 as described above. In addition, tissue was reacted with the IREG1 antibody that had been incubated overnight with 1 mg of the IREG1 immunizing peptide. To examine antibody staining, the tissue was covered with a coverslip using a fluorescence antifade mounting medium. Images were captured on an Olympus microscope equipped with a BioRad MRC-1000 confocal system, using a 10X or 60X objective (BioRad, Hercules, CA). All images were obtained using the same laser intensity, so that staining patterns could be compared. Images were processed using Adobe Photoshop 5 (Adobe Systems, San Jose, CA).

**Statistical methods.** The results are expressed as means ± SEM. Data were analyzed by ANOVA and Tukey’s test using the Instat program (Graphpad Software, San Diego, CA). Differences were considered significant at P < 0.05.

**RESULTS**

**Total iron concentration in IEC-6 cells with iron loading.** Cellular iron levels measured by atomic absorption spectrophotometry showed that iron-deficient cells contained 789 ± 24 fmol iron/μg protein, whereas iron-loaded cells contained 5587 ± 96 fmol iron/μg protein and control cells contained 1636 ± 52 fmol iron/μg protein. All conditions were different from one another. Iron uptake was measured at varying pH conditions. When iron uptake was measured over the first 15 min, it was 20.1 ± 3, 11.2 ± 2 and 7.7 ± 2 μmol/(g protein × min) in iron-deficient, control and iron-loaded cells, respectively, all of which differed (Fig. 1).

**Iron Release by IEC-6 cells.** Iron was released from IEC-6 cells over time (Fig. 2). The rate of release was constant for all iron conditions, resulting in the release of 50% of the radiolabeled iron within 60 min. Cells in the stationary or exponential growth phase did not differ in rate of iron release. Addition of Cp or apotransferrin to the medium had no effect on the release of Fe (data not shown).

**Validation of IREG1 antibody.** To determine the presence of IREG1 antisera we used ELISA to show that there was immunoreactivity when the antisera were reacted with immunizing antigen. To rigorously test the specificity of the antisera, we overexpressed recombinant rat IREG1 in Cos7 cells.

![FIGURE 1](https://example.com/figure1.png)  
**FIGURE 1** The rate of uptake of ferrous iron [Fe(II)] in IEC-6 cells incubated with 1 μmol/L of Fe(II) under iron-deficient, control and iron-loaded conditions for up to 60 min. Values are means ± SEM, n = 3. Different letters indicate that the three conditions differed from one another, P < 0.05.

![FIGURE 2](https://example.com/figure2.png)  
**FIGURE 2** The rate of release of radiolabeled iron following the loading of iron-deficient, normal and iron-loaded IEC-6 cells with 1 μmol/L ferrous iron [Fe(II)] for 60min. Efflux was measured at varying times up to 60 min after loading. Values are means ± SEM, n = 3. The three conditions did not differ.
When the protein was immunoprecipitated and subjected to Western blot analysis using the antisera, a single band migrating at ~60 kDa was observed. The signal was markedly greater than in untransfected or vector alone transfected Cos-7 cells in which the signal was equally low (Fig. 3). In addition, the immunofluorescent signal seen with the IREG1 antibody on sections of tissue was identical to that previously reported (see below). This signal can be eliminated if the IREG1 antibody is incubated overnight with the immunizing peptide used to generate it, which indicates that the antibody is specific for rat IREG1 protein (Fig. 4C).

**Validation of the isolation of villous mucosa from crypt mucosa.** Protein obtained from crypt mucosa and IEC-6 cells reacted positively for PCNA. Mid-villous cells were negative for PCNA (Fig. 5).

**Protein expression in IEC-6 cells and duodenal mucosa.** DMT1 migrated predominantly as a band of ~66 kDa in IEC-6 cells although there were an additional two bands (iron-deficient cells) and one smaller sized band (control cells) (Fig. 6A). In villous mucosa, DMT1 migrated as a band at ~90 kDa (Fig. 6A). In addition, DMT1 expression in IEC-6 cells was inversely related to cellular iron loading (Fig. 6A). Ferritin was demonstrated as a ~20-kDa protein in both IEC-6 cells and rat intestinal scrapings (Fig. 6B). Ferritin levels were directly related to cellular iron loading (Fig. 6B). IREG1 was predominantly at ~60-kDa protein in IEC-6 cells and duodenal mucosa scrapings (Fig. 6C). In IEC-6 cells, IREG1 expression was unaffected by cellular iron loading (Fig. 6C).

**Glycosylation of DMT1 in vivo.** Digesting duodenal mucosal protein with glycosidase F resulted in a reduction of the 90-kDa band to ~66 kDa (Fig. 7), suggesting that the heavier 90-kDa band observed in vivo is due to glycosylation of the DMT1.

**Copper oxidase activity of IEC-6 cells.** Copper oxidase activity of 0.16 U was detected in the homogenate of IEC-6 cells, but no activity could be detected extracellularly. In addition, this activity was lost when the IEC-6 homogenate was boiled (data not shown).

**Localization of sucrase isomaltase, DMT1 and IREG1.** SI was not detected in IEC-6 cells. In whole mounts of duodenum, SI was not detected in the crypt region but its expression commenced at the crypt-villous junction where it localized strongly along the brush border throughout the length of the villus, but attained the highest concentrations in the mid-villous region (Fig. 8). DMT1 was detected on the IEC-6 cell membrane only when immunofluorescent preparation was carried out at 4°C (Fig. 9A). However, when the reaction was carried out at 37°C, DMT1 was also seen at intracellular locations, where it localized to vesicles resembling endosomes (Fig. 9B). IEC-6 cells fixed before immunofluorescent preparation showed the same pattern as that at 37°C (Fig. 9C).

In whole mounts of duodenum, DMT1 expression was studied in iron-deficient rats (Fig. 9A). Here, DMT1 was seen predominantly above the crypt-villous junction and reaching
highest levels in the mid-villous region; it fell, however, in more distal cells, but was still present in enterocytes of the villous-tip (Fig. 9D). In these cells, it was detected along the brush border as well as in the apical third of the cytoplasm (Fig. 9B). It was absent from the basal third of the cytoplasm. No fluorescence was seen when preimmune serum was substituted for the primary antibody (Fig. 9E).

In IEC-6 cells, IREG1 was detected only on the cell membrane when immunofluorescence preparation was carried out on viable cells at 4°C (Fig. 10A). However, when the reaction was carried out at 37°C, the protein was also seen in intracellular locations, where it localized to vesicles resembling endosomes (Fig. 10B). When cells were fixed before processing for immunofluorescence, the pattern of staining was similar to that at 37°C (Fig. 10C).

In duodenum, IREG1 expression was seen predominantly above the crypt-villus junction and was seen in enterocytes throughout the length of the villus. In these cells it was seen along the lateral and basal membranes as well as in the basal cytoplasm (Fig. 4A and B). IREG1 was not detected along the apical membrane (Fig. 4B).

Unfortunately, because the DMT1 and IREG1 antibodies were both produced in rabbits, it was not possible to do colocalization studies to determine whether these proteins colocalized to the same organelles in IEC-6 cells and from tissue.

**DISCUSSION**

We evaluated the use of IEC-6 cells as a model with which to study the mechanism of intestinal iron transport. These cells are derived from the rat small intestine and were produced from a single clone. Therefore they are homogeneous, contain the normal rat somatic karyotype and because they are nontransformed, they have a limited lifespan in culture (up to 50 passages). Due to the lack of staining for the villous enterocyte marker, SI, which was confirmed in this study, these cells are described as having an immature, crypt-like phenotype (21). Nonetheless, by Western blot analysis, we found that DMT1, IREG1 and Fn are expressed by IEC-6 cells. Because these proteins are reported to be expressed in villous enterocytes and not crypt epithelium as assessed morphologically here and elsewhere, it suggests that IEC-6 cells have a mature phenotype at least as far as iron absorption is concerned (22–25).

Because of the recognized role of DMT1 in the uptake of divalent metals and IREG1 in Fe release, we next examined whether these transport processes are present in IEC-6 cells. From preliminary studies, the addition of Fe(II) at a concentration known to saturate the transporter resulted in the uptake of Fe(II). Furthermore, our results are consistent with the requirement of a proton gradient for DMT1 to transport Fe(II) in the uptake step by showing this was highest at pH 5.5 and least effective at 7.5 (10). Analysis of the data for the uptake of iron over time showed that this was curvilinear, suggesting the coexistence of an efflux transporter. Indeed we found that cells preloaded with radiolabeled iron released 50% of this within 60 min, revealing an efflux transporter. Interestingly, the release of iron from these cells does not require the presence of Cp or apotransferrin, nor was iron efflux increased when these were added to the medium. This differs from hepatocytes and macrophages that require the ferroxidase activity of Cp for most efficient release of iron as evidenced by its retention in the absence of Cp (26). These results therefore appear consistent with the functioning of the Cp homologue hephaestin in the release of iron from the enterocyte (8). Hephæst in is predicted to contain a carboxy terminal transmembrane domain, suggesting that it operates extracellularly (8). We tested this possibility by determining protein-dependent copper oxidase activity both within and outside the cell and found it present only within IEC-6 cells. This suggests that hephaestin functions intracellularly and supports the observation that it occupies a perinuclear location in villous enterocytes (27).

The expression of DMT1, IREG1 and the likely presence of hephaestin in IEC-6 cells are consistent with these proteins functioning in the uptake and efflux transport pathways. These findings differ from the only other study assessing Fe(II) uptake in IEC-6 cells performed 10 years ago (28). They showed an uptake carrier in confluent cells, but there was no evidence of an efflux transporter. Here we show that these cells have both uptake and efflux transporters and have similar efficiencies in proliferating and stationary cells. Apart from the use of these cells in different states of proliferation, there appear to be no other major differences in the methodology of the two studies.

An important property of iron absorption is that it is regulated. In vivo iron absorption is increased by anemia, hypoxia, erythropoiesis and feeding diets low in iron; conversely it is inhibited by high iron stores (29–32). Variations in uptake of Fe(II) are mediated by alterations in the expression of DMT1, whereas IREG1 appears to respond only to...
severe iron deficiency (5,7,33). In view of this, we tested whether expression and function of these transport proteins in IEC-6 cells changed with variation in iron loading as is seen in vivo. DMT1 increased in proportion to uptake and this in turn was inversely related to cellular iron stores, a finding that supports studies using a human cell line (15,16). In addition to the ∼66-kDa product that can be predicted from the cDNA of DMT1, we also found two smaller products in iron-deficient cells and only one in control cells. Previously, it was suggested that DMT1 is produced de novo and is subject to rapid degradation if iron stores rise (34). It is therefore possible that these smaller bands represent degradation of DMT1 as a result of activity associated with the uptake of iron that would be greatest with iron deficiency. Interestingly, the larger mass of DMT1 obtained from in vivo preparations compared with IEC-6 cells can be explained by glycosylation of DMT1 in mucosal enterocytes. This is based on the reduction in size of DMT1 to that of IEC-6 cells after glycosidase F treatment.

Ferritin expression increased with cellular iron levels and is consistent with its role in iron sequestration to prevent oxidative damage. The expressions of DMT1 and Fn conform to the known molecular mechanisms regulating their expression. In the case of DMT1 and Fn, there is an IRE within the 3′ and 5′ untranslated regions (UTR) of the mRNAs encoding these genes, respectively. With a decrease in cytoplasmic iron levels, there is increased activity of iron responsive proteins (IRP) I

![Figure 9](https://academic.oup.com/jn/article-abstract/132/4/680/4687346)

**FIGURE 9** Confocal immunofluorescence microscopy of IEC-6 cells (A-C) and rat duodenum (D and E) detecting the expression of rat divalent metal transporter 1 (DMT1). In IEC-6 cells, staining was seen only at the cell membrane at 4°C (A) as well as intracellularly at 37°C (B). In cells fixed before preparation, staining was identical to that when viable cells were studied at 37°C (C). Low (D) and high (E) power photomicrographs detecting the expression of DMT1 in a frozen section of duodenum from a rat fed the iron-deficient diet. Staining was predominantly found along the apical membrane in villous enterocytes. Arrows in D demonstrate crypt-villus junction. Arrows in E demonstrate apical (right arrow) and basolateral (left arrow) membranes. Preimmune serum was substituted for the primary antibodies to test the specificity of the reaction and showed no reaction (F).

![Figure 10](https://academic.oup.com/jn/article-abstract/132/4/680/4687346)

**FIGURE 10** Confocal immunofluorescence microscopy of IEC-6 cells prepared for the expression of rat basolateral transporter (IREG1). Staining was only seen at the cell membrane at 4°C (A) as well as intracellularly at 37°C (B) and with fixed cells (C).
and II which leads to increased binding to IRE. This stabilizes the DMT1 transcript, enhancing the translation of DMT1 but reducing that of Fn. Thus, with cellular iron deficiency, increased expression of DMT1 leads to greater uptake of Fe(II) and because there is less Fn to sequester the iron, it is exported from the cell. Conversely, increased iron stores result in the degradation of DMT1 mRNA and inhibition of translation. The lack of detection of DMT1 in iron-loaded IEC-6 cells by Western blotting in this study supports this interpretation. Furthermore, because the DMT1 antibody used here recognizes both IRE and non-IRE forms of the protein, the non-IRE form would be expressed under iron-loaded conditions. Because no DMT1 was detected under these conditions, it suggests that DMT1 non-IRE is expressed at levels below detection or is absent from IEC-6 cells. In contrast to DMT1, gests that DMT1 non-IRE is expressed at levels below detection or is absent from IEC-6 cells. In contrast to DMT1,

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LITERATURE CITED