Overexpression of the Ferritin Iron-responsive Element Decreases the Labile Iron Pool and Abolishes the Regulation of Iron Absorption by Intestinal Epithelial (Caco-2) Cells*

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Mammalian cells regulate iron levels tightly through the activity of iron-regulatory proteins (IRPs) that bind to RNA motifs called iron-responsive elements (IREs). When cells become iron-depleted, IRPs bind to IREs present in the mRNAs of ferritin and the transferrin receptor, resulting in diminished translation of the ferritin mRNA and increased translation of the transferrin receptor mRNA. Likewise, intestinal epithelial cells regulate iron absorption by a process that also depends on the intracellular levels of iron. Although intestinal epithelial cells have an active IRE/IRP system, it has not been proven that this system is involved in the regulation of iron absorption in these cells. In this study, we characterized the effect of overexpression of the ferritin IRE on iron absorption by Caco-2 cells, a model of intestinal epithelial cells. Cells overexpressing ferritin IRE had increased levels of ferritin, whereas the levels of the transferrin receptor were decreased. Iron absorption in IRE-transfected cells was deregulated: iron uptake from the apical medium was increased, but the capacity to retain this newly incorporated iron diminished. Cells overexpressing IRE were not able to control iron absorption as a function of intracellular iron, because both iron-deficient cells as well as iron-loaded cells absorbed similarly high levels of iron. The labile iron pool of IRE-transfected cell was extremely low. Likewise, the reduction of the labile iron pool in control cells resulted in cells having increased iron absorption. These results indicate that cells overexpressing IRE do not regulate iron absorption, an effect associated with decreased levels of the regulatory iron pool.

Intestinal epithelial cells respond to a fall in body iron stores by increasing the absorption of dietary iron, so the extent of iron transport through the intestinal epithelium is inversely related to the content of body iron stores (1–3). Knowledge of the molecular mechanisms involved in the regulation of transintestinal iron transport through the intestine remains elusive, in part because of the heterogeneity in age and iron content of intestinal cells. The use of cultured human cell lines that undergo spontaneous differentiation to enterocytes helps to bypass this problem because they represent cell populations homogeneous in age. In particular, Caco-2 cells have been described as an excellent in vitro model of human enterocytes (4, 5). Caco-2 cells grown on bicameral inserts exhibit both apical iron uptake (3, 4) and transferrin-mediated basolateral iron uptake (6). Caco-2 cells reduce Fe3+ to Fe2+ in the apical medium, and this reduction correlates with increased iron uptake (7, 8). Moreover, the levels of intracellular iron (3) control the mechanisms responsible for the regulation of iron absorption through as yet unknown mechanisms.

Iron-regulatory proteins (IRPs) are cytosolic proteins that bind to structural elements, named iron-responsive elements (IREs). These IREs are present in the untranslated region of mRNAs that codify for ferritin, the transferrin receptor, and aminolevulinate synthase (9–16). Based on the structure of mitochondrial aconitase (17), IRP1 (relative mobility 98 kDa) has been proposed to possess four domains, in which domains 1, 2, and 3 are connected to domain 4 through a hinge region (for review, see Ref. 11). The activities of both IRP1 and IRP2 respond to cellular iron through different mechanisms. Low levels of intracellular iron cause IRP1 to bind to, and stabilize, transferrin receptor (TfR) mRNA and to bind to ferritin mRNA, diminishing its translation (9–11), whereas IRP2 activity is regulated through iron-induced IRP2 ubiquitination and proteasome degradation (16). Overexpression of a mutant IRP1 constitutively active in binding to IRE produced cells that express high levels of TfR despite iron repletion (18). This was the first direct demonstration of IRP1 involvement on the expression of proteins of iron metabolism.

Intestinal cells have an active IRE/IRP system (19–21). Caco-2 cells cultured to different intracellular iron concentrations regulate in a concerted way IRP1 and IRK2 activities, apical iron uptake activity, ferritin levels, and transferrin receptor density (19). Interestingly, a fraction of the IRP-2 activity in Caco-2 cells was unresponsive to iron overload, producing basal levels of apical iron uptake and TfR (19). IRP activity was found normal in individuals with hemochromatosis (20), and a decrease in ferritin expression took place in the duodenum from individuals with idiopathic hemochromatosis or iron deficiency anemia (21). High IRP activity was found in monocytes of patients with hereditary hemochromatosis, an indication that one characteristic of this disease might be a decrease of the labile iron pool (22). Hence, there is convincing evidence of the presence of an active IRE/IRP system in intestinal cells, but its role in the regulation of intestinal iron absorption is unclear.

Because intracellular iron is involved in regulating both IRP activity and iron absorption, we tested the hypothesis that iron absorption...
absorption is regulated by the IRE/IRP system. Reasoning that excess IRE should functionally abrogate the mRNA binding activity of IRPs, we generated Caco-2 cells that overexpressed IRE and characterized the ability of these cells to regulate iron absorption.

EXPERIMENTAL PROCEDURES

Reagents—Anti-human transferrin antibody was from Calbiochem. Fetal bovine serum, culture medium, dextrose, phosphate buffered saline (PBS), and antibiotics were purchased from Sigma. 59Fe and 55Fe, the ferric chloride form, [32P]ATP, and (a-32P)UTP were from New England Nuclear products. Culture plasticware and Transwell inserts were from Costar (Cambridge, MA). To eliminate contaminant iron, all buffer solutions were filtered through Chelex-100 (Sigma).

Cell Culture—Caco-2 cells, from the American Type Culture Collection (HTB37, Rockville, MD), were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Culture medium was changed every 2 or 3 days. Cells were treated with trypsin and replated once a week. For transport and binding experiments, cells were grown on 1 cm2 polycarbonate cell culture inserts, with 0.4-m pore size membranes. Cells grown for 12–14 days were used.

Subcloning of IRE and Cell Transfections—The DNA encoding for human RNA-binding protein IRE (11) was obtained by amplifying the polylinker segment of the pSPT-fer plasmid (10) by polymerase chain reaction using T7 and Sp6 as primers (Life Technologies, Inc). After restriction with EcoRl and Xhol, the main IRE segment, 5′-AATTCTGCTTACCA-CAGTGCTTGGACGGAATCCCUGGGATCT-3′, was cloned into the polylinker of pcDNA3 (Invitorgen, San Diego, CA), previously restricted with EcoRl and Xhol. Restriction analysis of pcDNA3 and pcDNA-IRE plasmid with HindIII and NotI demonstrated that the IRE was inserted in the plasmid. The plasmid obtained was named pcDNA-IRE.

Caco-2 cells grown to half-confluence (2–3 days after plating) were transfected with equal amounts of either pcDNA-IRE or pcDNA3 plasmids. LipofectAMINE (Life Technologies, Inc.) at 5 μg/ml of DNA was used for the transfections. The DNA/LipofectAMINE mixture was removed after 36 h of incubation at 37 °C. The cells were incubated overnight in DMEM and 10% FBS and then changed to a selection medium, DMEM with 10% FBS plus 0.4 mg/ml Geneticin (Life Technologies, Inc. G418). The cells were grown for three passages (one-week growth, trypsin treatment, and replating) under these conditions. Wild type Caco-2 cells did not grow under these conditions. Transfected cells were stored in liquid nitrogen.

Analysis of IRE and Ferritin Levels—Total cell RNA was isolated from Caco-2 cells as described elsewhere (23), and equal amounts of RNA were electrophoresed in 1.5% agarose under denaturing conditions. To confirm that each line contained equal amounts of RNA, the ribosomal content of each lane was determined with ethidium bromide. RNA, transferred to Hybond-N membranes (Amersham Pharmacia Biotech), was hybridized with a 32P-labeled IRE probe, consisting of a 28-mer antisense sequence of human IRE, end-labeled with [32P]ATP. As a positive control, the sense ferritin IRE sequence was electrophoresed and hybridized with the above probe.

Assay of IRE Activity—32P-Labeled IRE was prepared by in vitro transcription of pSPT-fer (10) using T7 RNA polymerase (Life Technologies, Inc.) and (a-32P)UTP. The IRE binding activity of IRPs was determined by a band shift assay as described (19), reacting 20 μg of cell extract/assay with 4 × 108 cpm of 32P-labeled IRE.

Determination of Intracellular Ferritin Levels—Intracellular levels of ferritin were determined in Caco-2 cell extracts containing different concentrations of iron, using a sandwich enzyme-linked immunosorbent assay as described (19). Polyclonal rabbit anti-human ferritin and peroxidase-labeled rabbit anti-human ferritin antibodies were purchased from Dako Corporation (Carpinteria, CA).

Measurement of Transferrin Receptor Density—Transferrin receptor density was determined in cell extracts by an enzyme-linked immunosorbent assay (24) using OKT9 anti-TR monoclonal antibody as primary antibody and peroxidase-labeled goat anti-mouse IgG (Sigma) as secondary antibody.

Equilibrium Binding of Caco-2 Cells with [55Fe]—Cells with known concentrations of intracellular iron were obtained as described (3). Briefly, Caco-2 cells were seeded at 5 × 104 cells/cm2 for 24 h and incubated for a week in low iron Iscove medium (Life Technologies, Inc.) and 10% low iron serum ([iron] < 0.3 μmol/liter) (1), supplemented with variable amounts of Fe as the complex FeCl3·nH2O or FeCl2·nH2O. After the period of the cells reached confluence, 2 × 105 cells/cm2 were used. The cells were trypsin treated and seeded at a density of 1 × 106 cells/flask and cultured as above. After a second trypsin treatment, the cells were plated on 1 cm2 polycarbonate inserts (Transwell, COSTAR, Cambridge, MA) and were cultured in media as before for 12–14 days, with change of media every 3 or 4 days. When needed, the insert-grown cells were transfected with pcDNA3-IRE 7 days before the experiment. Measuring the transepithelial electrical resistance with an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) monitored the formation of a cell monolayer in the inserts. The total intracellular iron concentration was estimated from the specific radioactivity of the 55Fe in the medium and a cell volume of 5 μl/cell inserts (3).

Cell Extracts—To prepare cell extracts, cells were treated with lysis buffer (50 μl of 106 cells of 10 mM HEPES, pH 7.5, 3 mM MgCl2, 40 mM KCI, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.7 μg/ml pepstatin A, 5% glycerol, 1 mM dithiothreitol, 0.6% Triton X-100). The mixture was incubated for 15 min on ice and sedimented for 10 min at 10,000 g. The supernatant was stored at 70 °C, and aliquots were used for the determination of ferritin, THR density, IRP activity, and IRP immunodetection.

For 55Fe flux experiments we used 3Fe-equilibrated cells (see above), grown on 1 cm2 inserts transfected with either pcDNA3 or pcDNA3-IRE. At this time there were, on average, 600,000 cells/insert, and the transepithelial electrical resistance was 250–300 ohm cm2. To ensure integrity of the cell barrier, we measured the transepithelial electrical resistance at the beginning and at the end of each experiment, discarding all inserts with transepithelial electrical resistance lower than 220 ohms cm2 at the end of the experiment. Cells were incubated at 37 °C for 30–120 min with 10 μM Fe3+, as the 55Fe/NTA complex, added to the apical medium. The incubation medium was low iron Iscove medium. Iron uptake was stopped by washing the inserts three times with ice-cold saline supplemented with 1 μM EDDA. 55Fe radioactivity in the cells, and in the basolateral media, was measured in a Cobra II gamma counter (Packard Instrument Co., Meriden, CT). Uptake was expressed as the rates (mol of 55Fe × h−1 × insert−1), estimated by linear regression fitting of the data.

Determination of the Labile Iron Pool—The intracellular pool of iron concentration of Caco-2 cells was determined as described by Epstein et al. (26). Briefly, Caco-2 cells were cultured on coverslips for 10 days in DMEM, 10% FBS. Calcine-AM (0.5 μM, Molecular Probes, Eugene, OR), was then loaded into for 5 min at 37 °C. After washing the calcine that was not internalized, the cells were transferred to a cuvette containing 3 ml of MOPS saline (20 mM MOPS-OH, 150 mM NaCl, 1.8 mM CaCl2, 5 mM glucose, pH 7.4) and 5 μl of anti-calcine antibody (the kind gift of Dr. Z. I. Cabantchik). After determination of the basal calcine fluorescence (excitation 488 nm, emission 517 nm), the fluorescence of the calcine-iron complex was generated by addition of 100 μM SIH. The increase in fluorescence thus obtained was directly proportional to the iron labile pool. In the experiments indicated in the text, the cells were preincubated for 2.5 h either in iron-rich medium (DMEM, 10% FBS + 10 μM FeNTA) or in iron-poor medium (Iscove, 10% low iron FBS) prior to calcine loading.

Data Analysis—Variables were tested in triplicate wells, and experiments were repeated at least twice. The variability between experiments was <20%. Curve fitting was done using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA).

RESULTS

Cellular Levels of IRE, Ferritin, and TfR in IRE-transfected Cells—The cellular level of IRE was determined in cells transfected with pcDNA3-IRE and in cells transfected only with pcDNA3 (Fig. 1). After hybridization with 32P-antisense IRE, cells transfected with plasmid alone showed one band of about 800 nucleotides, most probably corresponding to ferritin mRNA (26). Cells transfected with pcDNA3-IRE evidenced a band of about 800 nucleotides and a lower band of about 200 nucleotides (Fig. 1A). This lower band most probably corresponds to IRE. It is unknown by its hybridization with the antisense IRE. An assay of IRE activity showed decreased activity in IRE-transfected cells compared with cell transfected with pcDNA3 or not transfected (Fig. 1B). The decreased activity could be caused by competition of 32P-IRE binding by unlabeled endogenous IRE.

In fact, little or no decrease in IRP activity was observed when 2 μg of cell extract was used per assay instead of the 20 μg used in the present assay (not shown).

The IRE/IRP System Regulates Iron Absorption
FIG. 1. Characterization of IRE-transfected cells. Panel A, Northern blot of pcDNA3 (control) and pcDNA3-IRE-transfected cells. Insert-grown Caco-2 cells were transfected with either pcDNA3 or pcDNA3-IRE. After 7 days in culture, total RNA was obtained and separated in denaturant agarose gels. The proteins were blotted to Hybond-N membranes and were hybridized with a 32P-labeled IRE probe, in the antisense sequence of ferritin IRE, end-labeled with [α-32P]ATP. Left lane, sense IRE sequence; center lane, cells transfected with pcDNA3; right lane, cells transfected with pcDNA3-IRE. Panel B, band shift assay of pcDNA3-transfected (lanes 1 and 4), pcDNA3-IRE-transfected (lanes 2 and 5), and wild type (lanes 3 and 6) Caco-2 cells, in the absence (lanes 1–3) or presence (lanes 4–6) of β-mercaptoethanol (β-ME). Panel C, 55Fe content in control and IRE-transfected cells, after culture of cells for 7 days in media containing low (0.5 μM), medium (2 μM), and high (5 μM) 55Fe. Panel D, ferritin levels of control and IRE-transfected cells grown as in panel C. Panel E, TfR levels in control and IRE-transfected cells grown as in panel C. After a week in culture with different concentrations of 55Fe in the culture media, the intracellular concentration of 55Fe in control cells varied according to a previously established pattern (3). For the experiment shown in Fig. 1C, the intracellular iron concentrations in cells grown in culture media containing 0.5, 2, or 5 μM 55Fe were 21.6, 62.3, and 243.5 μM, respectively. In contrast, the intracellular concentration of 55Fe in IRE-transfected cells remained relatively constant, regardless of the concentration of iron in the culture. Intracellular 55Fe concentrations of 207.1, 210.1, and 238.3 μM were found for cells grown in 0.5, 2, or 5 μM 55Fe, respectively (Fig. 1C). At low intracellular iron concentration, the cell ferritin concentration in IRE-transfected cells was higher than in control cells, as expected from IRE binding to active IRP and hence inhibiting IRP binding to the IRE motif in ferritin mRNA. Only at high intracellular iron levels was the ferritin content equal in control and IRE-transfected cells (Fig. 1D). At low intracellular iron levels, TfR levels were about 2-fold lower in IRE-overexpressing cells compared with control pcDNA3-transfected cells (Fig. 1E), probably reflecting the lack of stabilization of TfR mRNA. Furthermore, TfR levels in IRE-transfected cells did not change in response to changes in intracellular iron, whereas control cells showed a significant decrease with increasing cell iron (Fig. 1E). These results indicate that in relation to control cells, cells transfected with pcDNA3-IRE up-regulated their ferritin content, down-regulated their TfR levels, and both ferritin and TfR levels did not change with changes in cellular iron.

IRE-transfected Cells Have Deregulated Iron Transport Activity—Prior evidence indicates that IRP-1 activity, IRP-2 mass, TfR density, ferritin levels, and transepithelial iron transport respond similarly to changes in intracellular iron concentration (19). These results suggest that a common iron responsive factor regulates both intracellular iron levels and iron absorption by Caco-2 cells. Thus, it was of interest to study apical iron uptake and transepithelial iron transport as a function of intracellular iron concentration in IRE-transfected cells. For this purpose, control and transfected cells were grown in bicameral inserts, and their capacities for apical 59Fe uptake and transepithelial 59Fe transport activities were compared (Fig. 2). The rate of 59Fe uptake by IRE-transfected cells was about twice as large as that of control cells (Fig. 2A). Most of the
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Fig. 3. Determination of the labile iron pool. Wild type Caco-2 cells (A–C), Caco-2 cells transfected with pcDNA3 (D), or cells transfected with pcDNA3-IRE (E), were grown in glass coverslips for 10 days in DMEM, 10% FBS. The labile iron pool was then measured either directly (A, D, and E) or after incubation for 2.5 h in a iron-poor medium (B) or a iron-rich medium (C). SIH is a membrane-permeant iron chelator that takes iron from the calcein-iron chelate thus increasing calcein fluorescence. Therefore, the level of the cellular labile iron pool is directly proportional to the increase in SIH-induced calcein fluorescence. Shown are representative traces of intracellular calcein fluorescence. The right column shows the mean ± S.D. of fluorescence changes in arbitrary fluorescence units (ΔAFU), for six determinations.

The increased apical iron uptake could be caused by increased expression of a putative apical iron transporter or increased activity of the transporter. In the latter case, it is possible that the high levels of ferritin and the low levels of TIR induced by IRE overexpression resulted in low levels of the regulatory iron pool, which in turn should result in the shifting of the chemical equilibrium toward the entry of iron during apical iron uptake. Hence, we determined the regulatory, or labile, iron pool of cells subjected to varied manipulations (Fig. 3). Whereas wild type and pcDNA3-transfected cells presented a sizable labile iron pool (Fig. 3, A and D), cells transfected with pcDNA3-IRE had a very small iron labile pool (Fig. 3E). The labile iron pool was diminished in cells preincubated in low iron medium (Fig. 3B) and was increased in cells preincubated in high iron medium (Fig. 3C). Mean values, in arbitrary fluorescence units, for five independent determinations were 190, 70, 338, 184, and 22, for normal, high iron, low iron, pcDNA3-transfected, and pcDNA3-IRE-transfected cells, respectively.

The decrease in the labile iron pool could be the cause of the increased iron uptake observed in IRE-transfected cells, because a low intracellular iron pool should drive the transport of iron toward the cell interior. To test this hypothesis, the regulatory iron pool of control cells was decreased by preincubation of the cells in iron-free culture medium prior to the 59Fe transport assay. Preincubation of Caco-2 cells in iron-free media induced a doubling in the rate of apical to basolateral 59Fe transport after 5 h of preincubation, whereas preincubation of IRE-transfected cells did not induce it (Fig. 4). Hence, manipulation of the regulatory iron pool induced in Caco-2 cells a quick response in the rate of apical iron uptake and transepithelial iron transport. This response was abrogated in Caco-2 cells overexpressing IRE.

DISCUSSION

The cellular level of iron is regulated by the activity of proteins known as IRPs. IRPs bind to hairpin motifs present in the mRNA of several key proteins of iron metabolism, such as ferritin and TIR, modulating their translation. Because both the mRNA binding activity of IRPs and iron uptake by intestinal epithelial cells are regulated by the cellular level of iron, in this study we investigated if the activity of the IRE/IRP system regulates intestinal iron absorption. To that end, we overexpressed IRE in Caco-2 cells, reasoning that by binding to available IRE, excess IRE should functionally decrease or eliminate IRP mRNA binding activity. This approach was preferred to the expression of dominant negative IRP, because intestinal cells have both IRP1 and IRP2, so cells should be transfected with both IRP1 and IRP2 dominant negative genes to abrogate IRP activity.

We found that regardless of the extracellular iron concentration present during cell culture, cells overexpressing IRE presented constitutively high levels of intracellular iron, probably the result of high ferritin levels (Fig. 1). But, contrary to expectations, apical 59Fe uptake was higher in IRE cells than in control cells (Fig. 2). Because the passage of iron from the lumen of the intestine into the enterocyte is mediated by one or more membrane iron transporters (27–29), the increased iron uptake observed in IRE cells could be the result of increased...
expression of the transporters or their increased activity. Two mammalian membrane iron transporters have been cloned, DCT1, also named Nramp2, (27, 28) and SFT (29). DCT1 is an electrogenic cation-H⁺ co-transporter with high levels of transcripts in kidney and the microvilli region of intestine (27). STF is an iron transporter identified by expression cloning of a K562 library, found in perinuclear vacuolar structures resembling recycling endosomes (29). The tissue and cellular locations of DCT1 and SFT indicate that the primary function of STF may be the acquisition of transferrin-derived iron through the endocytosis process, whereas the function of DCT1 may be the apical transport of iron by intestinal epithelial cells. Although the mRNA of STF does not have an IRE motif, alternative splicing of DCT1 produces a DCT1 without IRE and a DCT1 with one IRE motif in its 3′-untranslated region (30). If, by analogy with TfR mRNA, the binding of IRP stabilizes DCT1(IRE) mRNA, then decreased translation of this mRNA should be expected in Caco-2 IRE cells.

The results found in this work indicate that apical transport activity was increased in Caco-2 cells overexpressing ferritin IRE. Therefore, any effect of IRE overexpression on decreasing DCT1(IRE) mass was overwhelm by its effects on other component(s) involved in the iron absorption process. Indeed, the high levels of ferritin and the low levels of TfR induced by IRE overexpression resulted in very low levels of the labile iron pool. This decrease should result in a favorable chemical gradient for the entry of iron during apical iron uptake. This favorable gradient may underlie the observed increase in apical iron uptake by Caco-2-IRE cells. Nevertheless, the possible participation of some yet not described IRE-containing element that might exert a negative control on iron absorption cannot be discarded.

Decrease of the labile iron pool after a short preincubations in low iron media produced a marked increase in transepithelial iron transport. A correlate to these findings is found in the observation that rats subjected to short-term dietary iron depletion respond with a quick increase in iron absorption, as if they were anemic (31). So it is possible that the lowering of the labile iron pool sets in the cells an “anemia signal,” triggering, as a response, increased iron absorption.

In summary, Caco-2 cells overexpressing IRE presented elevated levels of ferritin and diminished levels of TfR, as expected if excess IRE should bind to active IRP and abolish IRP control of ferritin and TfR synthesis. Moreover, IRE-overexpressing cells presented constitutively low levels of the labile iron pool and high rates of apical iron uptake and transepithelial iron transport. Thus, the present results indicate that the IRE/IRP system regulates intestinal iron absorption through the regulation of the labile iron pool.

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