Dietary iron induces rapid changes in rat intestinal divalent metal transporter expression

KWO-YIH YEHT,1,3 MARY YEH,1,3 J. ABRA WATKINS,1,3
JUAN RODRIGUEZ-PARIS,1,3 AND JONATHAN GLASS1,3
1Section of Hematology/Oncology, Departments of Medicine and 2Molecular and Cellular Physiology, and 3Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130

Received 11 November 1999; accepted in final form 25 May 2000

Yeh, Kwo-Yih, Mary Yeh, J. Abra Watkins, Juan Rodriguez-Paris, and Jonathan Glass. Dietary iron induces rapid changes in rat intestinal divalent metal transporter expression. Am J Physiol Gastrointest Liver Physiol 279: G1070–G1079, 2000.—The divalent metal transporter (DMT1, also known as NRAMP2 or DCT1) is the likely target for regulation of intestinal iron absorption by iron stores. We investigated changes in intestinal DMT1 expression after a bolus of dietary iron in iron-deficient Belgrade rats homozgyous for the DMT1 G185R mutation (b/b) and phenotypically normal heterozygous littermates (+/b). Immunofluorescent staining with anti-DMT1 antisera showed that DMT1 was located in the brush-border membrane. Duodenal DMT1 mRNA and protein levels were six- and twofold higher, respectively, in b/b rats than in +/b rats. At 1.5 h after dietary iron intake in +/b and b/b rats, DMT1 was internalized into cytoplasmic vesicles. At 1.5 and 3 h after iron intake in +/b and b/b rats, there was a rapid decrease of DMT1 mRNA and a transient increase of DMT1 protein. The decrease of DMT1 mRNA was specific, because ferritin mRNA was unchanged. After iron intake, an increase in ferritin protein and decrease in iron-regulatory protein binding activity occurred, reflecting elevated intracellular iron pools. Thus intestinal DMT1 rapidly responds to dietary iron in both +/b and b/b rats. The internalization of DMT1 may be an acute regulatory mechanism to limit iron uptake. In addition, the results suggest that in the Belgrade rat DMT1 with the G185R mutation is not an absolute block to iron.

enhanced green fluorescent protein-divalent metal transporter-1 fusion protein; subcellular localization; iron regulatory protein activity electrophoretic mobility shift assays; ferritin expression

Iron is a nutrient required for a wide range of critical proteins, such as electron transfer proteins, hemoglobin, and ribonucleotide reductase for metabolism, growth, and proliferation (8, 29). To combat the inherent toxicity of iron in the promotion of oxidation (18, 21) and the insolubility of Fe3+, eukaryocytes have evolved unique mechanisms for cellular iron uptake and transport (8, 29), including the regulation of intestinal absorption of dietary iron by body iron status.

When body iron stores are low, iron absorption is high, and when body iron stores are high, iron absorption is low (29). The mechanisms underlying the regulation of intestinal iron absorption by body iron stores are now being elucidated. Recently (16), the cDNA has been cloned for divalent metal transporter DMT1 (also known as natural resistance-associated macrophage protein 2 (NRAMP2) or DCT1), an intestinal iron transporter that is upregulated in rats fed an iron-deficient diet. In two animal models, the microcytic anemic mouse and the Belgrade rat, a defect in intestinal iron absorption (9, 28) occurs because of a common missense mutation, G185R, in the DMT1 gene (12, 13). Recently, DMT1 has been demonstrated in the intestinal brush-border membrane (BBM) and has been observed to increase with iron deficiency (5, 32). Furthermore, in mice with a knockout of the hereditary hemochromatosis gene, which produces a mouse model for hereditary hemochromatosis, there is an increase of intestinal DMT1 expression (14). The result is an increase of dietary iron absorption despite elevated serum transferrin saturation and body iron stores (14). Thus DMT1 appears to be directly involved in the iron uptake at the BBM of the absorptive cells.

It is now known that intestinal expression of DMT1 mRNA and protein is regulated by iron status (16, 32). However, it is unknown whether coordinated changes in DMT1 mRNA and protein occur rapidly in response to dietary iron intake or only after there are alterations in the total iron stores of the organism. Because the major DMT1 mRNA isoform expressed in the intestine contains an iron-regulatory element (IRE) in the 3'-untranslated region (3'-UTR) (14, 16), it is likely that intestinal DMT1-IRE mRNA levels are negatively regulated by iron through inactivation of iron-regulatory protein (IRP). In an analogous situation, IRP acting on a 3' IRE is the mechanism that rapidly regulates transferrin receptor expression (27). In contrast, decreased IRP binding to the IRE in the 5'-UTR of ferritin mRNA derepresses ferritin mRNA translation (2, 34). Thus the IRP pathway may quickly up- or down-regulate iron-regulatory protein activity.
regulate intestinal DMT1 mRNA levels followed by parallel changes in DMT1 protein levels. To test this possibility, we determined the duodenal DMT1 mRNA and protein levels and IRP binding activity at defined times after a bolus iron feeding in phenotypically normal heterozygous (+/b) and anemic homozygous (b/b) Belgrade rats. The use of b/b rats also allowed us to examine whether the DMT1 mRNA is upregulated in iron deficiency and whether the expression of DMT1 after iron feeding shows the same pattern as the phenotypically normal +/b rats, which have iron uptake indistinguishable from +/- rats (11, 28). In addition, the b/b rats permitted us to examine whether the mutant DMT1 protein was appropriately localized in the enterocyte for dietary iron uptake. In the present study, we have produced rabbit antibodies specific to DMT1 and found that DMT1 is expressed in the BBM and to lesser degree in basolateral membrane of enterocytes of both the +/b and b/b rats. We also demonstrate that dietary iron induced in both +/b and b/b intestinal epithelium an unexpected initial increase of intestinal DMT1 protein levels while rapid decreases of DMT1 mRNA were occurring. The finding that dietary iron decreases mucosal IRP binding activity, reduces DMT1 mRNA, and increases ferritin protein levels in both +/b and b/b rats indicates that the IRP pathway in the regulation of enterocyte iron homeostasis occurs normally in b/b rats.

MATERIALS AND METHODS

Materials. Tris, hydrochloric acid, phenylmethylsulfonyl fluoride (PMSF), HEPES, dithiothreitol, sucrose, glycerol, Nonidet P-40, leupeptin, pepstatin, aprotinin, Triton X-100, and ferrous sulfate were purchased from Sigma Chemical (St. Louis, MO). DMEM, fetal bovine serum (FBS), penicillin, streptomycin, 1-glutamine, and lipofectamine were from GIBCO BRL (Gaithersburg, MD). p-Enhanced green fluorescent protein (EGFP) vectors were from Clontech (Palo Alto, CA). SDS, acrylamide, bisacylamide, and agarose were obtained from Bio-Rad Laboratories (Richmond, CA). Guanidine thiocyanate and b-mercaptoethanol were from Eastman Kodak (Rochester, NY). [32P]UTP was from NEN (Boston, MA). Hybond N nylon membrane and enhanced chemiluminescence Western blotting kits were from Amersham (Arlington Heights, IL). QuickHyb rapid hybridization solution, p-CR Script cloning kit, Prime-It II random primer labeling kit, and RNA transcription kit were from Stratagene (La Jolla, CA).

Animals. Phenotypically normal heterozygous (+/b) and anemic homozygous (b/b) Belgrade rats were bred and maintained in the animal quarters of Louisiana State University Health Sciences Center. Both the heterozygous and homozygous rats were obtained by mating homozygous (b/b) males with heterozygous (+/b) females. The homozygous (b/b) pups were identified by high reticuloocyte counts and low hematomas. The rats used for the study were weaned on a standard Harlan Tekled 22/5 rodent diet for 2 mo. Hepatic ferritin levels in b/b rats vs. +/b rats verified the iron-deficient state of the b/b rats. In b/b rats, hepatic ferritin levels were 15 ± 3 µg/gm wet wt (n = 3) compared with 211 ± 52 µg/gm wet wt (n = 3) in +/b rats. Before studies of DMT1 expression, rats housed in individual cages were fasted overnight with 5% glucose saline solution ad libitum. The rats were fed a piece of chow (250 mg) containing a defined amount of iron as FeSO4 at a dose of 50 µg iron/g body wt. Usually the chow was consumed within 5 min, and the rats were killed 0, 1.5, 3, and 6 h after dietary iron intake. The duodenum (from the pylorus to the ligament of Treitz) was removed and rinsed with ice-cold PBS. The duodenal mucosa was scraped and used for analysis.

Preparation of rabbit anti-DMT1 antibodies. To produce antibodies against DMT1 protein, we synthesized two polypeptides with amino acid sequences deduced from the rat DMT1 cDNA-IRE clone (16). Polypeptide 1, from 334 to 348 amino acids (CRNSSSPPHLFPND), is located in the presumed fourth external loop shared by the two isoform proteins encoded by both DMT1-IRE and DMT1-non-IRE mRNAs. Polypeptide 2 consists of amino acids from 540 to 553 (CGRS-VSISKVILSE) near the COOH terminal of the protein isoform encoded by DMT1-IRE mRNA. The amino acid sequences of both of these regions are amino acid sequences highly preserved in mammals with little sequence homology to NRAMP1. The polypeptides were covalently linked to keyhole limpet hemocyanin (KLH), emulsified with Freund's complete adjuvant, and then inoculated intradermally into New Zealand White rabbits. The rabbits were boosted with polypeptide-KLH conjugate emulsified in incomplete Freund's adjuvant on days 40 and 80 after the first injection. Antisera were collected 10 days after each boost for determination of titers and specificity. Indirect ELISA determination of anti-peptide antibody titers found that polypeptide 2-induced antiserum (designated as anti-540) showed about three- to fourfold higher titers than that of polypeptide 1-induced antiserum (designated as anti-334).

To verify that the antisera were specific to DMT1, we constructed an EGFP-DMT1 fusion gene. The EGFP in the expressed fusion protein served as an NH2-terminal tag of the DMT1. For the construction of the EGFP-DMT1 fusion gene, we cloned the rat DMT1 cDNA by RT-PCR using pCR-Script cloning kits (Stratagene). This clone (pScript-DMT1) was sequenced and confirmed to contain the correct DMT1 insert of 1751 bp from –3 to 1748 nucleotides (with the coding region extending from 1 to 1686). The pScript-DMT1 plasmid was digested with EcoRI, and the DMT1 cDNA (from –3 to 1748 plus an additional 31 bp from the plasmid) was gel purified and inserted inframto EcoRI linearized pEGFP-C1 vector (Clontech). The functional regions of the fusion gene construct were sequenced and confirmed to be ligated in-frame.

Cell culture and transfection. COS-1 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% l-glutamine. Approximately 1 × 105 cells/plate were seeded in 60-mm plates 16 h before transfection. Cells were either not transfected or transfected with pEGFP or pEGFP-DMT1 by lipofectamine according to the manufacturer's suggested protocol (GIBCO BRL). At 72 h after transfection, cells were rinsed with PBS and examined under an Olympus AX70 Provis fluorescence microscope with Princeton Instrument Micro Max charge-coupled device camera and IP Lab software. Cells were then lysed in RIPA buffer (0.15 mM NaCl, 10 mM NaPO4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 µg/ml apro- tinin, 1 µg/ml leupeptin, and 1 mM sodium orthovanadate) (pH 7.4) containing 1% Triton X-100. The total cell lysate was subjected to Western blot analysis for the determination of the reactivity of rabbit antibodies to EGFP-DMT1 fusion protein.

Indirect immunofluorescent staining. Intracellular location of DMT1 was examined by indirect immunofluorescent staining methods. A 1-cm duodenal segment (5 cm below the pylorus) from rats killed at 0 and 1.5 h after iron intake was...
embedded in OCT compound (Miles, Elkhart, IN), frozen, and sectioned at 5-μm thickness. Sections were mounted on the slide and stained as described previously (33). Briefly, tissue sections were 1) fixed in −20°C acetone for 10 min, 2) washed twice with PBS, 3) preincubated with 10% control rabbit serum at room temperature for 10 min, washed once with PBS, and incubated with anti-540 or anti-334 antisem (1:400 dilution) for 1 h, 4) washed three times with PBS for 10 min each time, 5) incubated with FITC-conjugated goat-IgG anti-rabbit IgG (1:250 dilution) and propidium iodide for 30 min, 6) washed three times with PBS, and 7) mounted in Immunomount and observed with the Olympus AX70 Provis fluorescent microscope as noted above.

Northern blot analysis. Total RNA was isolated from the duodenal mucosa as described previously (33). For Northern blot analysis, 10 μg of total RNA were size-fractionated by electrophoresis on a denaturing 1.0% agarose-formaldehyde gel, and the RNA was transferred to a Hybond N nylon membrane by vacuum. RNA blots were air dried, cross-linked in an ultraviolet chamber, hybridized with labeled probes using QuikHyb hybridization solution, and washed under high stringency (Stratagene). The radioactive signals were stored on a phosphor screen, visualized with a laser scanner using a Storage Phosphor imaging system (Molecular Dynamic, Sunnyvale, CA), and quantitated with ImageQuant software.

The 32P-labeled DMT1, ferritin H, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) riboprobes were prepared by in vitro transcription of linearized rat intestinal DMT1, H ferritin (33), and pTri-GAPDH clone (Ambion, Austin, TX) in the presence of [32P]UTP (NEN). Mucosal ferritin H levels were measured for positive control of translation derepression after dietary iron intake. Mucosal GAPDH mRNA levels were determined for normalization of the amount of RNA loaded onto gels for Northern blot analysis.

Western blot analysis. The duodenal mucosa was washed once in PBS and homogenized in RIPA buffer containing 1% Triton X-100. After centrifugation at 10,000 × g for 5 min at 4°C, the supernatant was transferred to a fresh tube. Protein concentrations in supernatants were determined by the method of Bradford (4). Aliquots of the total cell extract containing 15 μg of protein were subjected to SDS-PAGE under reducing conditions (33) and transferred to nitrocellulose membranes. Molecular weight standards (Santa Cruz Biotech, Santa Cruz, CA) were run in parallel. Membranes were blocked with 5% nonfat powdered milk in a solution of Tris-buffered salt with Tween 20 for 1 h at room temperature for 2 h, incubated with anti-540 (1:5,000), anti-334 (1:2,500), or anti-ferritin (1:8,000) for 16 h at 4°C, washed three times for 10 min each with TBST, and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies for 1 h at room temperature. Immunoreactive proteins were detected by enhanced chemiluminescence Western blotting system (Amersham) and quantified by transmittance densitometry using volume integration with ImageQuant application software.

Determination of IRP binding activity by electrophoretic mobility shift assay. 32P-labeled RNA containing the IRE was prepared by in vitro transcription of the linearized duodenal ferritin H cDNA clone (33). The transcription reaction was carried out in a reaction mixture containing 1.5 mM each of ATP, GTP, and CTP and 50 μCi [32P]UTP (800 Ci/mmol, NEN) with T7 RNA polymerase. The [32P]RNA was purified after urea gel electrophoresis. To prepare the cytoplasmic extract, the mucosal scrape was homogenized in 10 vol of ice-cold lysis buffer (10 mM HEPES, pH 7.5, 3 mM MgCl2, 40 mM KCl, 5% glycerol, 0.3% Nonidet P-40, and 0.5 mM PMSF) with a Dounce homogenizer. The cytoplasmic extract in the supernatant was collected after centrifugation at 13,000 g for 10 min at 4°C and stored at −70°C. Interaction of RNA and protein was analyzed by electrophoretic mobility shift assay (EMSA). Briefly, aliquots of cytoplasmic extracts containing 20 μg of protein were added to a reaction mixture containing 10 mM HEPES, pH 7.5, 3 mM MgCl2, 40 mM KCl, 5% glycerol, 2 mM DTT, and 1 × 105 cpm [32P]RNA probe. After 10 min of incubation at room temperature, RNase T1 (1 U/reaction) and heparin (5 mg/ml) were added into the reaction mix and incubated for 15 min to degrade unbound probe and displace nonspecific protein–RNA interaction. Protein–RNA complexes were resolved on a 5% nondenaturing PAGE. Radioactive signals were detected by PhosphorImager analysis.

Statistical analysis. ANOVA was computed from the experimental data among animal groups. When the F value obtained from ANOVA was significant, Bonferroni’s test was applied to test for differences among groups. P < 0.05 was considered to be significant.

RESULTS

Characterization of antibodies against DMT1. To verify that polypeptides 1 and 2 induced rabbit antisera that reacted against DMT1, we constructed an EGFP-DMT1 fusion gene in the pEGFP-C1 plasmid (Fig. 1A) and transiently expressed the EGFP-DMT1 fusion protein in COS-1 cells. For controls, COS-1 cells were either not transfected or transfected with the pEGFP-C1 plasmid. The nontransfected cells exhibited no fluorescence (not shown). Western blot analysis of cell lysates showed that only the antisera against GFP reacted to a 28-kDa EGFP protein in the cells transfected with the EGFP plasmid alone. In the cells transfected with EGFP-DMT1, a 55-kDa protein was recognized by anti-GFP, anti-540, and anti-334 antibodies (Fig. 1B), suggesting that both the anti-334 and anti-540 antisera recognized DMT1. To further confirm that both the anti-540 and anti-334 antisera detected the entire EFGP-DMT1 protein, an antisera was raised against a KLH-conjugated oligopeptide of amino acids 17–30 (DHGDSASLGAINPAC). This antisera, designated anti-17, reacted with the 55-kDa fusion protein (Fig. 1B), although it also recognized several minor bands. Thus the fusion protein expresses DMT1 as an intact molecule, and the antiserum, each raised to oligopeptides from a different region of DMT1, recognize DMT1.

To confirm that the anti-540 and anti-334 antisera were specific to intestinal DMT1, total cell lysates from the duodenal and proximal jejunal mucosa (5 cm below the ligament of Treitz) were used for Western blots. Anti-540 antisera reacted to a band with an apparent molecular mass of 43 kDa in both duodenal and jejunal cell lysates (Fig. 1C). The inclusion of 0.1 μg/ml polypeptide 2, but not polypeptide 1, during the first antibody incubation blocked the detection of the 43-
kDa protein (Fig. 1C). The anti-334 antiserum reacted to the 43-kDa band and to a faint 41-kDa band (Fig. 1C). The reactivity of the antiserum to both bands was blocked by polypeptide 1 but not polypeptide 2 (data not shown). Thus anti-540 is specific to the DMT1 protein produced by the DMT1-IRE mRNA, and anti-334 reacts to an additional band that probably is incompletely glycosylated or is partially degraded DMT1 protein.

Intracellular localization of DMT1 in COS-1 cells and rat enterocytes. Fluorescent microscopy showed that at 72 h the cells transfected with pEFGP exhibited strong, homogeneously distributed fluorescence (Fig. 2A). In cells transfected with pEFGP-DMT1 plasmids,
Strong fluorescence was located in the cell membrane with some fluorescence in cytoplasmic vesicles and perinuclear cytoplasm (Fig. 2B). Indirect immunofluorescent staining with anti-540 or anti-334 antisera was performed on duodenum from +/b and b/b rats. Previous studies (11, 28) showed that iron uptake in +/b rats was indistinguishable from that in +/+ rats. Hence, the heterozygote rats served as controls for the b/b rats in this and subsequent experiments. Strong immunoreactivity was located in the BBM of enterocytes in both +/b and b/b rats immediately after iron intake (time 0) (Fig. 2, C and D). Modest fluorescence was present in the basolateral membrane and in organelles adjacent to the basal membrane (Fig. 2, C and D). The intensity of the immunoreaction in the BBM appeared to be stronger in b/b rats than in +/b rats, an observation supported by Western blot analysis (see below). Preimmune serum did not show any fluorescence (data not shown). At 1.5 h after iron intake, DMT1 was relocalized with large numbers of immunoreactive cytoplasmic vesicles appearing in the apical cytoplasm of both +/b (E) and b/b (F) rats. The same results were observed using anti-334 antiserum (data not shown).
and basal membranes (Fig. 2F). The DMT1-positive vesicles were not found to be released from the enterocytes.

The induction of vesicle formation could occur either by exposure to iron alone or to the chow containing the iron. To distinguish between these two possibilities, in situ duodenal loops in+/b rats were continuously infused with PBS alone or with 1 mM FeSO₄ at a rate of 6 ml/h for 1.5 h. Only in the enterocytes exposed to iron was there the appearance of numerous DMT1-reactive vesicles in the apical cytoplasm and to a lesser degree adjacent to the basolateral membrane (data not shown). Hence, DMT1 vesicle formation can be ascribed to the exposure to iron and not to feeding per se.

Effect of dietary iron intake on mucosal DMT1 mRNA levels. The presence of an IRE in the 3'-UTR of DMT1 suggests that increased cellular iron will result in lower levels of DMT1 mRNA. Trinder et al. (32) demonstrated by in situ hybridization decreased levels of mRNA in control rats compared with iron-deficient rats. We were interested in determining whether a single bolus of dietary iron would alter DMT1 mRNA levels and whether any effect would be seen in the iron-deficient b/b rats, which exhibit a defect in iron uptake. Northern blots of total RNA from+/b intestine with ³²P-labeled DMT1 riboprobes showed the two previously described 4.5- and 3.5-kb DMT1 mRNA isoforms (Fig. 3). A bolus of dietary iron resulted in a gradual decrease of both DMT1 mRNAs in+/b rats throughout the 6 h of analysis (Fig. 3). In+/b rats, the basal levels of both DMT1 mRNAs were about sixfold higher than those of b/b rats. After dietary iron intake, these levels decreased rapidly during the first 1.5 and 3 h to reach the same low levels at 3 and 6 h as in+/b rats (Fig. 3). In contrast to DMT1, the mucosal H ferritin mRNA levels were not different in the+/b and b/b rats and were not changed after dietary iron intake (Fig. 3A).

Effect of dietary iron intake on mucosal DMT1 protein levels. A lack of coordinated change in mucosal DMT1 protein and mRNA levels occurred after dietary iron intake. In+/b rats, dietary iron induced a transient two- and fourfold increase, rather than the anticipated decrease, of DMT1 protein at 1.5 and 3 h, respectively, after iron intake (Fig. 4). By 6 h, the proteins had decreased to slightly less than baseline levels (Fig. 4). The basal level of mucosal DMT1 protein was about twofold higher in b/b rats than in+/b rats, a relatively smaller difference than seen at the mRNA levels. In the b/b rats, dietary iron also rapidly induced a 2.5-fold increase of DMT1 protein at 1.5 h, after which the protein levels decreased steadily, reaching basal levels at 6 h after iron intake (Fig. 4). To verify that the changes in the DMT1 protein were related to increased intracellular iron, mucosal ferritin levels were determined. Intestinal ferritin increased steadily, resulting in an increase of 5- and 120-fold in+/b and b/b rats, respectively, at 6 h after iron intake (Fig. 4). Because mucosal ferritin mRNA levels were not changed, the increase of ferritin protein appears to be the result of iron-induced translational derepression of ferritin H mRNA. It is worth noting that the basal mucosal ferritin level was barely detectable in b/b rats and the increased ferritin protein at 6 h was still significantly lower than that in+/b rats (Fig. 4).
Effect of dietary iron intake on intestinal IRP1 and IRP2 binding activity. IRP plays a critical role in the regulation of cellular iron homeostasis (10). The decrease of mucosal DMT1 mRNA and increase of ferritin levels after dietary iron intake might be mediated by IRP. Measurement of IRP activity by EMSA showed that in+/b rats, dietary iron intake caused steady decreases in both IRP1 and IRP2 binding activities (Fig. 5). In b/b rats, both IRP1 and IRP2 binding activities were higher than those of+/b rats at 0 h, suggesting diminished intracellular iron pools. During the first 3 h after dietary iron intake, both IRP1 and IRP2 binding activity decreased rapidly in the b/b rats (Fig. 5). As a result, IRP1 and IRP2 activities at 3 and 6 h after iron intake were similar between+/b and b/b rats (Fig. 5).

DISCUSSION

The expression of DMT1 mRNA has been shown to be upregulated by low iron status (16). The effect of iron levels on the expression of intestinal DMT1 has been explored in several animal models as a function of the iron status of the animals (5, 32). The current studies explored the more rapid changes that occur with feeding of a bolus of iron. These studies were undertaken to determine whether changes of expression and/or localization of DMT1 could provide a more acute regulatory mechanism for iron uptake. Three findings in this study were unexpected: 1) dietary iron stimulated internalization of DMT1; 2) dietary iron transiently stimulated synthesis of DMT1 protein; and 3) enterocytes from b/b rats were able to respond to dietary iron. To gain more understanding about the
expression and localization of DMT1 protein in the enterocyte, we produced rabbit antisera that detected both DMT1 isoforms (anti-334) and an antisera specific for the isoform produced by the DMT1-IRE mRNA (anti-540). The specificity of these antisera to DMT1 is supported by the evidence that 1) the EGFP-DMT1 fusion protein expressed in COS-1 cells was recognized by anti-GFP, anti-334, and anti-540 antisera as a single protein band, 2) the anti-GFP but not the anti-334 and anti-540 antisera reacted to a 28-kDa GFP band in COS-1 cells transfected with the pEGFP vector, 3) both anti-334 and anti-540 antisera reacted with a 43-kDa protein in cell lysates of intestinal mucosa and the antigen-antibody reaction was abolished only in the presence of the respective polypeptide, and 4) subcellular relocation of immunoreactive organelles occurred after dietary iron intake, indicating that the immunoreactive protein responds to cellular iron uptake. In addition, another antisera raised by immunization of the NH2-terminal region of DMT1 (amino acids 17–30) also reacted to the same EGFP-DMT1 fusion protein band (Fig. 1). The predicted molecular mass of DMT1 is 62 kDa. The DMT1 protein estimated by SDS-PAGE in the present study was only 43 kDa. The large number of hydrophobic transmembrane domains in the intestinal DMT1 protein might contribute to high electrophoretic mobility. The increase of electrophoretic mobility is known for several transporters; for example, the molecular masses of the glucose transporter GLUT-1 and the zinc transport facilitator ZnT-1 are both estimated to be 55 kDa, but the apparent molecular masses determined by SDS-PAGE are only 35 and 38 kDa, respectively (25, 26). Various molecular masses have been estimated for DMT1 as determined by the mobility on SDS-PAGE (5, 15, 31). Using anti-Flag antibodies, the Flag-tagged DMT1 expressed in human embryonic kidney (HEK) cells exhibited an apparent molecular mass of 63 kDa (31). Recombinant DMT1 expressed in various cell types showed heterogeneous molecular mass from ~70 to 100 kDa (15). The endogenous DMT1 expressed in the duodenum of iron-deficient mice showed major 80–100 kDa bands and two minor bands of ~36 and 42 kDa (5). The variation in the molecular mass of the 80–100 kDa NRAMP2 was attributed to differences in glycosylation (5). It is unclear whether posttranslational modification of the DMT1 produced in various cell types and in the duodenum of different animal species can account for the variation of DMT1 mobility during denaturing PAGE.

Our data with indirect immunofluorescent staining showed that BBM had the strongest DMT1 immunoreactivity in the enterocytes, with some reactivity at the basolateral membrane. The expression of DMT1 protein in the BBM is consistent with its function for dietary iron uptake by enterocytes and is in agreement with recent reports (5, 32). In contrast to other studies, however, we observed no DMT1 in the cytoplasm until the animals ingested a bolus of iron. This difference probably occurred because in our study the animals were fasted before examination of the epithelium. Most interestingly, the present study for the first time demonstrated that dietary iron intake induced the formation of DMT1-associated vesicles. With overnight fasting, essentially all of the DMT1 was detected in the BBM or basolateral membrane. By 1.5 h after a bolus of dietary iron, a significant portion of DMT1 appeared in internalized vesicles. With the appearance of DMT1 in cytosolic vesicles, there was an apparent decrease of DMT1 in the membranes, indicating that DMT1 was being redistributed from the membrane to vesicles. Because the production of DMT1 was significantly increased at 1.5 h after dietary iron intake, it is possible that some of the cytosolic DMT1 was also derived from newly synthesized protein. To be determined is whether the internalization of DMT1 is a regulatory mechanism or if DMT1 and newly absorbed iron are internalized together as part of the iron-uptake process. In the b/b rats, DMT1 was also expressed in the BBM, indicating that the G185R mutation does not alter intracellular targeting of DMT1 to the BBM of enterocytes. The mutant G185R DMT1 protein has been shown to be normally located on transfection into HEK cells (31). In response to dietary iron, the G185R DMT1 was also internalized. There were differences in DMT1 internalization noted between the +/b and b/b rats. Although the formation of DMT1 vesicles in the apical cytoplasm of the enterocytes was similar in the +/b and b/b rats, in the area adjacent to the basolateral membrane DMT1 aggregates formed after dietary iron were increased in number and size in b/b rats compared with the +/b rats. Further studies are needed to determine whether the larger aggregates are related to the iron deficiency in the b/b rats or to altered properties of internalized vesicles because of the G185R mutation in DMT1.

Northern blot analysis showed two DMT1 mRNA isoforms, a major band of 4.5 kb and a minor band of 3.5 kb, expressed in both +/b and b/b rats. The expression of two different sizes of DMT1 mRNA in rat duodenum is consistent with previous reports (16). Both of the 4.5- and 3.5-kb DMT1 mRNA species in +/b and b/b rats showed a conspicuous parallel decrease after dietary iron intake, consistent with the two mRNAs containing an IRE in the 3′-UTR. However, changes in mRNA levels could result from other mechanisms, including altered transcription. Studies are currently underway to determine which of the two isoforms is translated and whether the 3.5 kb-DMT1 mRNA is a cleaved portion of the 4.5-kb DMT1 mRNA. The basal levels of both mRNA isoforms were upregulated in the b/b rats. The increase of DMT1 mRNA expression appears to be the result of iron deficiency (5, 32). This interpretation is supported by the data that the intestinal ferritin level, a marker of cellular iron status, was barely detectable in b/b rats. The decrease in the b/b rats of mucosal DMT1 mRNA levels after dietary iron intake suggests that the block of iron uptake imposed by the DMT1 G185R mutation may not be complete. As a consequence with the high concentrations of dietary iron (50 μg/g in a single bolus), the intracellular iron pool even in the b/b rats must have increased. The increase of intracellular iron is
evident by the decrease of IRP1 and IRP2 binding activities and the increase of mucosal ferritin levels. Although the b/b rats respond to a bolus of iron, neither the IRP binding activity nor the ferritin levels reach levels achieved in the phenotypically normal +/b rat. Hence, after a single bolus of iron, the b/b enterocytes are still relatively iron depleted. It is possible that an alternative iron-transport pathway such as the integrin-mobilferrin pathway (6) triggered the response of DMT1 in the b/b rats. However, the internalization of DMT1 as well as the other observed changes so closely paralleled those in the +/b rats that it is more likely that the high concentration of iron in the bolus (50 μg/g) allowed some iron to be transported through the mutated protein.

We found that the IRE-binding activities of IRP1 and IRP2 were significantly higher in b/b than +/b enterocytes. The high IRP activity appears to be related to the lower iron status of the b/b rats, because both IRP1 and IRP2 binding activities were reduced to the level found in +/b rats at 3 h after dietary iron intake (Fig. 5B). Iron modulates IRP1 binding activity by inactivation and IRP2 binding activity by degradation through the proteasome pathway (17, 19, 20, 22). These two regulatory processes seemed to proceed approximately equally, as the rate of decrease for both IRP-binding activities occurred in parallel in both +/b and b/b rats. The reduction of IRP-binding activity fits the hypothesis that IRP activity regulates DMT1 mRNA levels through the 3' IRE (10). However, that either IRP will bind to the DMT1-IRE has yet to be shown and effects on transcription or other mechanisms for increased mRNA turnover need to be excluded. For example, there are four monomeric AUUUAA sequences in the AU rich 3'-UTR of the DMT1-IRE mRNA (15, 24). The AUUUAA-rich elements have been demonstrated to be coupled to translational degradation of mRNAs (1, 23, 30).

In the b/b rat duodenum, the DMT1 protein levels were twofold higher than in the +/b rats. We observed a greater difference in the levels of DMT1 mRNA in the duodenum of the b/b rats compared with +/b rats. The relatively greater increase in DMT1 mRNA vs. protein suggests that the DMT1 mRNA is overexpressed and weakly translated under low-iron status in b/b rats. This explanation is supported by the data that a bolus dose of dietary iron induced a rapid and transient increase of DMT1 protein. A gradual and steady increase of ferritin H protein also occurred after dietary iron intake, confirming that the intracellular iron pools were increased and that the pattern of rapid increase is DMT1 specific. The specificity for iron inducing the transient increase of DMT1 protein was seen also by feeding the rats a dietary bolus of zinc. Zinc, which is also transported by DMT1 (16), produced the same internalization of DMT1 but did not stimulate DMT1 protein synthesis (data not shown). The mechanism for the transient increase of DMT1 protein is as yet unknown. Because the animals were starved overnight, it is intriguing to implicate nutritional factors that could affect translation of DMT1 mRNA. One such regulatory factor could be the heme-regulated eIF-2α kinase that is activated under conditions of heme deficiency, resulting in the phosphorylation and inactivation of eIF-2α (7). With an influx of iron with feeding, cellular heme levels would increase and eIF-2α kinase activity would decrease with a subsequent increase of eIF-2α activity and DMT1 mRNA translation. The heme-regulated eIF-2α kinase has recently been shown (3) to be present in many tissues. We have found that the kinase mRNA is expressed in the small intestinal epithelium (unpublished data).

In short, the studies presented here demonstrate that after dietary iron intake a series of events occur in the intestinal epithelium, including translocation of DMT1 to the cytosol, a transient increase of DMT1 protein in association with a decrease of DMT1 mRNA, and a progressive decrease in IRP activity. The net result of these changes would be to limit the influx of iron during feeding and to prevent the overexpression of DMT1 in an iron-rich milieu.

We thank Dr. K. Tatchell and Mr. A. Bloecher for assistance in using the Olympus AX70 Provis fluorescent microscope. This study was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Research Grants DK-37866 and DK-41279 and by the Feist-Weiller Cancer Center at Louisiana State University Health Sciences Center.

REFERENCES


34. Yeh KY, Yeh M, and Glass J. Glucocorticoids and dietary iron regulate postnatal intestinal heavy (h) and light (l) ferritin expression in rats. *Am J Physiol Gastrointest Liver Physiol* 278: G217–G226, 2000.