Expression and regulation of SLC39A family zinc transporters in the developing mouse intestine

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Abstract

Several ZIP genes (SLC39A family of metal transporters) play roles in zinc homeostasis. Herein, the temporal and spatial patterns of expression of the mouse ZIP1, 3, 4, and 5 genes in the developing intestine and the effects of maternal dietary zinc deficiency on these patterns of expression were examined. ZIP1 and ZIP3 genes, conserved members of the ZIP subfamily II, were found to be coexpressed during development. Expression of these genes was detected on day 14 of gestation in smooth muscle and the pseudostratified endoderm. By 5 days post-partum, prominent expression became restricted to muscle and connective stroma. In contrast, expression of ZIP4 and ZIP5 genes, members of the ZIP subfamily called LIV-1, coincided with epithelial morphogenesis. ZIP5 expression was detected on d16 of gestation and localized to the basolateral membranes of the single-layered epithelium. ZIP4 expression was detected on d18 of gestation and localized to the apical membrane of villus epithelial cells. When dams were fed a zinc-deficient diet beginning at parturition, ZIP4 expression in the nursing neonate was greatly induced. In contrast, neonatal ZIP5 expression remained unchanged, but this protein was removed from the basolateral membrane of the enterocyte. These responses to dietary zinc deficiency mimic those found in the adult intestine. These studies reveal cell-type-specific expression of ZIP genes during development of the intestine, and suggest that the mouse intestine can elicit an adaptive response to dietary zinc availability at birth.

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Introduction

Zinc is an essential metal that serves structural and/or catalytic roles in hundreds of peptides, including zinc-finger-containing proteins that constitute the most abundant protein superfamilly in the mammalian genome (Ravasi et al., 2003). Thus, zinc deficiency causes profound physiological effects, particularly during periods of rapid growth. Fetal demands for nutrients, including essential metals, increase dramatically during late gestation, and zinc deficiency during pregnancy can exert embryotoxic and teratogenic effects (Hurley, 1981).

Suckling mammals have a high absorptive capacity for trace minerals, including zinc (Pacha, 2000), and reduced zinc levels in the milk are deleterious or even lethal (Huang and Gitschier, 1997). However, little is known about specific zinc transport proteins and their regulation or function in the developing mammalian intestine.

Two major families of mammalian zinc transporters have been identified (Guerinot, 2000; Taylor and Nicholson, 2003; Palmiter and Huang, 2004). Solute-linked carrier 30A (SLC30A) family members, named ZnTs, function in zinc efflux and compartmentalization (Palmiter and Huang, 2004). Ten members of that family have been identified in mice (Seve et al., 2004). In contrast, members of the SLC39A family, named ZIPS, function in the uptake of zinc and other metals (Taylor and Nicholson, 2003). In mice and humans, 14 members of the ZIP family of metal ion uptake transporters have been identified (Eng et al., 1998; Guerinot, 2000; Taylor and Nicholson, 2003). These
proteins have eight predicted transmembrane domains, and spanning domain IV contains conserved histidyl, seryl, and glycyl residues in a predicted amphipathic α-helix. ZIP proteins have been implicated in early development of the Zebrafish embryo (Yamashita et al., 2004) and zinc homeostasis in humans (Wang et al., 2002; Kury et al., 2002).

The ZIP family can be subdivided into four subfamilies based on structural homology (Taylor and Nicholson, 2003). We have been studying the three members of subfamily II (ZIP1-3) (Dufner-Beattie et al., 2003a, 2005, in press; Wang et al., 2004a) and two of the 9 members of the LIV-1 subfamily (ZIP4 and 5) in mice (Dufner-Beattie et al., 2003b, 2004). Each (ZIP1-5) can function as a zinc-specific transporter in transfected cells (Gaither and Eide, 2000, 2001; Dufner-Beattie et al., 2003a,b; Wang et al., 2004b). Subfamily II members (ZIP1, 2, and 3) share a conserved 12-amino acid signature sequence in transmembrane domain four and have been highly conserved, with human and mouse peptides sharing amino acid sequence identities ranging from 83% to 93%. ZIP1 and ZIP3 gene expression can be detected in many embryonic tissues as well as in the preimplantation mouse embryo (Dufner-Beattie et al., 2005, in press), but ZIP1 gene expression is generally the more robust (Dufner-Beattie et al., 2003a). Much less is known about the expression of the ZIP2 gene. Active expression of this gene in mice has been detected in only a few cell-types (Dufner-Beattie et al., 2003a), and its function is under investigation. Targeted deletion of the ZIP1 and/or ZIP3 genes renders mice more susceptible to dietary zinc deficiency during pregnancy, but they are otherwise apparently normal when dietary zinc is adequate (Dufner-Beattie et al., in press). Thus, these proteins appear to play a critical role in the adaptation to dietary zinc deficiency rather than in the acquisition of dietary zinc.

Members of the LIV-1 subfamily in mice and humans share a peptide motif in transmembrane domain 5 (HEPXEXGD-FAXLXXG) (Taylor and Nicholson, 2003). Recent studies demonstrated that human ZIP4 (SLC39A4) is mutated in the genetic disorder of zinc metabolism acrodentriatic enteropathia (Wang et al., 2002; Kury et al., 2002). During periods of zinc deficiency, the mouse ZIP4 mRNA is induced and this protein is recruited to the apical surface of enterocytes (Dufner-Beattie et al., 2003b, 2004). The intracellular trafficking of ZIP4 is also regulated, and zinc stimulates the endocytosis of ZIP4 (Kim et al., 2004; Dufner-Beattie et al., 2004). Thus, ZIP4 plays a key role in zinc homeostasis by regulating the acquisition of dietary zinc. We recently found that the mouse ZIP5 gene is also actively expressed in tissues involved in zinc homeostasis (intestine, visceral endoderm, pancreas), but is not induced during zinc deficiency. Instead, ZIP5 is localized to the basolateral surface of these cells under zinc-replete conditions and is internalized during periods of dietary zinc deficiency (Dufner-Beattie et al., 2004).

In adult mammals, intestinal enterocytes absorb dietary zinc, but a significant amount of zinc is also released back into the intestinal lumen if dietary zinc is replete (Hambidge and Krebs, 2001). The pancreas also plays a role in zinc homeostasis (McClain, 1990), and a significant amount of zinc is released from the pancreas into the intestinal tract when dietary zinc is replete (King et al., 2000; Hambidge and Krebs, 2001). Under conditions of zinc deficiency, the intestinal absorption of zinc is enhanced and the excretion of zinc by enterocytes and pancreatic acinar cells is attenuated (Hambidge and Krebs, 2001). Thus, we have suggested that ZIP5 may function in the removal of excess zinc from the body, an action antagonistic to that of ZIP4.

Herein, we describe the temporal and spatial patterns of expression of these ZIP genes during fetal and neonatal development of the mouse intestine. The results demonstrate that ZIP1 and ZIP3 expression correlates with stromal maturation, whereas ZIP4 and ZIP5 expression occurs in the developing fetal epithelium. Adult patterns of temporal and spatial ZIP gene expression are established during late gestation in the intestine, and at birth the mouse intestine can elicit an adaptive response to dietary zinc deficiency by the reciprocal regulation of ZIP4 and ZIP5 localization and the reciprocal regulation of ZIP4 and metallothionein-I (MT-I) mRNA abundance.

Materials and methods

Animal care and use

All experiments involving mice were conducted in accordance with NIH guidelines for the care and use of experimental animals, and were approved by the Institutional Animal Care and Use Committee. CD-1 mice (48–60 days old) were purchased from Charles River River Laboratories (criver.com). ZIP1 knockout mice have been described previously (Dufner-Beattie et al., 2005), and ZIP4 knockout mice are described in detail elsewhere (Dufner-Beattie et al., in press). Mouse diets were purchased from Harlan Teklad (Teklad.com) and have been described in detail previously (Dalton et al., 1996; Dufner-Beattie et al., 2003a,b, 2004). Zinc levels in the diets were as follows: zinc-deficient (ZnD), 1 ppm Zn\(^{2+}\); zinc-adequate (ZnA), 50 ppm Zn\(^{2+}\).

To investigate developmental patterns of ZIP gene expression, mice were mated and day 1 of gestation (d1) was considered the day of the vaginal plug. Fetal intestines were collected on d14 to d20. After parturition, dams and pups were examined on different days of lactation (up to 20 days), as indicated in figure legends and Results. The day of parturition was considered lactation day 0 (0 dpp) and at birth the mouse intestine can elicit an adaptive response to dietary zinc deficiency by the reciprocal regulation of ZIP4 and ZIP5 localization and the reciprocal regulation of ZIP4 and metallothionein-I (MT-I) mRNA abundance.

Northern blot analysis

Frozen intestines were powdered in liquid nitrogen using a mortar and pestle on dry ice. Intestine powder (100 mg) was homogenized using a Polytron homogenizer (brinkmann.com) in 1.5 ml Trizol reagent (Invitrogen.com) to extract RNA following the manufacture’s protocol, as described previously (Andrews et al., 2001). Total RNA (5 μg) was size fractionated by agarose-formaldehyde gel electrophoresis, transferred and cross-linked to nylon membranes. Membranes were hybridized and washed under stringent conditions, as described (Dalton et al., 1994, 1996). Hybrids were detected by autoradiography with intensifying screens at ~70°C. Duplicate gels were stained...
with acridine orange or the same membrane was rehybridized with a β-actin probe to monitor RNA loading and integrity.

The mouse MT-I, β-actin, ZIP1 to 5 probes were labeled as described previously (Dalton et al., 1996; Dufner-Beattie et al., 2003a,b, 2005). Labeled ZIP cDNA probes were generated using [32P]dCTP and the Random Primers DNA Labeling System (Invitrogen). Labeled cRNA probes for MT-I and β-actin were transcribed in vitro, as described previously (Dalton et al., 1996). Probes had specific activities of about 1 to 3 × 10^9 dpm/μg.

Preparation of membranes and Western blot analysis

Membrane proteins were prepared using a modified protocol (Rodriguez et al., 2004; Sagawa et al., 2003; Krotova et al., 2003), as described previously (Dufner-Beattie et al., 2004). Intestine (100 mg) was homogenized using a polytron homogenizer in 1.5 ml of ice-cold lysis buffer [20 mM Tris−HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 mM NaF, 5 mM Na3VO4, and Protease Inhibitor Cocktail] containing a Protease Inhibitor Cocktail (roche-applied-science.com). The homogenate was centrifuged at 500×g for 10 min at 4°C to pellet insoluble debris. The supernatant was concentrated and centrifuged at 100,000 × g for 30 min at 4°C to pellet membranes. The membrane pellet was resuspended in wash buffer [150 mM NaCl, 10 mM phosphate buffer (pH 7.0), 1 mM DTT, 1 mM PMSF, 2 mM NaF, 5 mM Na3VO4, and Protease Inhibitor Cocktail] and collected by centrifugation at 20,000 × g for 10 min at 4°C. This washing process was repeated three times. The membrane pellet was then resuspended in 200 μl of RIPA buffer [150 mM NaCl, 50 mM Tris−HCl (pH 7.4), 1% Nonidet P-40, 1% sodium deoxcholate and 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 mM NaF, 5 mM Na3VO4, and Protease Inhibitor Cocktail] and collected by centrifugation at 20,000 × g for 10 min at 4°C. This washing process was repeated three times. The membrane pellet was then resuspended in 200 μl of RIPA buffer [150 mM NaCl, 50 mM Tris−HCl (pH 7.4), 1% Nonidet P-40, 1% sodium deoxcholate and 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 mM NaF, 5 mM Na3VO4, and Protease Inhibitor Cocktail] and sonicated and allowed to sit on ice for 60 min. This solution was then centrifuged at 20,000 × g for 10 min at 4°C to pellet insoluble debris. The supernatant was collected and its protein concentration determined using a BCA Protein Assay Kit (piercenet.com).

Membrane proteins (40 μg) were heated in 1× SDS sample buffer at 37°C for 30 min before being resolved on a 10% SDS-polyacrylamide gel and transferred to polyvinylidine difluoride membranes. The membranes were blocked overnight at 4°C in blocking solution (150 mM NaCl, 10 mM Tris−HCl (pH 7.4), 0.1% Tween-20, 5% (w/v) nonfat dry milk) and then incubated with primary antibody diluted in blocking solution (1:2000) for 1 h at room temperature. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000) in blocking solution for 2 h at room temperature. Immunoreactive bands were visualized using ECL Plus Western Blotting Detection System (amershambiosciences.com). As positive controls for ZIP4 and ZIP1 proteins, the plasmids pcDNA3.1Puro(+)-mZIP4HA (Dufner-Beattie et al., 2003b) and pcDNA3.1Puro(+)-mZIP1HA (Dufner-Beattie et al., 2003a), respectively, were stably transfected into HEK293 cells (Wang et al., 2003a), were gradually to moderate abundance by d20 (the day of parturition). Immunoreactive bands were visualized using ECL Plus Western Blotting Detection System (amershambiosciences.com). As positive controls for ZIP4 and ZIP1 proteins, the plasmids pcDNA3.1Puro(+)-mZIP4HA (Dufner-Beattie et al., 2003b) and pcDNA3.1Puro(+)-mZIP1HA (Dufner-Beattie et al., 2003a), respectively, were stably transfected into HEK293 cells (Wang et al., 2004a) and a total protein lysate was prepared in RIPA buffer and heated in 1× SDS sample buffer.

Polyclonal antisera against mouse ZIP4 and ZIP5 were raised in rabbits against the following peptides: (AEETPELLNPETRRL) and (ASEPEVQGQSASAGQK), respectively. Antibodies were purified by affinity chromatography (Wang et al., 2002; Dufner-Beattie et al., 2004). Previous studies have confirmed the specificity of these antisera (Dufner-Beattie et al., 2003b, 2004). Polyclonal antisera against mouse ZIP1 was generated commercially by Covance Research Products (crpcinc.com) in rabbits against a peptide (RRSGANHEA-SASGQK) and purified by affinity chromatography. As a negative control, the ZIP1 antisera was neutralized by preincubation for 2 h at room temperature with 6 × 10^−5 M peptide before use. In addition, specificity of the ZIP1 antisera was examined by Western blotting of intestinal membrane proteins from wild-type and ZIP1 homozygous knockout mice.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed using the Zymed Histostain-SP kit (zymed.com) for rabbit primary antibody and DAB staining, as described (Dufner-Beattie et al., 2004). Tissues were fixed overnight in Bouin’s fixative at 4°C, embedded in paraffin, and sectioned. Sections were deparaffinized, and subjected to antigen retrieval in 10 mM citrate, pH 6.0 at 95°C for 15 min (Shi et al., 1993). Sections were then treated with 1% peroxide for 10 min, blocked with 10% normal goat serum for 20 min, and incubated for 1 h at room temperature with primary antisera (ZIP5; 1:300 dilution: ZIP4; 1:600 dilution). Brown deposits indicated sites of antibody binding. Sections were not counterstained.

Immunofluorescence microscopy was performed using tissues fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde at 4°C overnight, infiltrated stepwise with gradations of sucrose (Barthel and Raymond, 1990), infiltrated with sucrose-tissue freezing medium (1:2 v/v), and quick frozen in a dry ice-ethanol bath. Cryosections (5 μm) were immersed in PBS containing 50 mM lysine, permeabilized in PBS containing 0.1% Tween 20 and 0.1% Triton X-100, and blocked with 10% normal goat serum. Sections were then incubated with a 1:200 dilution of anti-EGFP antibody conjugated to Alexa 594 (probes.com) washed in PBS, and mounted in SlowFade Light with DAPI (probes.com). Antibody binding was detected using a Texas Red cube in a DN4000B Leica fluorescence microscope coupled with a DC500 digital camera (leica.com). Red fluorescence indicated antibody binding whereas blue fluorescence revealed nuclei.

Results

Unique temporal and spatial expression of mouse ZIP genes in the fetal and neonatal intestine

Northern blotting was used to examine the relative abundance of several ZIP mRNAs in the fetal and neonatal intestines. We have previously shown that ZIP1 to 5 are each expressed in the adult mouse intestine (Dufner-Beattie et al., 2003a,b, 2004), and expression of these genes does not vary significantly along the duodenal–colonic axis (data not shown). In the fetal and neonatal intestine, ZIP1, ZIP4, and ZIP5 mRNAs were readily detected by Northern blotting (Fig. 1). In contrast, ZIP3 and ZIP2 mRNAs were not. Only after prolonged exposure of Northern blots could we detect ZIP3 mRNA in the adult intestine (Dufner-Beattie et al., 2003a), and the blots shown here were not exposed that long. Thus, ZIP3 mRNA is rare in the intestine, but ZIP2 mRNA is even less abundant and we have not detected ZIP2 mRNA in any tissues using Northern blotting. We did not attempt to monitor changes in these mRNAs further, but the expression of these genes (ZIP1–3) was examined as described below.

ZIP1 mRNA was present throughout fetal and neonatal development. Abundance of this mRNA increased modestly after d16 of gestation and remained essentially unchanged throughout the neonatal period. In contrast, ZIP4 mRNA was not detected before d18 of gestation, after which it increased gradually to moderate abundance by d20 (the day of parturition). Within 5 days post-partum, ZIP4 mRNA abundance in the neonatal intestine had decreased, but these levels rebounded by 15 days. ZIP5 mRNA was detected as early as d16 and also tended to increase in abundance just before parturition after which it remained constant, and at adult levels (in Fig. 1, compare the fetal (left), neonatal and adult (right) blots). MT-I mRNA became highly abundant in the mouse intestine just before birth and remained very abundant until near weaning (21 dpp), as has been reported in the rat (Mengheri et al., 1993).

Expression of the ZIP1 and ZIP3 genes was further examined by monitoring the expression of EGFP knock-in alleles in mice homozygous for targeted deletions of these genes (Dufner-Beattie et al., 2005, in press). In these mice, EGFP was knocked-in, such that its expression is driven by the endogenous ZIP1 or ZIP3 promoter. EGFP was detected in frozen sections of the
developing intestine using an anti-GFP antibody (Figs. 2 and 3). Sections from wild-type mice served as controls for the specificity of the antisera (Fig. 2, bottom). Low levels of EGFP were detected in the fetal intestines in both ZIP1 and ZIP3 knock-in mice. On d14 of gestation, EGFP was detected in smooth muscle and the pseudostratified endoderm. Fluorescence was more intense in sections from ZIP1 knock-in mice than in those from ZIP3 knock-in mice. By d16, the expression of both of these genes was most prominent in muscle and the developing stroma. After parturition, EGFP was highly abundant in the connective stroma and muscle, but essentially absent from the developing villus and crypt epithelial cells. Thus, ZIP1 and ZIP3 genes are coexpressed very early during development of the mouse intestine, and their expression becomes restricted to the stromal and muscle layers. Our preliminary studies of ZIP2 knockout mice did not detect EGFP expression in the intestine (unpublished results).

ZIP4 and ZIP5 proteins were localized by immunohistochemistry in the developing intestine using anti-peptide antibodies specific for these mouse proteins, as described previously (Dufner-Beattie et al., 2003b, 2004). Neither of these proteins was detected in the d14 intestine (Fig. 4). At this stage of development, the intestine consists of pseudostratified endoderm and a stromal layer (Figs. 4D and G). By d16, the intestinal endoderm converts to a single-layered epithelium and on d18 the formation of primitive crypts and villi is at a very early stage. ZIP5 was detected on the basolateral membranes of the newly formed epithelial cells on d16 and d18 (Figs. 4C and B), but ZIP4 was not detected on d16 (data not shown) and was barely detectable on the apical membrane of the villus epithelium on d18 (Fig. 4F). In contrast, by 1 dpp ZIP5 immunostaining revealed a crypt to villus axis with this protein being most prominent in the crypts (Fig. 4A). ZIP5 localization was restricted to the basolateral membranes, as is seen in the adult intestine (Dufner-Beattie et al., 2004). On 1 dpp, ZIP4 immunostaining was restricted to the apical membranes of the villus epithelial cells and was not apparent in the crypts (Fig. 4E). A similar pattern of ZIP4 apical localization is found in the adult intestine under conditions of dietary zinc deficiency (Dufner-Beattie et al., 2003b). Thus, mouse ZIP4 and ZIP5 genes are coexpressed during development and differentiation of the intestinal epithelium, and are differentially located on the apical and basolateral membranes, respectively, at the time of parturition.

The neonatal intestine can elicit an adaptive response to maternal dietary zinc status

The adult intestine undergoes an adaptive response to dietary zinc. ZIP4 mRNA is induced during zinc deficiency and this protein becomes localized to the apical membrane of enterocytes. In contrast, ZIP5 mRNA is not altered by dietary zinc, but this protein is removed from the basolateral membrane during zinc deficiency (Dufner-Beattie et al., 2004). To examine the adaptive response of the neonatal intestine to dietary zinc, dams were fed a ZnD diet beginning at birth, and the patterns of intestinal ZIP gene expression in the nursing neonatal pup intestine were determined.

As shown in Fig. 5A, dietary zinc deficiency significantly induced ZIP4 mRNA in both the dam and neonatal intestine. This response was apparent in the adult intestine within 2 days of initiation of the ZnD diet, whereas this response in the neonatal intestine lagged behind that of the dam. Dietary zinc status did not alter the abundance of ZIP1 or ZIP5 mRNAs in the dam or neonatal intestine, but MT-I mRNA abundance in the neonatal intestine was dramatically reduced. MT-I gene expression is a

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Fig. 1. Northern blot detection of ZIP1, 4, and 5 mRNA in the developing mouse intestines. Intestines were collected from fetuses (left panel) on the indicated day of gestation (day 1 = vaginal plug) or from suckling neonatal pups and lactating dams (right panel) on the indicated days of lactation (day 0 = day of parturition). Total RNA was extracted and analyzed by Northern blotting using the indicated probes.
sensitive indicator of embryonic zinc status (Andrews et al., 2001).

Western blot analysis of intestinal membrane proteins from the dam and neonate revealed that ZIP4 protein abundance dramatically increased during dietary zinc deficiency (Fig. 5B), coincident with increased ZIP4 mRNA. In contrast, ZIP1 protein abundance remained unchanged. The specificity of this ZIP1 antiserum was confirmed by Western blotting of intestinal membrane proteins from wild-type and ZIP1 knockout mice, as well as by analysis of cells expressing HA-tagged ZIP1, and preneutralization of the antiserum with peptide (Fig. 5C). However, an abundant nonspecific band was also detected by this anti-serum (not shown) which precluded its use for immunolocalization. Unfortunately, we have not been able to generate useful ZIP3 or ZIP2 antisera for protein localization or Western blotting studies, and we were unable to detect ZIP5 in the intestine membrane preparations by Western blotting.

The effects of dietary zinc deficiency on the cellular localization of ZIP4 and ZIP5 proteins in the neonatal intestine were examined using immunohistochemistry (Fig. 6). Dams were fed the ZnA or ZnD diet beginning at parturition and the intestines from the nursing pups were collected on day 5 post-partum. Relatively low levels of ZIP4 were detected on the apical surfaces of enterocytes from the neonatal intestine under zinc adequate conditions. Under these conditions, during the first 5 days of nursing, ZIP4 mRNA abundance was reduced (Fig. 5A) and the abundance of this protein on the apical membrane was reduced (compare Fig. 4E and Fig. 6A). In contrast, under conditions of dietary zinc deficiency, ZIP4 protein was readily detected on the apical membranes of enterocytes from the neonatal intestine, whereas ZIP5 protein was removed from the basolateral membranes (Fig. 6). Thus, the mouse intestine can elicit an adaptive response to dietary zinc status at the time of birth.

**Discussion**

We describe here the temporal and spatial patterns of expression of zinc transporters during development and differentiation of the mouse intestine. Specifically, these studies...
focused on several members of the SLC39A family of transporters (ZIPs) that have recently been shown to function in zinc uptake. The ontogeny of various intestinal transport capacities in mammals has been studied for many years, and it is known that suckling mammals have a high absorptive capacity. Specific carriers for iron, copper, and zinc have been postulated in the immature intestine, but an understanding of the molecular mechanisms responsible for changes in transport activity in mammals has only recently begun to be elucidated (Pacha, 2000).

Within the past decade, our understanding of the mechanisms regulating the homeostasis of zinc has advanced significantly.

Fig. 3. Delineation of ZIP3 gene expression in the developing intestine by immunofluorescence localization of EGFP expressed from a knock-in allele. Intestines were harvested from homozygous ZIP3 knockout mice on the indicated day of gestation (d14–d18; panels A–C) and day post-partum (1 dpp–10 dpp; panels D–F). Tissues were fixed and cryosections were stained using an anti-GFP antibody conjugated with Alexa 594. EGFP staining is indicated in red, and nuclei were counterstained with DAPI (blue) in the neonatal intestine sections. In panel A, a cross-section of the d14 intestine reveals the pseudostratified endoderm layer surrounding the lumen, and the outermost layers of the stroma consisting of developing smooth muscle.

Fig. 4. Immunohistochemical localization of ZIP4 and ZIP5 during intestinal development. Intestines were collected from fetuses on day of gestation (d14 to d18) or from neonatal pups 1 day post-partum. Intestines were fixed, embedded in paraffin, and processed for immunohistochemistry using the ZIP4 antiserum or the ZIP5 antiserum. Brown deposits indicate positive immunostaining. Panel G is a phase contrast micrograph of this longitudinal section of the d14 intestine. The pseudostratified endoderm lines the lumen.
Zinc homeostasis is controlled by uptake, efflux, and storage mechanisms. Uptake is regulated by members of the ZIP family, whereas efflux is controlled by members of the ZnT family (Kambe et al., 2004). In addition, members of the ZIP and ZnT families are involved in the intracellular storage and release of zinc. A significant amount of total cellular zinc is bound to metallothioneins that function to chelate zinc and provide a labile pool of biologically active zinc during periods of zinc deficiency (Dalton et al., 1996). Previous studies have demonstrated the expression of \( \text{ZIP} \) and \( \text{ZnT} \) genes in the developing rodent intestine (Nishimura et al., 1989; Mengheri et al., 1993; Barila et al., 1994; Liuzzi et al., 2003). Expression of the \( \text{ZIP} \) and \( \text{ZnT} \) genes is responsive to zinc (Langmade et al., 2000; Andrews, 2000), and both are actively expressed in the fetal intestine. The \( \text{ZIP} \) gene is essential early during embryonic development of the mouse (Andrews et al., 2004), and the \( \text{ZnT} \) gene functions in the mammary gland to provide zinc in the milk (Huang and Gitschier, 1997) which is essential for viability of the suckling pup (the lethal milk mutation).

Results reported herein document that several \( \text{ZIP} \) genes are also actively and differentially expressed in the developing intestine. The \( \text{ZIP} \) and \( \text{ZnT} \) families are involved in the intracellular storage and release of zinc. A significant amount of total cellular zinc is bound to metallothioneins that function to chelate zinc and provide a labile pool of biologically active zinc during periods of zinc deficiency (Dalton et al., 1996). Previous studies have demonstrated the expression of \( \text{ZnT} \), \( \text{ZIP} \), and \( \text{MT-I} \) genes in the developing rodent intestine (Nishimura et al., 1989; Mengheri et al., 1993; Barila et al., 1994; Liuzzi et al., 2003). Expression of the \( \text{ZnT} \) and \( \text{MT-I} \) genes is responsive to zinc (Langmade et al., 2000; Andrews, 2000), and both are actively expressed in the fetal intestine. The \( \text{ZnT} \) gene is essential early during embryonic development of the mouse (Andrews et al., 2004), and the \( \text{ZnT} \) gene functions in the mammary gland to provide zinc in the milk (Huang and Gitschier, 1997) which is essential for viability of the suckling pup (the lethal milk mutation).

Fig. 5. Effects of maternal dietary zinc-deficiency on \( \text{ZIP} \), \( \text{ZnT} \), and \( \text{MT-I} \) mRNA and protein levels in the intestines from the dam and the sucking neonatal pup. Lactating dams (6 per group) were fed either a zinc adequate (A) or a zinc-deficient (D) diet beginning the day of parturition (0 day of lactation) and intestines from the dams and their pups were collected and pooled on the indicated days of lactation. (A) RNAs were extracted and analyzed by Northern blotting using the indicated probes. (B) Membrane proteins were prepared and analyzed by Western blotting using the indicated antisera. Controls are proteins extracted from HEK293 cells (-) stably transfected (+) with \( \text{ZIP}4 \) or \( \text{ZIP}1 \) expression vectors. Only the relevant portions of the blots are shown. (C) The specificity of the \( \text{ZIP}1 \) antiserum was further examined by Western blotting of intestinal membrane proteins from homozygous knockout mice (KO) or wild-type mice (WT) and by preneutralization of the antiserum with peptide, as indicated. The predicted ~35 kDa \( \text{ZIP}1 \) band is shown, but a prominent and much larger nonspecific band is not shown. As above, only the relevant portion of the Western blot is shown.

Fig. 6. Effects of maternal dietary zinc-deficiency on \( \text{ZIP}4 \) and \( \text{ZIP}5 \) localization in the intestines of the nursing neonatal pup. Lactating dams (6 per group) were fed either a zinc-adequate (ZnA; panels A and C) or a zinc-deficient (ZnD; panels B and D) diet beginning at parturition and intestines from their nursing pups (samples used in Fig. 4) were collected on lactation day 5. Paraffin sections were prepared and processed for immunohistochemistry using the \( \text{ZIP}4 \) antiserum (panels A and B) or the \( \text{ZIP}5 \) antiserum (panels C and D). Brown deposits indicate positive immunostaining.
function of these proteins is not in the direct acquisition of zinc from the gut lumen. Studies of knockout mice suggest that these genes serve redundant or additive roles during the stress of severe dietary zinc deficiency (Dufner-Beattie et al., in press) and studies of ZIP1 and 3 localization and zinc uptake activity in transfected cells demonstrate that these proteins are recruited to the cell surface during zinc-deficiency (Wang et al., 2004a). However, human ZIP1 has been localized to plasma membranes of prostate cells (Costello et al., 1999). Dietary zinc levels do not affect the relative abundance of mouse ZIP1 or ZIP3 mRNA in the adult intestine (Dufner-Beattie et al., 2003a), and as shown herein, ZIP1 protein levels remain unchanged in the adult and neonatal intestine during zinc deficiency. Whether the cellular localization of these proteins (ZIP1 and ZIP3) is regulated by zinc in the intestine remains to be determined. Unfortunately, our antisera against these proteins have not proven useful in detecting their subcellular distribution. Clearly, the detection of EGFP in the intestine of knock-in mice cannot address the post-transcriptional regulation of ZIP1 and 3 during development.

Expression of the ZIP4 and 5 genes is restricted to the intestinal epithelium in the developing gut as it is in the adult (Dufner-Beattie et al., 2004). ZIP5 was localized to the basolateral membranes of the newly formed simple epithelium, the formation of which is accompanied by polarization of the epithelial cells and the creation of apical and basolateral membrane domains (Pacha, 2000). Thus, ZIP5 gene expression and the basolateral localization of this protein are very early events during differentiation of the intestinal mucosa. The ZIP4 gene was not activated until later in gestation, and this protein was localized to the apical membranes of the epithelium. It is interesting to note that the temporal–spatial patterns of expression of the glucose transporters, Glut 2 and Glut 5, are remarkably similar to those of ZIP4 and ZIP5. Glut 2 is detected on the basolateral membranes of the epithelium on d16 and Glu5 has been localized to the apical membrane after d18 (Pacha, 2000). This suggests that the transport activities of the basolateral membrane domain of the intestinal enteroocyte mature before those of the apical membrane domain.

Adult patterns of expression and regulation of these mouse ZIP genes are established at or within a few days of parturition which is consistent with the concept that they serve important roles in zinc homeostasis. We have hypothesized that they have opposing functions in the intestine: ZIP4 being critical for the acquisition of zinc from the food; ZIP5 being involved in removing excess zinc from the body when it is not needed (Dufner-Beattie et al., 2004). Thus, when zinc is replete, ZIP4 protein is rare on the apical surface of enterocytes, whereas ZIP5 protein is localized on the basolateral surface of these cells and on that surface of pancreatic acinar cells. In contrast, when zinc is limiting, ZIP4 protein is localized on the apical surface and ZIP5 protein is removed from the basolateral surface of the enterocyte and acinar cell. This pattern of regulation was observed in the newborn intestine when the mother was fed a zinc-deficient diet.

The presence of ZIP4 on the apical surface of the intestinal epithelium late in gestation may indicate a role for this protein in absorption of zinc from swallowed amniotic fluid. Similarly, the apical localization of ZIP4 at birth suggests that in mice this protein plays a critical role in absorption of zinc from the milk, as it must in humans (Wang et al., 2002). After several days of nursing, the apical localization of ZIP4 and ZIP4 mRNA abundance were diminished in the neonatal intestine, which is indicative of the effects of dietary zinc adequacy in adult mice (Dufner-Beattie et al., 2003b). Milk is known to be rich in zinc and other essential metals (Lommerdahl et al., 1981).

A novel outcome of our studies was the demonstration that the neonatal intestine can adapt to maternal dietary zinc levels as does the adult intestine, by changes in expression and/or cellular localization of ZIP4, ZIP5 and MT-I. When zinc was depleted from the maternal diet, ZIP4 mRNA abundance increased in the maternal intestine within a day whereas this response occurred in the neonatal intestine a day or two later coincident with a dramatic decrease in MT-I mRNA levels. Thus, changes in gene expression in the neonatal intestine are a remarkably sensitive indicator of maternal zinc status. Zinc can apparently be rapidly depleted from milk when the maternal diet is deficient in this essential metal. Thus, the ability of the neonatal intestine to adapt to zinc availability at birth is physiologically important.

Little is known about mechanisms that regulate the expression of mammalianZIPs. However, a significant role for post-transcriptional regulation has been revealed for ZIP1–5 (Dufner-Beattie et al., 2003b, 2004; Kim et al., 2004; Wang et al., 2004a). An observation worth noting here is that ZIP4 mRNA abundance dictates the abundance of this protein during zinc-deficiency in the adult and the neonatal intestine. This result is inconsistent with a significant translational control of ZIP4 expression. In contrast, ZIP5 mRNA levels are unchanged in response to dietary zinc, but this protein appears to be degraded during zinc deficiency, at least based on immunohistochemical detection. These results highlight the fact that multiple mechanisms regulate the expression of mouse ZIP genes and suggest that zinc homeostasis is tightly regulated.

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References