The Adaptive Response to Dietary Zinc in Mice Involves the Differential Cellular Localization and Zinc Regulation of the Zinc Transporters ZIP4 and ZIP5*

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The ZIP5 gene encodes a protein closely related to ZIP4, a zinc transporter mutated in the human genetic disorder acrodermatitis enteropathica. Herein, we demonstrate that mouse ZIP5 and ZIP4 genes are co-expressed in several tissues involved in zinc homeostasis (intestine, pancreas, embryonic yolk sac). However, unlike expression of the ZIP4 gene, which is induced during periods of zinc deficiency, ZIP5 gene expression is unaltered by dietary zinc. Immunohistochemistry localizes ZIP5 to the basolateral surfaces of enterocytes, acinar cells, and visceral endoderm cells in mice fed a zinc-adequate diet. However, this protein is removed from these cell surfaces and internalized during dietary zinc deficiency. In contrast, ZIP4 is induced and recruited to the apical surface of enterocytes and endoderm cells during zinc deficiency. In the pancreas, ZIP4 is expressed in β-cells, whereas ZIP5 is expressed in acinar cells. These results suggest that the function of ZIP5 is antagonistic to that of ZIP4 in the control of zinc homeostasis; rather than functioning in the acquisition of dietary zinc, as does ZIP4, ZIP5 may function in the removal of zinc from the body. Thus, during periods when dietary zinc is replete, ZIP5 may function to remove zinc from the blood via the pancreas and intestine, the major sites of zinc excretion in mammals, whereas the acquisition of dietary zinc by intestinal ZIP4 would be minimal. In contrast, during periods of dietary zinc deficiency when secretion of zinc by the pancreas and intestine is minimized, ZIP5 is removed from the cell surface, and the intestinal uptake of zinc is augmented by induction of ZIP4.

Members of the ZIP superfamily of metal ion uptake transporters (solute carrier family 39A) are found in all eukaryotes (1–3). In mice 14 members of the ZIP family have been identified based on sequence homology, and almost all of these genes are conserved in humans (1, 4, 5). ZIP proteins have eight predicted transmembrane domains, and transmembrane domain IV contains conserved histidyl, seryl, and glycyl residues in an amphipathic α-helix. These proteins often contain an intracellular loop between transmembrane domains III and IV and an extracellular amino terminus that is histidine-rich and may play roles in metal transport. Members of the ZIP superfamily have been shown to transport zinc, iron, or manganese into cells (6) and have been implicated in early development of the zebrafish embryo (7) and zinc homeostasis in humans (8, 9). Despite this growing body of knowledge, little is known about the structure and function of most members of this diverse family of proteins.

The ZIP family can be subdivided into four subfamilies, named Subfamilies I and II, gufA, and LIV-1 (5). The three members of subfamily II (ZIP1–3) have been well conserved in mammals, and each functions as zinc-specific transporter (10–12). However, the physiological roles of these transporters remain to be determined. The LIV-1 subfamily in mice contains 9 members (5) of which only three (ZIP4, -6, and -7) have been characterized in any detail (13–15). The founding member of this family, LIV-1 (ZIP6; SLC39A6), was identified as a breast cancer-associated protein whose expression is induced by estrogen in breast cancer cells (13, 16). In transfected cells, ZIP6 localizes to the plasma membrane and may function to increase zinc uptake (13). Very recently, ZIP7 was found to localize to intracellular membranes in transfected cells and to enhance zinc accumulation (14). However, ZIP6 expression appears to be at its lowest level in the human intestine, and ZIP7 appears to be fairly ubiquitously expressed at low levels (13, 14). This suggests that they do not play key roles in the acquisition of dietary zinc.

In contrast, ZIP4 (SLC39A4) is mutated in the human genetic disorder of zinc metabolism acrodermatitis enteropathica (8, 9). ZIP4 is also well conserved during evolution, and the mouse ZIP4 gene is induced, and this protein is recruited to the apical surface of enterocytes and embryonic visceral endoderm cells during periods of zinc deficiency (15). The intracellular trafficking of ZIP4 is also regulated (e.g. zinc stimulates the endocytosis of ZIP4) (17). Thus, ZIP4 plays a key role in zinc homeostasis by sensing dietary zinc levels.

Herein, we studied ZIP5 (SLC39A5), a member of the mouse LIV-1 subfamily that is closely related to ZIP4. We demonstrate that the mouse ZIP5 gene is most 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 but is internalized during periods of dietary zinc deficiency. In related studies, it was recently demonstrated that mZIP5 functions as a bona fide zinc transporter that is specific for zinc as a substrate (18). Our studies suggest that ZIP5 plays a central role in mammalian zinc homeostasis by antagonizing the actions of ZIP4.

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Materials and Methods

Animal Care and Use; Dietary Zinc Manipulation

All experiments involving mice were conducted in accordance with National Institutes of Health 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 Breeding Laboratories (criver.com). Mouse diets were purchased from Harlan Teklad (Teklad.com) and have been described in detail previously (12, 15, 19). Zinc levels in the diets were as follows: zinc-deficient (ZnD), 1 ppm Zn; zinc-adequate (ZnA), 50 ppm Zn.

To examine the tissue-specific expression of ZIP5, CD-1 female or male mice (6 per group) maintained on ZnA feed were killed, and the indicated tissues were harvested and snap-frozen in liquid nitrogen for subsequent extraction of RNA and Northern blot analysis. Pancreas RNA was extracted from fresh tissue.

To examine the effects of zinc on ZIP5 expression, female mice (six mice per group) were subjected to dietary zinc deficiency during pregnancy as described previously (15, 20).

Computer Analyses of Sequence Data

Multiple sequence alignments were performed using the Vector NTI Suite Program (Invitrogen.com).

RNA Extraction and Northern Blot Hybridization

Tissue RNAs were isolated as described in detail previously (21, 22). Total RNA (3 μg) was size-fractionated by agarose formaldehyde gel electrophoresis, transferred, and cross-linked to nylon membranes. Northern blot membranes were hybridized and washed under stringent conditions as described (19, 22, 23). 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, and ZIP4 probes were as described (12, 15, 19). The protein-coding region of ZIP5 mRNA (GenBank™ accession numbers A008448 and A007473) was amplified by reverse transcription-PCR from mouse intestinal RNA using Invitrogen’s Reverse Transcription-PCR System according to the manufacturer’s instructions (invitrogen.com). Primers specific to the ZIP5 cDNA are

| Forward Primer | Reverse Primer |
|---------------|---------------|
| 5'-GGGAATTCGTCTCAAAGGAAATAGAAGT-3' | 5'-CGGGAAGGGTACAGTTGCAGACATGTGA-3' |

The product was digested with EcoRI and SpeI, and the purified product was ligated into EcoRI-digested pcDNA3.1Puro(+) (24) along with annealed oligonucleotides (5'-CTAGGGCGTTCAATCAATGGTTCGAGATTAGGTGAT-3' and 5'-CAATTCTCAAGCAATCTGGAACATCATATGGATAGCCA-3') that contained SpeI and EcoRI compatible termini and encode a carboxyl-terminal hemagglutinin (HA) tag. The product (pcDNA3.1Puro(+) + ZIP5HA) was confirmed by DNA sequencing. Probes were labeled using the Random Primers DNA Labeling System according to the manufacturer’s instructions. Probes had specific activities of ~10^9 dpm/μg.

Western Blotting

ZIP4 and -5 Antibodies—A rabbit polyclonal antisera was generated against the following mZIP5 peptide: (C)ASEPEVQGQRENRQS. A rabbit polyclonal antisera was generated against the following mZIP5 peptide: (C)ASEPEVQGQRENRQS. The peptide was coupled to keyhole limpet hemocyanin and used to immunize rabbits as described previously for ZIP4 (8).

Preparation of Membrane and Cytosolic Proteins—Membrane proteins were prepared using a modified protocol (25–27). Maternal proximal small intestine or embryonic visceral yolk sac (~0.1 g) was collected and homogenized using a Polytron homogenizer in 1 ml of ice-cold lysis buffer (20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonil fluoride, 2 mM NaF, 5 mM Na2VO4 containing a Protease Inhibitor Mixture (roche-applied-science.com). The homogenate was centrifuged at 500 × g for 10 min at 4 °C to pellet insoluble debris. The supernatant was collected and centrifuged at 100,000 × g for 30 min at 4 °C to pellet membranes. The supernatant (cytosolic fraction) was recovered, and the membranes were rinsed with wash buffer (150 mM NaCl, 5 mM phosphate buffer (pH 7.0), 1 mM dithiothreitol, 1 mM phenylmethylsulfonil fluoride, 2 mM NaF, 5 mM Na2VO4, and Protease Inhibitor Mixture) and collected by centrifugation at 20,000 × g for 10 min at 4 °C. The membrane pellet was then resuspended in 200 μl of radiolmmune precipitation assay buffer buffer mix (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonil fluoride, 2 mM NaF, 5 mM Na2VO4, and Protease Inhibitor Mixture), sonicated, and allowed to sit on ice for 30–60 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 was determined using a BCA protein assay kit (piercenet.com).

Immunoblotting—Membrane proteins (25 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene 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 (1:500 for ZIP5 and 1:1000 for ZIP4 and PC1) for 1 h at room temperature. The membranes were then incubated with horse-radish peroxidase-conjugated secondary antibody diluted in blocking solution for 30 min at room temperature. Immunoreactive bands were visualized using ECL Plus Western blotting detection system with Hyperfilm ECL (amershambiosciences.com). As a positive control for ZIP5, the plasmid pcDNA3.1Puro(+) + mZIP5HA was transiently transfected into HEK293 cells and 48 h posttransfection a total protein lysate was prepared in 1× SDS sample buffer. As a negative control, the antiserum was each neutralized by preincubation for 2 h at room temperature with 6 × 10^-5 M peptide before incubation with the membranes.

Immunohistochemistry

Immunohistochemistry was performed using the Zymed Laboratories Inc. Histostain-SP kit (zymed.com) for rabbit primary antibody and diaminobenzidine substrate staining. 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 buffer (pH 6.0) at 95 °C for 6–8 min (28, 29). Sections were then treated with 1% peroxide for 10 min, blocked with 10% normal goat serum for 10 min, and incubated for 1 h at room temperature with the primary antiserum (ZIP5, 1:300 dilution; ZIP4, 1:600 dilution). Controls included antisera neutralized by incubation for 2 h at room temperature with 6 × 10^-5 M peptide before application to the tissue sections, use of preimmune serum, and omission of primary antiserum (results not shown). All of these controls confirmed the specificity of the antisera.

Immunofluorescence

Tissues were fixed overnight in 4% paraformaldehyde, and 5-μm cryosections were prepared as described (30). Sections were incubated in phosphate-buffered saline (150 mM NaCl, 5 mM phosphate buffer (pH 7.0)) containing 50 μM lysine for 20 min, permeabilized by incubation for 20 min in phosphate-buffered saline containing 0.1% Tween 20 and 0.1% Triton X-100, and then blocked using 10% normal goat serum or 10% normal donkey serum during insulin detection. Co-expression of insulin and mZIP4 in the pancreas was examined by immunofluorescence using guinea pig anti-porcine insulin (DakoCytomation.com) detected with donkey fluorescein isothiocyanate-labeled anti-guinea pig serum (Jackson Immunmo.com) and rabbit anti-mouse ZIP4 incubated with biotin-labeled anti rabbit anti-serum (Jackson Immuno.com) and detected using Qdot-605-streptavidin conjugate (Qdols.com) with a TRITC filter set. Nuclear staining was achieved using 4,6-diamidino-2-phenylindole.

Accession Numbers

GenBank™ accession numbers for mouse the ZIP5 cDNA are AC008448 and AK007473 and those for the mouse ZIP5 gene are AC124670 and AC122159.

Results

Identification of the Mouse ZIP5 Gene and mRNA and Evolutionary Conservation of the Predicted ZIP5 Peptide—While searching the mouse-translated non-redundant data base for the ortholog of the human acrodermatitis enteropathica gene

1 The abbreviations used are: ZnD, zinc-deficient; ZnA, zinc-adequate; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate.
ZIP4, a cDNA was identified that encoded a protein with significant amino acid similarity to the carboxyl-terminal half of ZIP4. When aligned with mouse ZIP4, this protein was found to be 36% similar to ZIP4 across the entire length of the protein, but when only the carboxyl-terminal halves of the proteins were aligned, the similarity increased to 49%. Like ZIP4, this protein is also predicted to contain a peptide motif specifically found in transmembrane domain 5 of the other members of the LIV-1 subfamily (HEPXHE GDPAX LLXX G) (5). In addition, this protein was predicted to have eight transmembrane domains, as expected for a ZIP family member (Fig. 1 A). Based on these results, the gene encoding this protein was referred to as ZIP5 (SLC39A5), which was found later to be consistent with the nomenclature used on the NCBI website. Alignment of mouse and human ZIP5 revealed 87% amino acid similarity, with amino acid differences scattered throughout the protein (Fig. 1 A).

There were two entries in the mouse non-redundant database that encoded the ZIP5 protein, AK008448 and AK007473. These sequences were used to search the mouse expressed sequence tag (EST) data base, identifying an additional 49 base pairs at the 5' end of the cDNA, although it has not yet been experimentally determined whether this represents the actual 5' end of the transcript. There were several notable differences between AK008448 and AK007473. First, there were three single nucleotide differences within the coding region. Two of these altered the encoded amino acid, whereas the third was silent (Fig. 1 A). A survey of the entire EST data base revealed that each of these differences was present in several ESTs, suggesting that they likely represented polymorphisms rather than mutations or sequencing errors. The second difference between AK008448 and AK007473 was the deletion of 175 base pairs in AK08448 relative to AK007473, presumably due to the splicing in of a noncoding exon. Several other sequences demonstrated identical deletions, and an additional set of ESTs contained an extended deletion, again suggesting that these changes were not mutations or sequencing errors. These differences probably reflect alternate splicing that occurs within exon 2 but that does not alter the protein-coding sequence.

The mZIP5 gene was identified by using the 5' end-extended cDNA sequence to search the mouse high-throughput genomic sequences data base. The intron-exon structure of the gene was determined by aligning the gene with the cDNA and identifying splice donor and acceptor consensus sequences. From this alignment, it was determined that the two deletions noted previously in the cDNA sequences were not due to the splicing out of exons but, rather, due to the use of cryptic splicing signals (Fig. 1 B). In addition, as was seen with the mZIP4 gene (15), the mZIP5 gene contained 12 exons and was relatively short, spanning 5300 base pairs (Fig. 1 B). Using the MapViewer application on the NCBI server, the mZIP5 gene was localized to chromosome 10D3.

**FIG. 1.** Sequence alignment of the predicted mouse and human ZIP5 proteins and structures of the mouse ZIP5 gene and cDNA. A, optimal amino acid sequence alignment of the predicted mouse (m) and human (h) ZIP5 proteins. Shaded amino acids are identical, and shaded boldface amino acids are similar between these peptides. The eight putative transmembrane domains (TM1–8) are indicated by solid lines above the amino acid sequences. The LIV-1 family consensus sequence in TM5 is boxed (5). Black diamonds demarcate residues that were polymorphic in the mouse data base. Residue 137 was either leucine (shown) or arginine, whereas residue 332 was either glycine (shown) or valine. B, the full-length mouse ZIP5 cDNA was identified by searching the NCBI mouse translated non-redundant and EST databases. Three mRNAs, two with alternate splicing in exon 2 were identified, but each encodes the same predicted peptide. Positions of translation start and stop codons are shown. The mZIP5 gene was subsequently identified by comparing the mZIP5 cDNA sequence against the NCBI mouse high-throughput genome sequence data base. Intron-exon structure of the gene was delineated by comparing the cDNA sequence with the genomic sequence. kb, kilobase.
The Mouse ZIP5 Gene Is Expressed in Tissues Involved in Zinc Homeostasis—Northern blotting was used to examine the expression of the mZIP5 gene in total RNA extracted from several adult organs as well as from the embryonic visceral yolk sac (Fig. 2). The ZIP5 and ZIP4 transcripts were readily detectable in the proximal and distal small intestine as well as in the embryonic visceral yolk sac. However, ZIP5 mRNA was also detected in the pancreas and kidney, whereas ZIP4 mRNA was not.

Mouse ZIP5 Gene Expression Is Not Altered during Zinc Deficiency—To determine whether ZIP5 gene expression is responsive to dietary zinc, as we have shown previously for the ZIP4 gene (15), we examined the effects of zinc deficiency during pregnancy on the abundance of ZIP5 and ZIP4 transcripts in the maternal intestine (Fig. 3A) and embryonic visceral yolk sac (Fig. 3B). Pregnant mice were fed a ZnA or ZnD diet beginning on day 8 of pregnancy, and the maternal small intestine was harvested every 24 h thereafter (days 9–15). The relative abundance of MT-I mRNA was also examined because the transcription of this gene is diminished during dietary zinc deficiency (21). In mice fed the ZnD diet, ZIP5 mRNA abundance was unaffected, whereas ZIP4 mRNA levels were detectably increased by 24 h of zinc deficiency and continued to increase dramatically for 5 days (Fig. 3A). MT-I mRNA abundance during this period was severely reduced. Similar overall effects of zinc deficiency on these mRNAs were noted in the visceral yolk sac, and by 5 days of a zinc-deficient diet, ZIP5 mRNA levels remained unchanged, whereas ZIP4 mRNA was dramatically induced, and MT-I mRNA was repressed (Fig. 3B).

ZIP5 Is Localized on the Basolateral Surfaces of Intestinal Enterocytes, Visceral Endoderm Cells, and Pancreatic Acinar Cells in Mice Fed a Zinc-adequate Diet but Not in Those Fed a Zinc-deficient Diet—The cellular localization of ZIP5 was examined in the intestine, pancreas, and visceral yolk sac using an anti-peptide antiserum generated against an mZIP5 peptide. Tissues were collected from the same pregnant mice fed the ZnA or ZnD diets described above (Fig. 3). Controls for these experiments included the use of pre-immune serum and peptide preneutralization of the antiserum. These parallel controls eliminated the immunostaining shown in Figs. 4–7 confirming the specificity of the antibody (see Fig. 6, panels F and G). Serial sections of these tissues were also incubated with the ZIP4 antisera. This protein was induced and recruited to the apical surface of enterocytes and endoderm cells under these conditions (15). Only basal levels of ZIP4 staining were detected in these cells in mice fed the ZnA diet, and the immunostaining was predominately intracellular with little evidence for membrane localization (Fig. 4, A and B). In contrast, ZIP5 was localized to the basolateral surfaces of enterocytes and endoderm cells under these conditions (Fig. 5, A–E). In the proximal intestine ZIP5 localization was prominent in the crypts but diminished toward the apical regions of the villi (Fig. 5, D and E). Remarkably, the basolateral localization of ZIP5 was lost during zinc deficiency (Fig. 5, F and G) in both the intestine and the visceral yolk sac. Examination of intestinal sections from mice fed the ZnD diet demonstrated that the basolateral staining of ZIP5 was lost, and the apical staining of ZIP4 was first detected by 3 days of zinc deficiency (day 11) of pregnancy.

ZIP5 was also localized to the basolateral surfaces of pancreatic acinar cells (Fig. 6C). These results do not exclude the possibility that some ZIP5 may also be located on the apical surface of these cells. Nonetheless, ZIP5 staining was specific to the membranes of acinar cells. In contrast, ZIP4 staining...
**FIG. 4. Effects of dietary zinc deficiency on mZIP4 protein localization in enterocytes and visceral endoderm cells.** Pregnant CD-1 female mice (6 mice per group) were fed the ZnA or ZnD diet (as indicated) beginning on day 8 of pregnancy, and tissues were harvested on day 13 (5 days of zinc deficiency). The embryonic visceral yolk sac (VYS; panels A and C) and the maternal proximal small intestine (Intestine; panels B and D) were harvested, fixed, and embedded in paraffin. Paraffin sections were then processed for immunohistochemistry using the mZIP4 antiseraum or the ZIP5 antiserum (see Fig. 5). Brown deposits indicate positive immunostaining. Endoderm and mesoderm cells of the embryonic visceral yolk sac are indicated, as is a blood island (BI). The basal and apical surfaces of the intestinal enterocytes are indicated. Arrowheads indicate apparently intracellular immunostaining of ZIP4 in ZnA endoderm cells and enterocytes (panel A and B). Strong apical staining of these cells is evident in the ZnD samples (panels C and D). Similar results were obtained previously (15).

**FIG. 5. Effects of dietary zinc deficiency on mZIP5 protein localization in the maternal intestine and embryonic visceral yolk sac.** Sections of the embryonic visceral yolk sac (VYS; panels A, C, and F) and the maternal proximal small intestine (Intestine; panels B, D, E, and G) were obtained as described in the legend to Fig. 4, and serial sections of those shown in Fig. 4 were processed in parallel for immunohistochemistry using the ZIP5 antiserum. Sections were not counterstained. Enterocytes and intestinal enterocytes are indicated as are apical and basal surfaces of these cells. In panel E the region of the intestinal crypts and apical regions of the villi are indicated. Panels A and B are 400× magnification. Other panels are at 200× magnification except for panel E, which is at 100× magnification.
was specific for the islets of Langerhans (Fig. 6A). Remarkably, pancreatic ZIP5 immunostaining was also lost from the acinar cell membranes during zinc deficiency. Concurrently intracellular staining for ZIP5 in acinar cells was increased. Although the overall intensity of immunostaining was apparently diminished during zinc deficiency (Fig. 6, D and E), ZIP5 immunostaining was specifically detected intracellularly in acinar cells from zinc-deficient mice (Fig. 6, F and G). ZIP4 immunostaining in the islets remained unchanged during zinc deficiency but was not apparently restricted to the apical surfaces of the cells. Remarkably, insulin and ZIP4 were found to co-localize in the islets, suggesting that ZIP4 is predominately expressed in β-cells (Fig. 7).

ZIP5 Protein Levels Remain Unchanged during Zinc Deficiency—To further examine the effects of zinc deficiency on ZIP5, cell membranes were prepared from the visceral yolk sac, and membrane proteins were analyzed by Western blotting using ZIP5 and ZIP4 antisera (Fig. 8, A and B). These tissue samples were collected from the same mice fed the ZnA or ZnD diet and used for Northern blotting and immunohistochemistry shown above. An extract from cells transfected with an HA-tagged ZIP5 expression vector served as a positive control.

**Fig. 6.** Effects of dietary zinc deficiency on mZIP5 and mZIP4 protein localization in the pancreas. Sections of the maternal pancreas were obtained from day 12 or 14 pregnant mice (as indicated) that had been fed a ZnA or ZnD diet beginning of day 8. The pancreas was harvested on the indicated day of pregnancy, fixed, and embedded in paraffin. Paraffin sections were then processed for immunohistochemistry using the mZIP4 antiserum (panels A and B) or the ZIP5 antiserum (panels C–G). Sections were counterstained briefly with hematoxylin except in panels F and G. Islets of Langerhans are indicated, as are acinar cells and pancreatic ducts. Panels A–G are at 100× magnification, and insets in panels C and D (higher magnification views of acinar cells) are at 400× magnification. The sections shown in panels F and G were incubated with ZIP5 antiserum that was neutralized by preincubation with excess peptide (panel F) or that was not neutralized (panel G), and the diamino-benzidine substrate histochemical staining reaction was allowed to proceed for an extended period to intensify the staining. Arrowheads demarcate specific intracellular staining of ZIP5 (panel G).

**Fig. 7.** Colocalization of pancreatic mZIP4 and insulin. Cryosections (6 µm) of an adult pancreas were prepared and incubated with the rabbit anti-mZIP4 antiserum and guinea pig anti-porcine insulin antiserum, and immunoreactivity was detected using immunofluorescence. mZIP4 was detected using Qdot 605-streptavidin conjugate (red), and insulin was detected using fluorescein isothiocyanate-labeled anti-guinea pig antiserum (green). The color yellow in the merged image demonstrates co-localization of these antigens. Nuclei were labeled with 4,6-diamidino-2-phenylindole (blue) as shown in the merged image.
Highly glycosylated forms of ZIP5 are also detected in the positive control lane (Fig. 8A). A protein that co-migrated with positive control ZIP5 and that was of the predicted apparent molecular mass of ZIP5 (~56 kDa) was detected in the visceral yolk sac extracts, and the relative amount of this protein remained essentially unchanged even up to 7 days of dietary zinc deficiency. In contrast, ZIP4 immunoreactivity on this Western blot was minimal in extracts from mice fed the ZnA diet and dramatically increased in extracts from mice fed the ZnD diet (Fig. 8B). In addition to the predicted ZIP4 peptide (71 kDa), a smaller ~35-kDa peptide was also detected. This may represent a proteolytic breakdown product of ZIP4. It is present in very small amounts in the ZnA sample, is induced during zinc deficiency, and prneualization of the antiserum using the ZIP4 peptide eliminated its immunoreactivity (results not shown). The identity of the ~62-kDa protein band that is invariant in this Western blot is not known. Taken together these results suggest that the cellular localization of ZIP5 but not the amount of this protein is dramatically changed in response to zinc deficiency.

**DISCUSSION**

Eukaryotic cells adapt to the environmental/dietary zinc levels to maintain a constant state of cellular zinc nutrition (31–33). In adult mammals, the intestinal enterocyte is the site of absorption of dietary zinc, but under zinc-replete conditions a significant amount of zinc is also released from enterocytes back into the intestinal lumen (33). In addition, the pancreas plays a central role in zinc homeostasis (34), and a significant amount of zinc is released from the pancreas into the intestinal tract when dietary zinc is replete (32, 33). 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 (33). Pregnancy superimposes increased demands on the zinc homeostatic mechanisms, particularly during the last half of pregnancy, coincident with rapid growth of the embryo. This in turn exacerbates the effects of maternal dietary zinc deficiency (35–38). Zinc must traverse the visceral yolk sac and placenta in order to be utilized by the developing embryo and fetus, but little is known about how that process is regulated.

In fact, mechanisms underlying the mammalian adaptive responses to dietary zinc are in general poorly understood. Recent studies of mammalian zinc transporters suggest that members of the ZIP (SLC39A) (2, 3) and Znt (SLC30) superfamilies (39) of transporters play essential roles in zinc homeostasis. For example, mutations in ZIP4 (SLC39A4) cause acrodermatitis enteropathica (8, 9), a rare genetic disorder of zinc metabolism in humans (40). The lethal milk mutant mouse results from a defective Znt4 gene (SLC30A4) (41), which leads to reduced zinc concentrations in milk and subsequent neonatal lethality (42, 43). In addition, targeted deletion of the mouse Znt1 gene is lethal to the early embryo (44), deletion of mouse Znt5 causes osteopenia and male-specific sudden cardiac death (45), and deletion of Znt3 leads to the loss of zinc in synaptic vesicles (39).

Herein, we described the mouse ZIP5 gene (SLC39A5), which encodes a member of the LIV-1 subfamily of ZIP proteins. Our results indicate that ZIP5 fulfills many of the criteria expected for a protein that is involved in the regulation of zinc excretion during the adaptive response to changes in dietary zinc intake. In related studies it was found that mZIP5 functions as a zinc transporter that is specific for zinc as a substrate (18). Herein, we demonstrate that ZIP5 expression in vivo is most active in maternal enterocytes, embryonic visceral endoderm, and pancreatic acinar cells. Furthermore, we show that mZIP5 is localized to the basolateral surface under conditions of adequate dietary zinc but is removed from the basolateral surface of each of these cell types during periods of zinc deficiency. These results are consistent with the hypothesis that ZIP5 plays a role in removing zinc from the embryo and adult when dietary zinc is replete and that this function is attenuated during periods of dietary zinc deficiency.

This hypothesis suggests that ZIP4 and ZIP5 serve opposing functions in zinc homeostasis. ZIP4 is important for the acquisition of dietary zinc in humans and likely also performs this function in mice. In addition, mouse ZIP4 may also play a role in the acquisition of maternal zinc by the developing embryo. The ZIP4 gene is induced during zinc deficiency, and this protein is recruited to the apical surfaces of these cell types. Thus, during zinc deficiency there is a large increase in the amount of mZIP4 mRNA and protein as well as recruitment of this protein to the apical surfaces of enterocytes and endoderm cells. In contrast to our previous results, which suggested that mZIP4 is present on the apical membrane of the colonic epithelium (8), it should be noted that we have no evidence that mouse ZIP4 is localized to the apical surfaces of enterocytes and endoderm cells in mice fed a zinc adequate diet (15). This suggests that ZIP4 may function in the acquisition of dietary zinc particularly during periods of marginal zinc intake. We cannot, however, rule out the possibility that ZIP4 also exerts intracellular functions in these cell types. In contrast, ZIP5 is clearly localized on the basolateral surfaces of these cells when...
zinc is replete in the diet but is removed during periods of dietary zinc deficiency. The differential enterocytic localization and response of ZIP4 and ZIP5 to zinc is also relevant to a curious finding in patients with acrodermatitis enteropathica. Previously, Wang et al. (8) suggested that mutations in ZIP5 (referred to therein as hORF1) might be responsible for disease in those individuals for whom ZIP4 mutations could not be identified. However, detailed sequence analysis of DNA from such individuals failed to uncover any ZIP5 mutations.2 The results from this study provide a satisfying resolution to this paradox, but the basis for disease in other patients remains unsolved.

In contrast to the induction of ZIP4 gene expression in response to dietary zinc deficiency, ZIP5 mRNA levels were unaltered in the intestine and visceral endoderm under these conditions. Similarly, ZIP1, -2 and -3 mRNA levels in the intestine and visceral endoderm are unresponsive to dietary zinc status (12). However, the cellular localization of several ZIP proteins is influenced by dietary zinc. Our previous studies demonstrated that mouse ZIP1, -3, and -4 undergo zinc-induced endocytosis (17, 46). Under conditions of zinc deficiency the endocytosis of these proteins is reduced, leading to increased levels on the plasma membrane. This is reminiscent of the concept that both constitutive and inducible zinc transporters have multiple cellular functions, and that the regulation of iron uptake involves induction of the iron regulatory hormone hepcidin, which inhibits iron transport across the intestinal epithelium (49, 50). Perhaps a similar mechanism functions to regulate ZIP5 trafficking.

Zinc homeostasis in mice involves the uptake, efflux, and storage of this essential metal. Several members of the ZIP and Znt family of transporters are now known to also be expressed in mouse enterocytes and visceral endoderm cells and are, therefore, also likely to play important roles in zinc homeostasis. The effects of mutations in human ZIP4 can be partially ameliorated by increased dietary zinc intake, consistent with the concept that both constitutive and inducible zinc transporters function in the intestine. We have shown that ZIP1, -3, -4, and -5 are all expressed in the intestine and visceral yolk sac (12, 15), but our evidence suggests that of these proteins, only ZIP5 and ZIP4 are significantly expressed in the pancreas. ZIP5 is known to be expressed in acinar cells, but many have not been examined to date. Previous studies demonstrated that pancreatic metallothioneins may also play a role in zinc excretion. These genes are highly expressed in the mouse pancreas, and recent studies from our laboratory suggest the possibility that zinc-metallothionein complexes may be released from the pancreas into the gut (53). Transgenic mice that overexpress metallothionein specifically in pancreatic acinar cells show increased resistance to the effects of dietary zinc deficiency (54). Thus, metallothioneins may be involved in release of zinc from the pancreas. Among the Znt family, Znt5 is known to be abundantly expressed in the human pancreas (51), which strongly suggests that this gene is active in acinar cells, which represent the vast majority of cells in this organ. However, immunolocalization of Znt5 revealed staining in secretory granules of β-cells in the islets as well as in some acinar cells (51). Other studies report that Znt1 to 4 are also expressed in pancreatic β-cells, although the localization of these proteins has not been determined (55). Remarkably, we found that ZIP4 is also abundant in β-cells. Thus, ZIP4 may play a role in the uptake of zinc into β-cells, which require it to properly package insulin (56). Ultimately, resolving the biological functions of ZIP4 and -5 will require genetic manipulation of these genes in mice. Such studies are under way.

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The Adaptive Response to Dietary Zinc in Mice Involves the Differential Cellular Localization and Zinc Regulation of the Zinc Transporters ZIP4 and ZIP5
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