The Acrodermatitis Enteropathica Gene ZIP4 Encodes a Tissue-specific, Zinc-regulated Zinc Transporter in Mice*

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The human ZIP4 gene (SLC39A4) is a candidate for the genetic disorder of zinc metabolism acrodermatitis enteropathica. To understand its role in zinc homeostasis, we examined the function and expression of mouse ZIP4. This gene encodes a well conserved eight-transmembrane protein that can specifically increase the influx of zinc into transfected cells. Expression of this gene is robust in tissues involved in nutrient uptake, such as the intestines and embryonic visceral yolk sac, and is dynamically regulated by zinc. Dietary zinc deficiency causes a marked increase in the accumulation of ZIP4 mRNA in these tissues, whereas injection of zinc or increasing zinc content of the diet rapidly reduces its abundance. Zinc can also regulate the accumulation of ZIP4 protein at the apical surface of enterocytes and visceral endoderm cells. These results provide compelling evidence that ZIP4 is a zinc transporter that plays an important role in zinc homeostasis, a process that is defective in acrodermatitis enteropathica in humans.

A long recognized disease of zinc metabolism is the human genetic disorder acrodermatitis enteropathica (AE)1 (1, 2). This autosomal recessive disorder causes classic symptoms of zinc deficiency (3), such as dermatological lesions, changes in the gastric mucosa associated with digestive system problems, lack of weight gain, and immune and reproductive problems (4–8). Remarkably, these symptoms can be ameliorated by dietary zinc supplement (4, 6, 9–11), consistent with the finding of reduced, but not eliminated, uptake of 65Zn by the intestine from AE patients (12, 13), and the reduced uptake and total content of zinc in AE fibroblasts (14, 15). Recent genetic mapping localized the AE gene to chromosome 8q24.3 (16) and led to its identification as a member of the ZIP superfamily (17, 18). That gene was named hZIP4 (the Human Genome Organization Nomenclature Committee named this gene SLC39A4).

ZIP4 was found to be expressed in enterocytes and to reside in the plasma membrane. Mutations in hZIP4 were detected in AE patients (17, 18), strongly suggesting that they cause this genetic disorder.

The recently identified ZIP superfamily of metal ion uptake transporters (19, 20) are found in all eukaryotes, and many of its members mediate zinc uptake. In yeast, ZRT1 encodes the high affinity zinc transporter, and ZRT2 encodes the low affinity zinc transport system. The Arabidopsis iron-regulated transporter gene (IRT1) encodes a metal transporter that has remarkable sequence similarity with the yeast ZRTs and with other Arabidopsis zinc transporters (19, 21). Thus, the acronym ZIP was adopted to reflect ZRT/IRT-related proteins. Many members of the ZIP gene superfamily have now been detected based on sequence homology with yeast and Arabidopsis ZIP genes (22, 23). The ZIP proteins typically have eight membrane-spanning domains, and spanning domain four contains fully conserved histidyl and glycylic residues in an amphipathic α-helix. These proteins also often have a histidine-rich intracellular loop between spanners three and four. These structural motifs are hallmarks of the ZIP superfamily (22, 23). Computer searches of the complete human genome sequence revealed ~12 ZIP genes (24). Three of these ZIP proteins (hZIP1–3) fall into a subfamily that shares a conserved 12-residue signature sequence. hZIP1 and hZIP2 function as zinc transporters, and hZIP1 is the major zinc uptake protein in K562 cells (25, 26). ZIP4 and ZIP5 also comprise a ZIP subfamily (17), but their metal transport properties have not yet been determined.

ZIP genes that encode zinc transporters can also be regulated by zinc. The yeast ZRT1 and ZRT2 genes are up-regulated in response to zinc deficiency (27). Transcription of these genes is controlled by the transcription factor Zap1p (28), and the activity of Zap1p is inhibited by zinc. In Arabidopsis, the ZIP1, ZIP3, and ZIP4 genes are zinc-regulated, consistent with a role in zinc uptake (e.g. in roots) (29). In mammals, dietary zinc also regulates zinc transport activities and zinc transporter gene expression in the intestines (30–35). For example, the mouse, rat, and human ZnT1 genes, which encode members of the cation diffusion facilitator family of proteins, are regulated by zinc (34–36). There is little information regarding metalloregulation of ZIP family members in mammals.

In support of the hypothesis that ZIP4 is the AE gene, the results presented herein demonstrate that mZIP4 encodes a zinc transporter, that this gene is expressed in intestine and embryonic visceral yolk sac, that ZIP4 protein localizes to the apical surface of enterocytes and visceral endoderm cells, and that the expression of this gene and its protein product is dynamically regulated by zinc.
Mouse ZIP4 Regulation and Function

EXPERIMENTAL PROCEDURES

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 Breeding Laboratories (Raleigh, NC). Mouse diets were purchased from Harlan Teklad (Madison, WI) and have been described in detail previously (37). Zinc levels in the diets were as follows: zinc-deficient (ZnD), 1 ppm zinc; zinc-adequate (ZnA), 50 ppm zinc; and zinc-excess (ZnE), 50 ppm zinc plus 250 ppm zinc in the drinking water. These diets each contained ~18 μg/kg copper and are otherwise identical.

To examine the tissue-specific expression of ZIP4, CD-1 female or male mice (6/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 ZIP4 expression, female mice were subjected to dietary zinc deficiency followed by either an injection of zinc or switching to ZnE conditions. Dietary zinc deficiency during pregnancy was induced as described previously (38). CD-1 female mice were mated with CD-1 male mice and on day 1 (vaginal plug) of pregnancy mice were placed in pairs in cages with stainless steel false bottoms to reduce recycling of zinc (39). Mice were provided access to the ZnA feed and deionized distilled water. Water bottles were washed in 4 M HCl and rinsed in deionized water to remove zinc (39). On day 8, the diet was changed to the ZnD diet (or, where indicated, mice were maintained on the ZnA diet). The visceral yolk sac and maternal small intestine were harvested on day 11 to 15 (6 mice/group) and either fixed for immunohistochemistry or snap-frozen in liquid nitrogen for Northern blot analysis. Where indicated, zinc-deficient day 14 pregnant mice were either injected intraperitoneally with ZnCl₂ (100 μmol/kg body weight) or switched to ZnE conditions, and the embryonic visceral yolk sacs and maternal small intestines were collected at the indicated times after zinc treatment. The maternal intestine was isolated as an intact tissue (not a mucosal scrap) but was subdivided as follows. The first three centimeters was considered the duodenum, and the remainder of the small intestine was divided into equal parts, which were considered the proximal (nearest the duodenum) and distal small intestine. Previous studies have documented that little anorexia occurs under these experimental conditions, and results using pair-fed controls do not differ from those obtained using mice allowed free access to feed (37, 38).

Nonpregnant female CD-1 mice were fed ZnA or ZnD feed for 2 weeks and then injected intraperitoneally with zinc or switched to ZnE conditions, as described above. The duodenum and proximal small intestine were harvested at the indicated times after zinc treatment.

RNA Extraction and Northern Blot Hybridization—Tissue RNAs were isolated as described in detail previously (36, 40). Total RNA (3 μg) was size-fractionated by agarose-formaldehyde gel electrophoresis, transferred, and cross-linked to nylon membranes (41). Northern blot membranes were hybridized and washed under stringent conditions as described (36, 37, 41). Hybrids were detected by autoradiography with subsequent exposure of the film to x-ray film. The mouse metallothionein-I (MT-I) and -II (MT-II) were used as calibrating markers for RNA loading. Multiple sequence alignments were performed using the Vector NTI Suite Program (Informax, Bethesda, MD).

RT-PCR Detection of ZIP4 Isoform mRNAs—RT-PCR was used to distinguish between mZIP4 mRNA isoforms that encode the long versus the short isoforms of this protein. Total RNA (1 μg), isolated from the maternal small intestine or embryonic visceral yolk sac harvested from the zinc-deficient pregnant mice described above, was DNase I-treated according to the instructions from the manufacturer (Invitrogen). DNase I was inactivated by addition of EDTA to 2.5 mM, followed by a 10 min incubation at 65 °C. Reverse transcription was subsequently carried out using Promega-RT reverse transcriptase (Promega). Samples were then amplified by PCR for 27, 30, or 35 cycles using Platinum Taq DNA polymerase (Invitrogen). The long isoform transcript was amplified using the primers mZIP4INT1(s) (5'-AGACTGCAAGCTCTAAACGGAAGCCG-3') and mZIP4INT2(as) (5'-AGTAGCTCTGTCATCAAGGAGTCC-3'), whereas the short isoform transcript was amplified using the primers mZIP4INT1(s) (5'-AACATGACAATAGATGCGTGGATAGAATCCATGTCG-3') and mZIP4INT2(as). The RT-PCR products of the long and short isoforms transcripts were 475 and 329 bp, respectively.

Immunohistochemistry—The rabbit polyclonal antiserum against a mZIP4 peptide was described previously (17). Immunohistochemistry was performed using the Histostain-SP kit (Zymed Laboratories Inc., San Francisco, CA) for rabbit primary antibody and AEC staining. Tissues were fixed overnight in 4% paraformaldehyde at 4 °C, embedded in paraffin, and sectioned. Sections were deparaffinized, treated with 1% peroxide for 10 min, blocked with 10% normal goat serum for 10 min, and then incubated for 1 h at room temperature with the mZIP4 antiserum at a 1:160 dilution. Where indicated, the mZIP4 antiserum was neutralized by incubation for 2 h at room temperature with 6 × 10⁻⁶ M peptide before application to the tissue sections. Other controls included omission of primary serum or the omission of peroxidase conjugate.

Expression Plasmid Construction—Mouse ZIP4 cDNA encoding the long isoform was cloned into an expression vector that was used for zinc uptake studies in transiently transfected cells. To subclone mZIP4, total RNA from mouse intestine (1 μg) was reverse transcribed using Promega-RT reverse transcriptase (Promega, Madison, WI), followed by amplification of the cDNA using Pfu DNA polymerase (Stratagene) with primers mZIP4-S/S (5'-GGGAGCTCAAGGACCCAGG-3') and mZIP4-A/S (5'-GGGGAGCTCAAGGACCCAGG-3'). The amplified product was digested with EcoRI and SalI and ligated into pCMVSPORT6 vector or pCMV-Sport6 expressing the mouse mZIP4 cDNA (pcMV-mZIP4). Transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the instructions from the manufacturer. Transfection efficiencies were typically ~60%. Between 36 and 48 h after transfection, the cells were used for zinc uptake assays.

45Zn Uptake Assays—Zinc uptake assays were performed essentially as described previously (25, 26). Cells were washed three times with uptake buffer (15 mM Hepes, 100 mM KCl, 50 mM NaCl, 1 mM MgCl₂, and 10 mM Cytosine, pH 7.0) and then prewarmed uptake buffer containing the specified concentration of 65ZnCl₂ (PerkinsElmer Life Sciences) was added. The cells were then incubated in a shak ing 37 °C water bath for 15 min unless indicated otherwise. Assays were stopped by the addition of an equal volume of ice-cold uptake buffer supplemented with 1 mM EDTA (stop buffer). Cells were collected on glass fiber filters (Type AE, Gelman Sciences) and washed three times in stop buffer (~10 ml of total wash volume). Parallel experiments were conducted at 0 °C to measure cell surface 65Zn binding, which was subtracted from the values at 37 °C to obtain net zinc uptake values. Cell-associated radioactivity was measured with a Packard Auto-Gamma 5620 γ-counter. Metal salts were obtained from Sigma. Stock solutions of the chloride salts (CoCl₂, CuCl₂, MgCl₂, MnCl₂, NiCl₂, and CdCl₂) and AgNO₃ were prepared at 100 mM concentration in distilled water. A ZnCl₂ stock was prepared at 100 mM in 0.2 N HCl, and an FeCl₃ stock solution was prepared at 50 mM in 0.1 N HCl. Sodium ascorbate (1 mM) was used to reduce Fe³⁺ to Fe²⁺. Ascorbate treatment alone did not alter zinc fluxes. Cells grown in the absence of zinc, exposed to zinc-free medium for uptake experiments were washed three times with ice-cold uptake buffer, resuspended in PBS buffer containing 0.1% SDS and 1% Triton X-100, for cell lysis, and then assayed for protein content using a Bradford assay kit (Bio-Rad). Zinc accumulation and uptake rates were normalized to protein concentrations of these cell lysates. Michaelis–Menten constants were determined by nonlinear regression of the data using Prism (version 3.0a for Macintosh, GraphPad Software, San Diego, CA).

GenBank™ Accession Numbers—GenBank™ accession numbers for the mouse ZIP4 ESTs were AK005535, AJ314527, and BB84544 for
RESULTS

Identification of the Mouse ZIP4 Gene and mRNA and Evolutionary Conservation of the Predicted ZIP4 Peptide—The predicted human ZIP4 protein sequence (17, 18) was used to search the mouse translated non-redundant data base on the NCBI server, and a 2264-bp cDNA containing a 660-amino acid open reading frame homologous to the long isoform of hZIP4 was identified (Fig. 1). Two ESTs extended the 5′ end of this mZIP4 cDNA an additional 439 bp. However, the 5′ end of the vast majority of mZIP4 ESTs corresponded to the 2264-bp transcript (Fig. 1A).

The mZIP4 gene was subsequently identified using this cDNA sequence to search the mouse high throughput genomic sequence data base on the NCBI server. Intron-exon structure was determined by comparing the cDNA and genomic sequences in conjunction with splice donor and acceptor consensus sequences (Fig. 1A). Like the human gene (17, 18), mouse ZIP4 consists of 12 exons separated by 11 introns and is quite compact, spanning only ~5 kb of DNA. The ZIP4 gene in humans is located on chromosome 8q24.3, whereas the mZIP4 gene is located in the syntenic region on mouse chromosome 15E1.

Alignment of the predicted amino acid sequences of the long isoform of mZIP4 and hZIP4 (17, 18) revealed 76% amino acid identity between these two proteins (Fig. 1B). The amino-terminal half of these proteins, which is predicted to be extracellular, is not as well conserved, although several conserved blocks of amino acids are present. The highest degree of similarity occurs in the carboxyl-terminal half of the protein, which contains the eight predicted membrane-spanning domains characteristic of ZIP proteins. The amino acid sequence within the predicted membrane-spanning regions is highly conserved; most of the amino acid differences between these proteins occur in the intervening loops. Interestingly, of the 11 amino acid changes found in various AE patients, 9 occur at residues that are also conserved in the mouse protein. These changes often convert an uncharged to a charged residue within the highly conserved transmembrane segments.

In humans, two ZIP4 mRNAs have been detected which are predicted to encode either a long (647-residue) or short (622-residue) isoform of this protein (18). These hZIP4 isoforms are identical in the carboxyl-terminal 583 residues, but the short form is predicted to have a 39-residue amino terminus encoded in intron 1 and to lack the 64 amino acids encoded in exon 1 (Fig. 1C). Similarly, two mZIP4 transcripts were identified in the mouse EST data base. The vast majority of mZIP4 ESTs correspond to the long isoform of this protein (Fig. 1B), and to date only two ESTs that correspond to a short form of mZIP4 have been entered into the EST data base. These mouse ESTs are predicted to encode a portion of a 613-residue protein with a unique 16-residue amino terminus (Fig. 1C). This amino terminus is encoded within intron 1, but the remaining 597 residues are identical between the long and short isoform of mZIP4. There is very little similarity between the amino-terminal amino acids of the short isoforms in human and mouse. The short form transcripts are predicted to originate from an alternate transcription start point within intron 1 (Fig. 1A), but are almost the same length as those that encode the long isoform. The functional significance of two isoforms of mZIP4 is unknown, but based on the relative abundance of these ESTs, the long isoform of mZIP4 is predicted to be far more abundant, and was therefore studied in more detail.

Mouse ZIP4 Can Function as a Zinc Transporter—Because of its homology with the ZIP family of proteins and the presence of hZIP4 mutations in AE patients, mZIP4 is predicted to function as a zinc transporter. To assess the potential role of mZIP4 in zinc transport, the ZIP4 open reading frame encoding the more abundant long isoform was cloned into a mammalian expression vector, pCMV-Sport6, allowing high level expression from the CMV promoter. This plasmid (pCMV-mZIP4) and the vector alone were transiently transfected into HEK293 cells, and these transfected cells were then assayed for 65Zn uptake activity (Figs. 2 and 3). Consistent with an ability of mZIP4 to transport zinc, cells expressing mZIP4 from the CMV promoter accumulated ~5-fold more 65Zn over a 60-min period than did the endogenous zinc transport activity assayed in cells transfected with the vector alone (Fig. 2A). Only low levels of zinc accumulation were detected when either of these transfected cell types were incubated with 65Zn at 0 °C. These results indicated that zinc accumulation by both the mZIP4-dependent activity and the endogenous system was temperature-dependent, and therefore likely to be transporter-mediated and not the result of zinc binding to the cell surface. Also consistent with zinc transport, mZIP4-dependent zinc accumulation was concentration-dependent and saturable. This activity showed Michaelis-Menten kinetics with an apparent K_m of 1.6 ± 0.1 μM and a V_max of 13.1 ± 0.2 pmol of zinc/min/mg of protein (Fig. 2B). The endogenous system in HEK293 cells had a similar apparent K_m (2.1 ± 0.2 μM) but a far lower V_max (4.1 ± 0.1 pmol of zinc/min/mg of protein).

To assess the specificity of the mZIP4-dependent activity for zinc over other possible substrates, we tested the effect of various metal ions on zinc accumulation by cells transfected with the mZIP4 expression vector. The metal ion specificity of the endogenous zinc uptake system was determined in cells transfected with the empty vector. Cells were assayed after incubation for 15 min with 1.5 μM 65Zn with or without a 10- or 50-fold excess of added competitor metal. As shown in Fig. 3A, the endogenous zinc uptake activity in HEK293 cells was strongly inhibited by excess zinc, copper, and cadmium and to a lesser extent by several other metal ions. In marked contrast, mZIP4-dependent uptake activity was strongly inhibited only by excess nonradioactive zinc and to a far lesser extent by other metals (Fig. 3B). These results indicate that mZIP4-dependent activity is distinguishable from the endogenous activity and suggest that mZIP4 is very specific for zinc as its substrate.

The Mouse ZIP4 Gene Is Expressed in a Tissue-specific Manner—Expression of the mZIP4 gene was examined by Northern blot hybridization to total RNA extracted from several organs from male and female CD-1 mice, as well as from extra-embryonic tissues that surround the developing mouse embryo (Fig. 4). In adult mice, ZIP4 transcripts were readily detectable in the stomach, intestine and liver, but not in the other organs examined. This mRNA was far more abundant in the small intestine than in the stomach and liver. Furthermore, analysis of the different regions of the intestines (duodenum, proximal and distal small intestine, colon) revealed that this mRNA is abundant throughout the intestinal tract (some data not shown). Northern blot analysis of RNA from the embryonic visceral yolk sac and placenta taken from midgestation mouse embryos revealed that mZIP4 mRNA is also highly abundant in the visceral yolk sac (Fig. 4), but not in the placenta (data not shown). These extra-embryonic tissues transport nutrients from the mother to the embryo. Thus, ZIP4 mRNA was by far the most abundant in tissues involved in zinc uptake and/or storage. RT-PCR analysis of RNA samples suggested that the mRNA encoding the long isoform of this protein is far more abundant than that encoding the short isoform in all of these tissues that actively express the mZIP4 gene (data not shown).
Mouse ZIP4 Gene Expression and Protein Localization Are Zinc-regulated. To determine whether ZIP4 gene expression is responsive to zinc, mice were subjected to dietary zinc deficiency and the abundance of ZIP4 mRNA was examined by Northern blotting, and ZIP4 protein was localized by immunohistochemistry (Figs. 5 and 6).

Mouse ZIP4 Regulation and Function

Fig. 1. Structures of mouse ZIP4 gene and cDNA, and sequence alignment of the predicted mouse and human ZIP4 proteins. A, the full-length mouse ZIP4 cDNA and gene identified in the NCBI mouse translated non-redundant data base and the NCBI mouse high-throughput genome sequence data base, respectively. Translation start and stop codons and the polyadenylation signal are shown. The gene is approximately 5 kb in length. B, optimal amino acid sequence alignment of the predicted human and mouse ZIP4 proteins. The predicted long isoforms are shown. The shaded amino acids are conserved between mouse and human ZIP4. The eight putative transmembrane domains (TM1–8) are indicated by solid lines above the amino acid sequences. Eleven mutations resulting in amino acid changes found in AE patients are indicated using the one-letter amino acid code and the number of the amino acid residue in hZIP4 that was mutated (17, 18). C, alignment of the amino termini of the predicted short isoform of hZIP4 and mZIP4. The short form alternate transcription start point in intron 1 of the mZIP4 gene is designated by an arrow in A.
5A, pregnant mice were fed a ZnA or ZnD diet beginning on day 8 of pregnancy, and the abundance of mZIP4 mRNA in the maternal small intestine and the embryonic visceral yolk sac was examined. In addition, the relative abundance of MT-I mRNA was examined because this gene is known to be down-regulated by dietary zinc deficiency in these tissues (40). Dietary zinc deficiency during pregnancy is one model system with which to examine the roles of zinc in gene regulation and development. The demand for zinc increases during midgestation as a result of the rapid growth of the embryo, thus accentuating the effects of zinc deficiency (38, 40). In mice fed the ZnD diet, ZIP4 mRNA dramatically increased in relative abundance in the intestine and visceral yolk sac, whereas MT-I mRNA abundance was reduced. This effect occurred rapidly in the intestine and was noted after 3 days on the ZnD diet (day 11 of pregnancy). In contrast, this effect occurred gradually in the visceral yolk sac and was maximal after 6 days on the ZnD diet (day 14 of pregnancy) (Fig. 5A). As above, RT-PCR analysis of these RNA samples suggested that mZIP4 transcripts encoding the long isoform of the protein were far more abundant than those encoding the short isoform (data not shown).

Metalregulation of mZIP4 was further examined in non-pregnant mice fed the ZnD diet for 2 weeks followed by an injection of ZnCl₂ or switching them to conditions of dietary zinc excess (ZnE) (Fig. 6A). Zinc uptake was measured and compared with cells incubated in the absence of inhibitor (C, hatched bars). All metals were present as the divalent cation. Fe(II) was obtained by mixing FeCl₃ with 1 mM sodium ascorbate prior to treating the cells. Ascorbate alone had no effect on ⁶⁵Zn accumulation (data not shown). Each point represents the mean in a representative experiment (n = 3), and the error bars indicate ±1 S.D.

Fig. 2. Characterization of zinc uptake kinetics in cultured cells transiently transfected with an mZIP4 expression vector. HEK293 cells were transiently transfected with either the vector pCMV-Sport6 or pCMV-mZIP4 (long isoform). Transfectants were cultured for 48 h prior to assay. A, zinc accumulation was assayed with pCMV-mZIP4 (triangles) or pCMV-Sport6 (circles) transfectants with 2 μM ⁶⁵Zn at 37 °C (filled symbols) or 0 °C (open symbols). B, concentration dependence of zinc uptake activity was determined over a range of ⁶⁵Zn concentrations in cells incubated for 15 min with labeled zinc. Each point represents the mean in a representative experiment (n = 3), and the error bars indicate ±1 S.D.

Fig. 3. Characterization of metal specificity of uptake in cultured cells transiently transfected with an mZIP4 expression vector. The ability of various metal ions to inhibit ⁶⁵Zn uptake by HEK293 cells transfected with an empty vector (A) or the mZIP4 expression vector (B) was determined. Either a 10-fold (open bars) or a 50-fold (filled bars) molar excess of the indicated metal ions was added to uptake buffer containing 1.5 μM ⁶⁵Zn, and the cells were incubated under these conditions for 15 min prior to washing and counting. Zinc uptake was measured and compared with cells incubated in the absence of inhibitor (C, hatched bars). All metals were present as the divalent cation. Fe(II) was obtained by mixing FeCl₃ with 1 mM sodium ascorbate prior to treating the cells. Ascorbate alone had no effect on ⁶⁵Zn accumulation (data not shown). Each point represents the mean in a representative experiment (n = 3), and the error bars indicate ±1 S.D.

Fig. 4. Northern blot detection of mZIP4 transcripts in mouse organs. The indicated mouse organs were collected, and total RNA was extracted and assayed by Northern blotting using a cDNA probe for mZIP4. Hybrids were detected by autoradiography (upper panel). Integrity and loading of RNA was confirmed by acridine orange staining of a duplicate gel (lower panel) and hybridization with an mZIP1 probe (data not shown). ZIP1 is expressed in most of these organs.
The effects of zinc on the localization and apparent abundance of mZIP4 protein were also examined in the intestine and visceral yolk sac from pregnant mice fed the ZnD diet. Immunohistochemical localization of ZIP4 protein in the intestine and visceral yolk sac revealed (Fig. 5B) intense staining at the apical surfaces of enterocytes and visceral endoderm cells from zinc-deficient mice on day 14 of pregnancy. In contrast, in pregnant mice fed the ZnA diet, more diffuse immunostaining of ZIP4 was apparent throughout the enterocytes and endoderm cells on day 14. The counterstain obscured the diffuse immunostaining in the visceral yolk sac from mice fed the ZnA diet. Examination of other tissues (liver, placenta) did not reveal significant immunostaining of mZIP4 under these conditions. However, the islets of Langerhans in the pancreas showed apical staining of many cells (data not shown). The effect of ZnE conditions on mZIP4 localization was then examined. Immunohistochemical localization of ZIP4 in the d14 visceral yolk sac from zinc-deficient mice revealed that an injection of zinc led to the rapid loss of staining at the apical surface of the endoderm cells and the apparent internalization of ZIP4 protein by 9 h. By 24 h after an injection of zinc or switching to ZnE conditions, there was a dramatic reduction in immunoreactive ZIP4 (Fig. 6C). Similar results were obtained with the intestine (data not shown). Thus, expression of the mZIP4 gene in the intestine and visceral yolk sac is remarkably responsive to zinc, as is the localization and abundance of this protein.

**DISCUSSION**

These studies were undertaken to examine the function and regulation of mouse ZIP4 because its human orthologue has recently been mapped to the genetic locus responsible for AE. AE is a recessive disorder of zinc metabolism, and hZIP4 was found to have mutations in patients with AE. It was predicted that the AE gene would encode a zinc transporter found on the apical surface of enterocytes that is regulated by zinc. Intestinal uptake of zinc is an essential component of mammalian zinc metabolism, and this process is responsive to dietary zinc, being induced during periods of zinc deficiency (30–35). Mouse ZIP4 was found to meet these criteria. Thus, it may play an important role in zinc metabolism and homeostasis.

The structure of ZIP4 places it as a member of a gene superfamily that encodes metal ion uptake transporters (19, 20). It was demonstrated herein that ZIP4 functions to preferentially transport zinc, unlike many other members of the ZIP superfamily, which are more promiscuous with regard to metal ion specificity (24, 42). Human ZIP1 and ZIP2, for example (25, 26), function as zinc transporters, but their uptake activity is inhibited by copper, iron, and cadmium. Zinc uptake by hZIP2 is also inhibited by magnesium and cobalt. By comparison, mouse ZIP4 is remarkably specific for zinc. The mechanism for this substrate specificity is not understood. Mutagenesis of Arabidopsis IRT1, an iron transporter with broad substrate specificity, revealed that the conserved histidyl, serine, and glycyl residues in spanning domain 4 are essential for all uptake activity, whereas residues in loop regions dictate substrate specificity (43). Human and mouse ZIP4 share significant amino acid similarity throughout the protein, but the eight predicted transmembrane domains are the most highly conserved, and many of the amino acid mutations found in AE patients are in these domains. Thus, the structure of the transmembrane domains must play a paramount role in ZIP4 function, consistent with the concept that these domains may form...
a channel through which zinc must pass. Whether AE mutations eliminate or reduce ZIP4 activity remains to be determined. The ZIP4 and ZIP5 subfamily of ZIPs is most similar to LIV-1 (17), which has been evolutionarily conserved between humans and mice. The functions of ZIP5 are unknown.

Based on the analysis of ESTs and RT-PCR studies, two ZIP4 mRNAs were identified in humans (17, 18) and in mice. These mRNAs are predicted to encode ZIP4 isoforms that are identical in the carboxyl termini (583 or 597 residues, respectively), but different in their amino termini (64 or 16 residues, respectively). These amino-terminal residues in the short isoform are encoded within intron 1 of the ZIP4 gene in both mice and humans, and share little sequence similarity between these species. To date it has not been formally demonstrated that two isoforms of this protein are actually translated in human or mouse cells. Although the short isoform of ZIP4 is predicted to lack an amino-terminal signal peptide, it could be localized to the plasma membrane using an internal signal peptide. Nevertheless, this protein is abundant on cells within the visceral yolk sac, the first site of erythropoiesis during development. ZIP4 also localizes to the apical surface of visceral endoderm cells and its abundance is regulated by zinc. Re-expression of ZIP4 is responsive to zinc is unknown. However, ZIP4 mRNA and protein abundance are regulated by zinc, and this protein accumulates to very high levels on the surface of enterocytes during periods of zinc deficiency.

We also provided evidence that ZIP4 may be important for the uptake of maternal zinc into the embryonic environment in mice. The ZIP4 gene is actively expressed during pregnancy in visceral endoderm cells that surround the developing embryo soon after implantation. These cells produce amniotic and serum proteins before the liver develops and are part of the visceral yolk sac, the first site of erythropoiesis during development. ZIP4 also localizes to the apical surface of visceral endoderm cells and its abundance is regulated by zinc. Remarkably, ZIP4 is not expressed in the placenta in humans or mice. Thus, our studies suggest that the visceral yolk sac also functions to sequester zinc from the mother during midgestation. It is interesting to note that MT-I gene expression in the visceral endoderm is constitutively high during development, and this expression is regulated by the zinc-sensing transcription factor MTF-1. Thus, we hypothesized previously that the visceral endoderm might be particularly rich in zinc (40).

Data presented herein suggest that zinc regulates mZIP4 at two levels: abundance of mZIP4 mRNA and apical localization of the mZIP4 protein. This complex regulation would allow for strict control of zinc uptake activity. The mechanisms of regulation of mZIP4 remain to be determined, but zinc induces rapid changes in ZIP4 mRNA and protein levels. Metalloregulation of genes involved in metal metabolism has been shown to involve transcriptional, as well as post-transcriptional mecha-
Many transition metals, including zinc, can regulate gene transcription by direct interactions with metal-sensing transcription factors that either repress or activate gene transcription. The transcription factor Zap1p regulates zinc transporter genes (and other genes) in yeast (28), and the transcription factor MTF-1 regulates zinc efflux from the cell (29). The regulation of gene expression by iron in mammals occurs predominantly through post-transcriptional mechanisms, including changes in mRNA stability and translation (30). This regulation involves metal-dependent interactions of iron-responsive protein-1 with iron-responsive elements in the untranslated regions of ferritin and transferrin mRNAs (31).

The finding that mZIP4 protein is rapidly lost from the apical surface in response to zinc suggests that the localization of this protein in the plasma membrane may be a metal-regulated process. Copper regulates trafficking of the human Menke's copper transporter, which functions to efflux copper from the cell. This protein is localized in the trans-Golgi network under conditions of low copper. In contrast, elevated extracellular copper induces a rapid relocalization of this protein to the plasma membrane (47–49). Thus, it is conceivable that ZIP4 trafficking may be zinc-regulated.

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