The Mammalian Zip5 Protein Is a Zinc Transporter That Localizes to the Basolateral Surface of Polarized Cells*

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The mouse and human Zip5 proteins are members of the ZIP family of metal ion transporters. In this study, we present evidence that mouse Zip5 is a zinc uptake transporter that is specific for Zn(II) over other potential metal ion substrates. We also show that, unlike many other mammalian ZIP proteins, the endocytic removal of mZip5 from the plasma membrane is not triggered by zinc treatment. Thus, the activity of mZip5 does not appear to be down-regulated by zinc repletion. Zip5 expression is restricted to many tissues important for zinc homeostasis, including the intestine, pancreas, liver, and kidney. Zip5 is similar in sequence to the Zip4 protein, which is involved in the uptake of dietary zinc. Co-expression of Zip4 and Zip5 in the intestine led to the hypothesis that these proteins play overlapping roles in the uptake of dietary zinc across the apical membrane of intestinal enterocytes. Surprisingly, however, we found that mZip5 localizes specifically to the basolateral membrane of polarized Madin-Darby canine kidney cells. These observations suggest that Zip5 plays a novel role in polarized cells by carrying out serosal-to-mucosal zinc transport. Furthermore, given its expression in tissues important to zinc homeostasis, we propose that Zip5 plays a central role in controlling organismal zinc status.

Zinc is an essential nutrient for all organisms because of the many important roles this metal plays. Therefore, organisms require efficient mechanisms to take up zinc from their diet or extracellular environment. Zinc can also be toxic if overaccumulated. Thus, precise regulatory mechanisms are also required to control this uptake to maintain an adequate supply of zinc while preventing its overaccumulation. At the cellular level, these homeostatic mechanisms include the regulation of zinc transport into and out of the cell, sequestration within intracellular organelles, and binding of the metal by intracellular macromolecules such as metallothioneins. At the organismal level, tissue-specific homeostatic roles are superimposed on these cellular mechanisms. Zinc homeostasis in mammals is primarily regulated through the control of zinc absorption in the intestine and the loss of endogenous zinc in the intestine and through both pancreatic and liver excretion (1–3). Under conditions of extreme zinc overload or deficiency, excretion of zinc through the kidney into urine is also a contributing factor to homeostasis.

Movement of zinc into and out of cells and subcellular organelles is mediated by zinc transporter proteins. In many organisms, zinc uptake is mediated by members of the ZIP family of metal ion transporters. For example, the Zup3 protein of Escherichia coli is involved in the uptake of zinc (4). In the yeast Saccharomyces cerevisiae, zinc acquisition is largely mediated by the Zrt1 and Zrt2 transporters (5, 6). In mammals, the Zip1, Zip2, Zip3, LIV-1 (Zip6), KE4 (Zip7), and BIGM103 (Zip8) proteins have been implicated in zinc uptake in a variety of cell and tissue types (7–13). The importance of ZIP transporters in dietary zinc uptake in mammals has recently become clear from studies of acrodermatitis enteropathica (AE). AE is a recessive inherited disease of humans that causes impaired absorption of dietary zinc (14–17). Moreover, fibroblasts isolated from AE patients have decreased zinc uptake activity (18, 19). These results suggested that AE is caused by mutations affecting a protein responsible for zinc transport into intestinal enterocytes and possibly other cells. Support for this hypothesis was obtained with the cloning of the AE gene, SLC39A4, which encodes a ZIP family zinc transporter designated Zip4 (20–22). Consistent with its proposed role in dietary zinc uptake, SLC39A4 mRNA was detected throughout the small intestine. Consistent with the control of zinc absorption, mouse Zip4 expression and localization is regulated by zinc status (22). Scl39A4 mRNA levels are elevated in the intestines of animals fed low zinc diets and reduced when dietary zinc is high. We have also recently shown that mZip4 activity is regulated post-translationally in cultured cells; zinc stimulates the endocytosis of the mZip4 protein thereby decreasing zinc uptake activity (23). This observation is consistent with in vivo data showing that, under zinc deficiency conditions, the mZip4 protein is localized to the apical surface of enterocytes and moves to intracellular sites upon zinc repletion (22). Thus, both transcriptional and post-translational mechanisms are likely to regulate Zip4 activity in vivo.

Some results indicate that at least one additional zinc transporter is present on the apical surface of intestinal enterocytes and involved in dietary zinc uptake. First, among the mutations in SLC39A4 gene associated with AE were several mutations that are likely to completely abolish the transport function of the Zip4 protein (20, 21, 24, 25) yet the symptoms of AE can be ameliorated by increased levels of dietary zinc. Furthermore, some AE mutations fail to map to the same chromosomal region as SLC39A4 (20). Gitschier and co-workers (20, 26) identified a possible candidate for this transporter, a ZIP pro-

* This work was supported by National Institutes of Health Grant DK50181 (awarded to Glen K. Andrews, University of Kansas Medical Center, and D. J. E.) and Grant GM56285 (to D. J. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: AE, acrodermatitis enteropathica; MDCK, Madin-Darby canine kidney cells; HA, hemagglutinin; CMV, cytomegalovirus; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; TPEN, N,N′,N″-tetraakis(2-pyridylmethyl)ethylenediamine; PNGase F, peptide N-glycosidase F.
tein that they named provisionally as “hORF1” that is now designated as “Zip5.” This proposed role was especially intriguing given that the Expressed Sequence Tag sequence databases suggested that the corresponding gene, SLC39A5, was expressed in the gastrointestinal tract. In this report, we describe the characterization of the Zip5 protein and address its possible role as an apical zinc uptake transporter. Our studies indicate that Zip5 is a zinc transporter, but, in contrast to our initial hypothesis, this protein is localized to the basolateral and not the apical plasma membrane of polarized MDCK cells. Therefore, we propose that Zip5 acts in vivo in serosal-to-mucosal zinc transport.

MATERIALS AND METHODS

Plasmids Used—The mZip5 cDNA cloned into pcDNA3.1 PurO (+) was provided by Jodi Dufner-Beattie and Glen Andrews (University of Kansas Medical Center). This construct has the hemagglutinin (HA) antigen epitope fused to the carboxyl terminus of the protein. The HA-tagged mZip4-expressing plasmid was described previously (22). Both genes were expressed in transfected cells from the vector’s CMV promoter.

Northern Blots and Tissue RNA Arrays—Human multiple tissue Northern blots and a multiple tissue expression array were purchased from BD Biosciences and probed with a 488-bp 32P-labeled probe containing SLC39A5 open reading frame nucleotides 103–509 according to the manufacturer’s instructions.

HEK293 Cell Culture and Transfection Methods—Human embryonic kidney cells (HEK293) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) plus 0.45% glucose under 5% CO2. All culture media contained 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 100 μM non-essential amino acids (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Zinc depletion of media using Chelex 100 (Sigma) was performed as previously described with minor modifications (27). Briefly, a solution of 2% (w/v) Chelex 100 resin in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum (Invitrogen) was added to cells with constant flow followed by filtration through a 0.2-μm filter. HEK2933 (2 × 106) cells were seeded in 25-mm² flasks and transfected with the plasmid DNAs using LipofectAMINE 2000 (Invitrogen). Stably transfected HEK293 cell lines were selected with 5.0 μg/ml puromycin (Sigma) 48 h after transfection.

Approximately 80% of cells in each population expressed the transgene as shown by immunofluorescence microscopy (data not shown). Cell viability was assessed with the CellTiter 96® AQueous One Solution Cell Proliferation Kit (Promega). The conversion of the substrate in the presence of oxygen is measured colorimetrically according to the manufacturer’s instructions.

Zn Uptake Assays—Transiently transfected HEK293 cells were used for Zn uptake assays as described previously (22). In brief, 48 h post-transfection, cells were washed once in uptake buffer (15 mM HEPES, 100 mM glucose, and 150 mM KCl, pH 7.0) and then added to prewarmed uptake buffer (0.45 mM glucose) in 96-well plates (2 × 105 cells per well for 5 days, and then cells were used to assay mZip-HA protein expression and localization as described above).

65Zn Uptake Assays—65Zn uptake assays as described previously (22). In brief, 48 h post-transfection, cells were washed once in uptake buffer (15 mM HEPES, 100 mM glucose, and 150 mM KCl, pH 7.0) and then added to prewarmed uptake buffer containing the specified concentration of 65ZnCl2 (PerkinElmer Life Sciences, Inc.) and incubated in a shaking 37 °C water bath for 15 min. Assays were stopped by adding an equal volume of ice-cold uptake buffer supplemented with 1 mM EDTA (stop buffer). Cells were then collected on nitrocellulose filters (Millipore; 0.45-μm pore size) and washed three times in stop buffer (10 ml of total wash volume). Cell-associated radioactivity was measured with a Packard Auto-Gamma 5650 /H9262 well-counter. In a parallel experiment, cells were washed three times with ice-cold PBS, fixed in 3.7% paraformaldehyde for 30 min at 4 °C, blocked, and incubated for 1 h at room temperature with 1:500 primary rabbit anti-HA antibody (Sigma), and then washed five times with PBS to remove unbound antibodies. The cells were then lysed by sonication in buffer containing 62 mM Tris-Cl (pH 6.8), 2% SDS, 5 mM dithiothreitol, and protease inhibitors (Roche Molecular Diagnostics). Lysates containing the solubilized anti-HA antibodies were then detected using anti-rabbit HRP antibodies (1:10,000). The protein detected in the mZip-HA lanes co-migrated with purified anti-HA antibody (data not shown).

Immunofluorescence Microscopy—To assay the surface levels of mZip-HA proteins, cells were grown in 24-well plates for 48 h on sterile glass coverslips. In some experiments, ZnCl2 or TPEN was added to the medium at the indicated concentrations and times. The cells were then washed three times with PBS on ice, fixed in 3.7% paraformaldehyde for 30 min at 4 °C, washed and incubated for 1 h at 37 °C to remove bound 65Zn-HA proteins, and then washed three times with PBS to remove unbound antibodies. The cells were then lysed by sonication in buffer containing 62 mM Tris-Cl (pH 6.8), 2% SDS, 5 mM dithiothreitol, and protease inhibitors (Roche Molecular Diagnostics). Lysates containing the solubilized anti-HA antibodies that were bound to the surface mZip-HA proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the anti-HA antibodies were then detected using anti-rabbit HRP antibodies (1:10,000). The protein detected in the mZip-HA lanes co-migrated with purified anti-HA antibody (data not shown).

mZip-HA Expression in MDCK Cells—Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, 10% fetal calf serum, 5 mM L-glutamine, and 100 μM non-essential amino acids (Invitrogen). Media containing 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mM L-glutamine, and 100 μg/ml sodium phosphate were then added to the medium at the indicated concentrations and times. The cells were then washed three times with ice-cold PBS, fixed in 3.7% paraformaldehyde for 30 min at 4 °C, and washed three times with PBS. Cells were blocked for 1 h with PBS plus 5% normal goat serum (Jackson ImmunoResearch Laboratories, Inc.) and 1% bovine serum albumin (Sigma). Cells were incubated for 1 h at room temperature or overnight at 4 °C with a 1:500 dilution of primary rabbit anti-HA antibody (Sigma). The cells were washed again with PBS followed by eight washes with PBS containing 1:500 primary rabbit anti-HA antibody and 1:10,000 dilution of ZnCl2 or TPEN secondary antibody conjugated with Alexa 488 (Molecular Probes). The cells were washed again with PBS and then examined with an Olympus IX70 microscope fitted with a Bio-Rad MRC-600 confocal laser. The intracellular distribution of mZip-HA proteins was examined in a similar manner using cells that were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Mouse anti-p230 was used as a marker of the trans-Golgi network (BD Biosciences). Detection of p230 was performed using an anti-mouse IgG antibody conjugated to Alexa 594.

mZip-HA Expression in MDCK Cells—Madin-Darby canine kidney (MDCK) cells were grown on Costar Transwell polycarbonate filters with a 0.4-μm pore size at starting densities of 2 × 105 cells per well for 5 days, and then cells were used to assay mZip-HA protein expression and localization as described above.

For immunoblot analysis of mZip5-HA apical and basolateral cell surface protein levels, MDCK cells were grown on Costar Transwell filters for 5 days, washed three times with cold PBS, and fixed with 3.7% paraformaldehyde in PBS for 30 min at 4 °C. The cells were then washed three times with PBS plus 5% normal goat serum and 1% bovine serum albumin. Cells were incubated 1 h at room temperature with 1:500 dilution of primary rabbit anti-HA antibody (Sigma) added to the apical or basolateral side of the monolayer. Control experiments indicated that the antibodies could not diffuse across the cell layer during this incubation. Surface-bound antibodies were then assayed by immunoblotting as described above.

RESULTS

Similarity between Mammalian Zip4 and Zip5 Proteins and Their Tissue-specific Patterns of Expression—The mouse SLC39A5 and human SLC39A5 genes encode Zip5, a previously uncharacterized member of the ZIP family. Mouse and human Zip5 are very similar in sequence to each other and also similar...
to mouse and human Zip4 (Fig. 1). Mouse and human Zip5 share 84% identity, whereas the Zip4 and Zip5 proteins are ~30% identical. Like other ZIPs, these proteins each have eight predicted transmembrane domains, and the similarity shared between them is greatest in this region. In addition, both Zip4 and Zip5 proteins have long amino-terminal domains. This domain is ~300 amino acids for Zip4 and about 200 amino acids for the Zip5 proteins. The amino terminus of Zip4 has been shown previously to be extracellular (23). Mammalian Zip4 and Zip5 proteins also have a conserved domain with the sequence HE\(X\)PHE\(X\)GD in predicted transmembrane domain 5. Although the function of this domain is unknown, it is conserved in many ZIP proteins (12).

To determine the tissue-specific pattern of Zip5 expression, we probed Northern blots of human mRNAs with a probe generated from the SLC39A5 gene. SLC39A5 expression was highest in liver, kidney, pancreas (Fig. 2A), and throughout the small intestine and colon (Fig. 2B) with little expression detected in other tissues. A similar pattern of expression was observed previously for mouse and human SLC39A4 mRNA (20–22). Thus, Zip4 and Zip5 are closely related proteins with similar tissue-specific patterns of expression.

Probing of a multiple tissue expression array with SLC39A5 indicated that this gene was also expressed in the spleen and appendix as well as fetal liver and fetal kidney (data not shown). No expression was detected in adrenal gland, bladder,
The positions of molecular weight markers are indicated. A, tissue Northern blot. and 3 protein. First, a band of the expected 59-kDa size was observed was likely the result of nicamycin (Fig. 3). mulated a major protein of plasmid expressing mZip5-HA from the CMV promoter accumulated a major protein of ~80-kDa molecular mass and a less abundant protein of 70 kDa. No proteins were detected in HEK293 cells transfected with the vector alone (Fig. 3A, lanes 1 and 2). The predicted molecular mass of mZip5-HA is ~59 kDa. The discrepancy between the observed and expected size was likely the result of N-glycosylation of the mZip5-HA protein. First, a band of the expected 59-kDa size was observed when cells were treated with the N-glycosylation inhibitor tunicamycin (Fig. 3A, lanes 4–6). Second, the predicted molecular mass was also observed when lysates were treated with PNGase F to enzymatically remove glycosyl groups (Fig. 3B, lanes 3 and 4). Three potential sites of N-glycosylation (Asn-49, Asn-91, and Asn-158) are found in the amino-terminal domain of mZip5 (Fig. 1). The only other potential site is Asn-390, which is predicted to lie within transmembrane domain 4. Therefore, these results are consistent with the topology shown in Fig. 3C in which the amino terminus of Zip5-HA is located on the extracytosolic face of the membrane. Additional data (see below) indicate that the carboxyl-terminal end of the protein is also extracytosolic.

mZip5 Is a Zinc-specific Transporter—To examine the ability of Zip5 to act as a zinc transporter, mZip5-HA was expressed in transiently transfected HEK293 cells. Pools of transfected cells were collected and assayed for protein expression and zinc uptake activity. Immunoblots of total lysates indicated that these cells accumulated abundant mZip5-HA protein (Fig. 4A). A substantial amount of this protein was located on the plasma membrane. This was shown using an immunoblotting assay to assess the levels of anti-HA antibodies bound to the surface of non-permeabilized HEK/mZip-HA cells (23, 28). The carboxyl-terminal epitope tag of each protein is predicted to be extracellular (Fig. 3C) and therefore exposed to antibody binding in non-permeabilized cells. Cells were grown in basal media, fixed with paraformaldehyde, and the intact cells were then probed with anti-HA antibodies to label mZip5-HA proteins on the cell surface. After extensive washing to remove unbound antibodies, the cells were lysed, and the anti-HA antibodies that had been bound to the cell surface were detected by immunoblotting. As shown in Fig. 4B, a significant amount of anti-HA antibody was bound to mZip5-HA-expressing cells. In contrast, little anti-HA antibody was recovered from vector transfectants. The steady-state distribution of total mZip5-HA will be explored in more detail later in this report.

Consistent with the ability of Zip5 to transport zinc, cells expressing mZip5-HA accumulated ~6-fold more zinc over a 60-min period than did the endogenous uptake activity assayed in vector-only transfectants (Fig. 4C). Only low levels of zinc accumulation were detected when these assays were conducted with chilled cells indicating that zinc accumulation by both mZip5 and the endogenous system present in HEK293 cells are temperature-dependent and therefore likely to be transporter mediated. As shown in Fig. 4D, uptake by the endogenous system and mZip5 was concentration-dependent and saturable. When assayed over a range of zinc concentrations, the uptake activity of mZip5 showed Michaelis-Menten kinetics with an apparent $K_m$ value of $1.7 \pm 0.2 \mu$M and a $V_{max}$ of $13.2 \pm 2 \text{ pmol/min/mg of protein}$. The endogenous system in HEK293 cells had an apparent $K_m$ value of $2.1 \pm 0.1 \mu$M zinc and a $V_{max}$ of $4.1 \pm 0.5 \text{ pmol/min/mg of protein}$. Finally, we reasoned that the increased zinc uptake ability observed for mZip5-HA-expressing cells could enhance the ability of these cells to grow under zinc-limiting conditions. mZip5-HA-expressing cells and vector-transfected cells were incubated in increasing concentrations of a zinc chelator, TPEN, for 2 days, and cell viability was then measured. Although both cell types lost viability with increasing concentrations of TPEN, the mZip5-HA cells had significantly increased resistance to the chelator (Fig. 4E). These results are consistent with the ability of mZip5 to transport zinc into the cell where it is then available for zinc-dependent processes.

To assess the substrate specificity of Zip5, we examined the effects of various metals on zinc accumulation by cells expressing mZip5-HA. We predicted that high levels of other substrates would compete with zinc and interfere with its uptake via mZip5-HA. HEK293 cells were transiently transfected with either the empty vector or the vector encoding the HA-tagged mZip5 protein. Pools of transfectants were assayed for zinc uptake activity with apparent $K_m$ levels of $65^{\text{Zn}}$ (1.5 $\mu$M) in the presence of either 10- or 50-fold molar excess of competitor metal. Zinc uptake by the endogenous system was strongly inhibited by excess zinc, copper, and cadmium and to a lesser extent by other metals (Fig. 5A). In contrast, zinc uptake by mZip5-HA was strongly inhibited by zinc and was far less sensitive to other metals (Fig. 5B). These results are consistent with the zinc-specific uptake ability of mZip5. A similar result was obtained with mZip1, mZip2, mZip3, and mZip4 suggesting that these proteins are all zinc-specific transporters (9, 22).

mZip5 Activity Is Not Down-regulated by Zinc Treatment—Our previous studies of yeast and mammalian zinc transporters indicated that these proteins can be regulated to decrease zinc uptake activity in response to zinc treatment. For example, we have shown that several mammalian ZIP proteins are down-regulated by zinc post-translationally. Endocytosis of mZip1, mZip3, and mZip4 is stimulated by zinc thereby decreasing plasma membrane protein levels and reducing zinc uptake activity (23, 28). To assess whether mZip5 is similarly regulated by zinc-stimulated endocytosis, we first examined mZip5-HA distribution in stably transfected HEK293 cells. For

![Fig. 2. Tissue-specific expression of the SLC39A5 mRNA. Analysis of SLC39A5 expression using human multi-tissue Northern blots. A, multi-tissue Northern blot. B, Northern blot of gastrointestinal tissues.](image-url)
Comparison, we also examined the localization of mZip5-HA in these experiments. Immunoblot analysis indicated that these stable transfectants expressed similar levels of mZip4-HA and mZip5-HA proteins (Fig. 6A). No proteins were detected in vector-transfected cells.

We recognize that the high level expression of mZip5 from the CMV promoter in these cells may alter the intracellular localization of the protein. Nonetheless, the subcellular distribution of mZip4-HA and mZip5-HA was assessed by immunofluorescence confocal microscopy to provide the foundation for studies of zinc-stimulated endocytosis in HEK293 cells. This same approach was used previously to show zinc-stimulated endocytosis of mZip1, mZip3, mZip4, and hZip4 (23, 28). Cells were grown in a zinc-replete basal medium and then fixed, permeabilized, incubated with anti-HA antibody, and subsequently incubated with a fluorophore-conjugated secondary antibody. No significant fluorescence was observed in vector-only transfectants (data not shown). Cells expressing mZip4-HA and mZip5-HA showed abundant staining (Fig. 6B, green). A large proportion of each protein was distributed in intracellular vesicles scattered throughout the cytoplasm. For mZip4-HA, these vesicles were especially concentrated in a perinuclear region that co-localized with a marker protein of the trans-Golgi network, p230 (Fig. 6B, red). The merged images are shown in the right-hand panel of Fig. 6B, and the overlap of mZip-HA and p230 is shown in yellow. mZip5-HA showed less co-localization with the trans-Golgi network and greater apparent dispersion of cytosolic vesicles than did mZip4-HA.

Clear labeling of the periphery of cells suggested that some of the mZip5-HA proteins were localized to the plasma membrane. To assess surface levels of these proteins in cells grown in basal media, immunofluorescence studies were performed using cells that were fixed but not permeabilized. No fluorescence was detected following surface labeling of vector-only transfectants (Fig. 6C). In contrast, fluorescence staining was clearly observed on the surface of mZip4-HA- and mZip5-HA-expressing cells grown in basal media. The absence of detectable surface antibody binding by vector-only transfectants demonstrated that nonspecific antibody binding was not occurring. Therefore, some fraction of mZip4-HA and mZip5-HA proteins are on the plasma membrane in cells grown in basal medium.

To assess the possible effects of zinc status on mZip5-HA localization, we first examined distribution of these proteins by immunofluorescence microscopy of permeabilized cells. As we have shown previously (23), zinc deficiency established by 1-h incubation in media containing 10 \( \mu \)M TPEN resulted in increased mZip4-HA surface levels (Fig. 7A). Zinc treatment (10 \( \mu \)M) had no effect on mZip4-HA distribution, because the level of zinc in basal medium is sufficient to stimulate mZip4-HA endocytosis (23). In contrast, treatment of mZip5-HA-expressing cells with TPEN or zinc did not noticeably alter the apparent steady-state distribution of the protein even after 24 h of exposure (Fig. 7A, data not shown). Immunoblots of total protein extracts from these cells indicated no changes in protein levels occurred under these treatment conditions (data not shown).

Similar results were obtained when plasma membrane levels of the mZip-HA proteins were assayed by immunoblotting of surface-bound antibodies. As previously shown (23), 1-h incubation in media made zinc-deficient by treatment with Chelex-100 resin resulted in an increased level of anti-HA antibody bound to mZip4-HA-expressing cells consistent with increased surface levels of this protein (Fig. 7B). Zinc deficiency induced by incubation with 2 or 10 \( \mu \)M TPEN also increased surface levels of mZip4-HA. In contrast, no change in surface levels of mZip5-HA protein was observed. Thus, unlike mZip1, mZip3, and mouse and human Zip4, the mZip5 protein is not down-regulated by zinc-stimulated endocytosis in cultured cells.

The inability of mZip5-HA to be down-regulated by zinc treatment suggested that overexpression of this protein in transfected cells may cause sensitivity to high zinc. Consistent with this prediction, growth of mZip5-HA cells was more inhibited by high zinc levels than were vector transfectants (Fig. 8A). Notably, at 200 \( \mu \)M zinc added to basal medium, vector transfectants were >80% viable, whereas mZip5-HA cells were <20% viable. In contrast, mZip4-HA-expressing cells were no more sensitive to high zinc than were vector transfectants, consistent with the ability of these cells to down regulate...
mZip4-HA activity post-translationally. Immunoblotting confirmed that surface levels of mZip5-HA were not altered in high zinc, whereas mZip4-HA levels were reduced (Fig. 8B).

mZip4 and mZip5 Are Targeted to Different Plasma Membrane Regions in Polarized Cells—The similar zinc uptake activities and tissue-specific patterns of expression suggested that the Zip4 and Zip5 proteins may perform overlapping functions. For example, in the small intestine, Zip5 could play a constitutive role in dietary zinc uptake across the apical membrane, whereas Zip4 is important only when dietary zinc is low. If so, we predicted that both mZip4-HA and mZip5-HA would localize to the apical membrane of polarized cells grown in culture. Renal epithelial MDCK cells were used in these experiments to examine the plasma membrane distribution of mZip4 and mZip5 in polarized cells. When grown on permeable supports, these cells form monolayers with defined apical and basolateral surfaces separated by tight junctions. We stably transfected mZip4-HA- and mZip5-HA-expressing plasmids into MDCK cells. Horizontal (x-y) sections through MDCK monolayers generated by immunofluorescence confocal microscopy detected both mZip4-HA and mZip5-HA proteins (green, Fig. 9, A and B, respectively). Nuclei were stained in these preparations with propidium iodide (red). No fluorescence was detected in vector-transfected cells (data not shown). Vertical (x-z and y-z) sections through the monolayer of mZip4-HA-expressing MDCK cells indicated localization of the protein at or just below the apical surface as was expected from its apical vector only or expressing mZip5-HA in the presence of 2 μM 65Zn at either 0 or 37 °C for the indicated times. D, the concentration dependence of zinc uptake activity was determined over a range of 65Zn concentrations with vector or mZip5-HA transfected cells incubated at 37 °C for 15 min. E, vector and mZip5-HA-transfected cells were grown in basal medium supplemented with the indicated concentration of TPEN for 2 d prior to analyzing cell viability. Each point represents the mean of a representative experiment (n = 3), and the error bars indicate ± 1 S.D.
Localization in intestinal enterocytes (Fig. 9A) (22). In contrast, no mZip5-HA protein was detected on the apical surface, but the protein was readily apparent on the lateral membrane between cells indicating a basolateral localization (Fig. 9B). To corroborate these conclusions, the cell surface expression pattern of mZip4-HA and mZip5-HA were also examined using immunoblotting of surface-bound anti-HA antibodies similarly to as described in Fig. 4B. In this case, however, antibodies were added to the medium in contact with either the apical or basolateral surfaces. Control experiments indicated that antibodies added to one side of the monolayer did not diffuse across to the other surface (data not shown). When anti-HA antibodies were added to the media on the apical surface of the cells, only mZip4-HA-expressing cells bound detectable amounts of antibody (Fig. 9C). In contrast, both mZip5-HA and mZip4-HA was detected when antibodies were added to the basolateral surface (Fig. 9D). Far less antibody was bound to the basolateral sur-
face of mZip4-HA cells than was bound to the apical surface. Therefore, these results demonstrate that mZip4-HA localizes predominantly to the apical surface while mZip5-HA localizes to the basolateral membrane.

**DISCUSSION**

There are 14 different ZIP proteins encoded by the human genome, and these are designated as Zip1–14 (26). These proteins all share the hallmark features of ZIP family transporters. Given this underlying similarity, a key question then is, “What are the different functions of these closely related proteins?” Studies of ZIP proteins in bacteria, yeast, and plants have provided several clues as to the possible roles of the mammalian ZIP transporters. First, ZIP proteins may play redundant or overlapping functions. For example, the yeast Zrt1 and Zrt2 proteins are both responsible for zinc uptake into cells (5, 6). Zrt2 has a lower affinity for substrate and therefore serves as a major source of zinc under conditions of moderate zinc availability. In contrast, Zrt1 has a higher affinity for zinc allowing it to more efficiently scavenge substrate when availability is low. Second, some ZIP proteins have been shown to utilize substrates other than zinc. The Irt1 protein of Arabidopsis is involved in iron uptake into the roots of this plant (29, 30). Although Irt1 is capable of zinc, manganese, and cadmium uptake as well (31), its primary role is iron acquisition. Third, ZIP proteins may function in the transport of metal ions across the membranes of intracellular organelles. For example, the yeast Zrt3 protein transports stored zinc from the lumen of the vacuole into the cytosol for utilization under zinc-limiting conditions (32). Also in yeast, the Atx2 protein may transport manganese across the Golgi membrane (33). These studies suggest some of the possible functions that may be performed by the large number of ZIP proteins encoded in mammalian genomes.

The mammalian Zip4 and Zip5 proteins are very similar in sequence. Moreover, Zip4 and Zip5 are expressed in many of the same tissues, including the proximal and distal intestine. We showed previously that Zip4 is a zinc-specific transporter (22) and, in this report, we show that Zip5 is also specific for zinc. These similarities raised the question as to what different roles these two proteins may be playing in the tissues in which both are expressed. Drawing from the work of Gitschier and co-workers (20), our initial hypothesis was that Zip4 and Zip5 had overlapping functions, i.e. both were involved in the uptake of dietary zinc across the apical membrane of intestinal enterocytes. In this model, Zip4 and Zip5 would be analogous to yeast Zrt1 and Zrt2. The existence of a second transporter for dietary zinc uptake in addition to Zip4 was suggested by the analysis of AE-associated mutations in patients with acrodermatitis enteropathica. Some AE-associated SLC39A4 mutations are deletions that cause frameshifting and premature translation termination and are therefore likely to cause total loss of Zip4 function (21). In addition, some AE-associated missense mutations also cause the complete loss of Zip4 function (25). Because AE can be successfully treated with dietary zinc supplements, these studies indicated that at least one other zinc transporter is present in the apical membranes of intestinal enterocytes. Gitschier and co-workers proposed that Zip5 played this role (20).
In disagreement with this model, however, we found that mZip4 and mZip5 proteins localize to different regions of the plasma membrane in polarized MDCK cells. Whereas mZip4 primarily localized to the apical plasma membrane, the mouse Zip5 protein was found only on the basolateral surface of these cells. The apical localization of mZip4 was predicted by its presence in the apical membrane of intestinal enterocytes under zinc-limiting conditions in vivo (22). Our cell culture results showing basolateral localization of mZip5 have been recently confirmed in situ; staining of mouse intestinal villi with an anti-mZip5 antibody also showed that mZip5 is found on the basolateral surface of intestinal enterocytes in vivo. These studies were performed using mice fed a standard diet. Thus, although we have not examined transcriptional control of Slc39A5 expression in this study, these data indicate expression of this gene in zinc-replete animals.

If mZip5 is not involved in the uptake of dietary zinc, what then is the function of this protein on the basolateral membrane of enterocytes? First, mZip5 may supply serum zinc to these cells for their own use when the dietary intake of zinc is low. For example, in animals fed a zinc-deficient diet, zinc mobilized from internal stores such as the liver may be taken up by the enterocytes via mZip5. Alternatively, excretion of endogenous zinc is one mechanism by which the body rid itself of excess zinc. mZip5 may play a role in zinc homeostasis by transporting excess zinc into the enterocyte for subsequent efflux into the gut lumen. Third, serosal-to-mucosal transport of zinc by mZip5 may be involved in enterocyte sensing of body zinc status. Several studies have indicated that intestinal absorption of zinc is regulated in response to dietary zinc availability (1, 3, 34). In addition, intestinal absorption is also responsive to body zinc stores. For example, Richards and Cousins (35) showed that intraperitoneal injection of zinc markedly decreased absorption when administered 18 h before an oral dose of 65Zn. Thus, zinc absorption is clearly responsive to body zinc status. Dufner-Beattie et al. (22) have recently shown that intraperitoneal injection of zinc markedly decreases mZip4 protein and mRNA levels over similar time periods. Thus, we propose that Zip5 may play an important role in communicating body zinc status to the enterocyte to control mZip4 activity and potentially other aspects of zinc homeostasis. The failure of mZip5 to be down-regulated by zinc treatment through post-translational mechanisms is consistent with these proposed roles in zinc sensing and excretion.

Zinc homeostasis in mammals is regulated through the control of zinc absorption in the intestine and the loss of exogenous zinc in the intestine as well as through both pancreatic and liver excretion. Under conditions of extreme zinc deficiency or excess, the kidney also plays an important homeostatic role. Intriguingly, we note that mZip5 is expressed specifically in all of the tissues that are critical to mammalian zinc homeostasis, i.e. intestine, liver, pancreas, and kidney. Zip5 may play similar roles in the liver, pancreas, and kidney as proposed above for the intestine. For example, the high level of Zip5 expression in the intestine and liver may provide serum zinc to these tissues for later excretion into the gut. Thus, we predict that Zip5 is a central player in mammalian zinc metabolism.

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**Note Added in Proof**—While this paper was in press, a related study of mZip5 was accepted for publication (Dufner-Beattie, J., Kuo, Y. M., Gitschier, J., and Andrews, G. K. (September 9, 2004) J. Biol. Chem. 10.1074/jbc.M409962200). Consistent with our results, these authors demonstrated basolateral localization of mZip5 protein in mouse enterocytes, acinar cells, and visceral endodermal cells. Moreover, they showed that neither Slc39A5 mRNA nor mZip5 protein levels were altered in mice in response to changes in dietary zinc. Also consistent with our results, mZip5 protein was abundant on the cell surface under zinc-replete conditions. Remarkably, however, Dufner-Beattie et al. found that plasma membrane levels of mZip5 protein decreased under zinc deficiency in vivo. These data are consistent with a role for mZip5 in zinc excretion.

**Acknowledgments**—We thank Yien-Ming Kuo and Jane Gitschier for sharing their results with us prior to publication and Glen Andrews and Jodi Dufner-Beattie for critical reading of the manuscript.

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**REFERENCES**

1. King, J. C., Shames, D. M., and Woodhouse, L. R. (2000) J. Nutr. 130, 13605–13606
2. Cousins, R. J. (1985) Physiol. Res. 65, 338–309
3. Krebs, N. F. (2000) J. Nutr. 130, 13745–13775
4. Grass, W., Wang, M. D., Rosen, B. P., Smith, R. L., and Rensing, C. (2002) J. Bacteriol. 184, 864–866
5. Zhao, H., and Eide, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2454–2458
6. Zhao, H., and Eide, D. (1996) J. Biol. Chem. 271, 23203–23210
7. Gaither, L. A., and Eide, D. J. (2000) J. Biol. Chem. 275, 5560–5564
8. Gaither, L. A., and Eide, D. J. (2001) J. Biol. Chem. 276, 22258–22264
9. Dufner-Beattie, J., Langmade, S. J., Wang, F., Eide, D., and Andrews, G. K. (2003) J. Biol. Chem. 278, 50142–50150
10. Franklin, R. B., Ma, J., Zee, J., Guan, Z., Kuboki, B. I., Feng, P., and Costello, L. C. (2003) J. Inorg. Biochem. 96, 435–442
11. Taylor, K. M., Morgan, H. E., Johnson, A., and Nicholson, R. I. (2003) Biochem. J. 377, 131–139
12. Taylor, K. M., and Nicholson, R. I. (2003) Biochim. Biophys. Acta 1611, 16–30
13. Begum, N. A., Kohayashi, M., Moriwaki, Y., Matsumoto, M., Toyoshima, K., and Seya, T. (2002) Genomics 89, 630–645
14. Barnes, P. M., and Mynahan, E. J. (1973) Proc. Royal Soc. Med. 66, 327–329
15. Neldner, K. H., and Hambidge, K. M. (1975) New Engl. J. Med. 292, 879–882
16. Lombeck, T., Schnippering, H. G., Ritzel, F., and Feinendegen, L. E., and Bremer, H. J. (1975) Lancet 1, 855
17. Weismann, K., Hse, K., Kudosen, L., and Sorensen, S. S. (1979) Br. J. Dermato- tol. 101, 573–579
18. Vasquez, F., and Griesser, A. (1995) Biol. Trace Elem. Res. 50, 109–116
19. Griesser, A., Lin, Y. F., and Muga, S. J. (1998) J. Biol. Chem. 273, 1–8
20. Wang, K., Zhou, B., Kuo, Y. M., Zemansky, J., and Gitschier, J. (2002) Am. J. Hum. Genet. 71, 66–73
21. Kurry, S., Drenes, B., Beizeau, S., Giraudeau, S., Kharfi, M., Kamoun, R., and Moisan, J. P. (2002) Nat. Genet. 31, 239–240
22. Dufner-Beattie, J., Wang, F., Kuo, Y., Gitschier, J., Eide, D., and Andrews, G. K. (2003) J. Biol. Chem. 278, 33474–33481
23. Kim, B. E., Wang, F., Dufner-Beattie, J., Andrews, G. K., Eide, D. J., and Petris, M. J. (2004) J. Biol. Chem. 279, 4522–4530
24. Kurry, S., Kharfi, M., Kamoun, R., Taeib, A., Mallet, E., Baudou, J. J., Glastre, C., Michel, B., Sebag, F., Brooks, D., Maudi, V., Sebag, C., Bezeau, S., and Moisan, J. P. (2003) Hum. Mutat. 22, 337–338
25. Wang, F., Kim, B. E., Dufner-Beattie, J., Petris, M. J., Andrews, G., and Eide, D. J. (2004) Hum. Mol. Genet. 13, 563–571
26. Eide, D. J. (2004) Pflugers Arch. 447, 796–800
27. Messer, H. H., Murray, E. J., and Goebel, N. K. (1982) J. Nutr. 112, 652–657
28. Wang, F., Dufner-Beattie, J., Kim, B. E., Petris, M. J., Andrews, G., and Eide, D. J. (2004) J. Biol. Chem. 23, 24631–24639
29. Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5624–5628
30. Vert, G., Grutz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M. L., Briat, J. F., and Curie, C. (2002) Plant Cell 14, 1223–1233
31. Korshunova, Y. O., Eide, D., Moisan, J. P., and Curie, C. (2002) Plant Physiol. 130, 1215–1223
32. Richards, M. P., and Cousins, R. J. (1975) Biochem. Biophys. Res. Commun. 64, 1215–1223