Zn$^{2+}$-stimulated Endocytosis of the mZIP4 Zinc Transporter Regulates Its Location at the Plasma Membrane*

Received for publication, October 1, 2003, and in revised form, October 29, 2003
Published, JBC Papers in Press, November 11, 2003, DOI 10.1074/jbc.M310799200

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Zinc is an essential nutrient for all organisms. Its requirement in humans is illustrated dramatically by the genetic disorder acrodermatitis enteropathica (AE). AE is caused by the reduced uptake of dietary zinc by enterocytes, and the ensuing systemic zinc deficiency leads to dermatological lesions and immune and reproductive dysfunction. The gene responsible for AE, SLC39A4, encodes a member of the ZIP family of metal transporters, hZIP4. The mouse ZIP4 protein, mZIP4, stimulates zinc uptake in cultured cells, and studies in mice have demonstrated that zinc treatment decreases mZIP4 mRNA levels in the gut. In this study, we demonstrated using transfected cultured cells that the mZIP4 protein is also regulated at a post-translational level in response to zinc availability. Zinc deficiency increased mZIP4 protein levels at the plasma membrane, and this was associated with increased zinc uptake. Significantly, treating cells with low micromolar zinc concentrations stimulated the rapid endocytosis of the transporter. Zinc-regulated localization of the human ZIP4 protein was also demonstrated in cultured cells. These findings suggest that zinc-regulated trafficking of human and mouse ZIP4 is a key mechanism controlling dietary zinc absorption and cellular zinc homeostasis.

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Zinc is an essential nutrient for all organisms because it is required by a variety of enzymes that are involved in critical areas of metabolism. However, zinc is also potentially toxic when allowed to accumulate beyond cellular needs. Thus, homeostatic mechanisms have evolved to precisely regulate intracellular levels of this nutrient. Genetic studies in bakers' yeast, Saccharomyces cerevisiae, identified the first eukaryotic zinc import protein, ZRT1 (1, 2). The ZRT1 protein shows significant sequence homology to the IRT1 protein from Arabidopsis thaliana, which is primarily an iron transporter that can also transport zinc (3). Both ZRT1 and IRT1 were the founding members of the ZIP (ZRT1-IRT1-like protein) superfamily of metal transporters that exist in all eukaryotes (for review, see Ref. 4). The hallmark features of ZIP proteins include eight transmembrane domains, the fourth of which contains fully conserved histidyl and glycyl residues in a putative amphipathic α-helix. In humans, 14 ZIP proteins have been identified by data base sequence comparisons (5), and at least two of these proteins, hZIP1 and hZIP2, function as zinc importers when expressed in K562 cells (6, 7). Recently, another ZIP family member, hZIP4 (SLC39A4), was shown to be mutated in the inherited disorder of zinc deficiency, acrodermatitis enteropathica (AE) (8, 9).

AE is characterized by symptoms of zinc deficiency (10), such as dermatological lesions, changes in the small bowel mucosa, reduced weight gain, and immune and reproductive dysfunction (11–14). The primary basis of AE is hypothesized to be the reduced uptake of dietary zinc by intestinal cells (15, 16) because patients respond positively to dietary zinc supplements (11, 12, 17, 18). Consistent with this hypothesis was the finding that the affected protein in AE, hZIP4, and the murine homolog, mZIP4, are most abundantly expressed in the small intestine (8), and the mZIP4 protein is located at the apical membrane of intestinal enterocytes in zinc-deficient mice (8, 19). Moreover, mZIP4 has been shown to stimulate zinc uptake when expressed in the human embryonic kidney cell line, HEK293 (19). Patients with AE provide in vivo evidence of the importance of hZIP4 in dietary zinc uptake; however, skin fibroblast cell lines derived from AE patients also display zinc deficiency phenotypes and have reduced zinc uptake (20, 21). These findings suggest that hZIP4 is likely to function in the uptake of zinc in a variety of cell types.

The importance of mammalian ZIP4 in dietary and cellular zinc uptake has prompted recent studies investigating whether the expression of the mZIP4 gene is regulated by zinc availability. Studies using mice have established that mZIP4 mRNA levels in the small intestine and embryonic visceral yolk sac are induced under conditions of zinc deficiency, suggesting that the transcription or stability of mZIP4 mRNA is regulated by zinc availability (19). In this study, we investigated whether the mZIP4 protein is regulated by zinc availability. Using transfected HEK293 cells, a functional hemagglutinin antigen (HA)-tagged form of the mZIP4 protein was localized in cytoplasmic vesicles within the perinuclear region of the cell. There was considerable overlap between mZIP4-HA and the transferrin receptor in recycling endosomes, and mZIP4-HA was shown to cycle via the plasma membrane. Significantly, zinc deficiency conditions resulted in the accumulation of the transporter at the plasma membrane, and this was associated with increased zinc uptake activity. The addition of low micromolar...
zinc concentrations stimulated the rapid endocytosis of mZIP4-HA. The endocytosis of mZIP4-HA was also stimulated by manganese, cobalt, and cadmium, although this required higher concentrations relative to zinc. Notably, a zinc-responsive localization was also found for the hZIP4 protein. These findings suggest that zinc-regulated trafficking of human and mouse ZIP4 is a key mechanism controlling dietary zinc absorption and cellular zinc homeostasis.

EXPERIMENTAL PROCEDURES

Reagents, Cell Lines, and Antibodies—HEK293 cells expressing either the HA-tagged mZIP4 protein or pcDNA3.1-purmycin vector, were isolated after transfection using the LipofectAMINE 2000 reagent (Invitrogen). The hZIP4 cDNA cloned into the pcDNA3.1-purmycin vector was kindly provided by Jane Gitschier (UCSF) and tagged at the carboxyl terminus with the HA epitope using PCR and standard molecular biology protocols. Purmycin-resistant cells were established by the selection of transfected cells with 2 μg/ml purmycin in the growth medium (Sigma). mZIP4-HA expression was analyzed by Western blotting and immunofluorescence microscopy using the polyclonal rabbit anti-HA antibody (Sigma). All cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin/10 μg/ml gentamicin (Invitrogen). The mZIP4-HA cells were cultured for 24 h in 6-well trays, washed twice with PBS on ice, and fixed for 10 min in 4% paraformaldehyde without subsequent permeabilization steps. Cells were then blocked using 3% skim milk in PBS and incubated with 5 μg/ml anti-HA antibody for 30 min at room temperature. Cells were washed five times in PBS to remove unbound antibodies and then lysed by sonication in SDS lysis buffer described above. Cell lysates containing the solubilized anti-HA antibodies that were bound to the mZIP4-HA protein at the plasma membrane were separated using 4–20% SDS-PAGE, transferred to nitrocellulose membranes, and the anti-HA antibodies were then detected using horseradish peroxidase-conjugated antibodies (1:5,000) by chemiluminescence (Roche Applied Science). Tubulin protein levels were detected on parallel immunoblots using anti-tubulin antibodies (1:1,400,000, Sigma).

Assay of Zinc-stimulated mZIP4-HA Endocytosis—The endocytosis of mZIP4-HA was determined by measuring the uptake of anti-HA antibodies added to the cultured medium of HEK/mZIP4-HA cells. Cells were pregrown in 6-well trays for 24 h in basal medium and then incubated for the indicated times at 37 °C in basal medium. Chelated medium, or TPEN-containing medium containing 5 μg/ml anti-HA antibodies and the indicated amounts of zinc or other metals. Cells were washed twice with 2 ml of PBS on ice, and surface-bound antibodies were removed by three washes in 2 ml of ice-cold acidic buffer (above). Cells were harvested by scraping into 1 ml of ice-cold PBS and pelleted by centrifugation at 1,600 × g. The cell pellets were solubilized in SDS buffer (above), and 20 μl of lysates containing internalized anti-HA antibodies were separated using 4–20% SDS-PAGE, transferred to nitrocellulose membranes, and detected by chemiluminescence using horseradish peroxidase-conjugated secondary antibodies, as described above.

Zn2+ Uptake Assays—HEK/mZIP4-HA and HEK-vector cells were seeded in 24-well poly-L-lysine-coated plates 48 h prior to zinc uptake assays. Cells were preincubated for 1 h in basal medium or medium supplemented with 10 μM ZnCl2, 10 μM TPEN, or 10 μM ZnCl2 plus 10 μM TPEN. Cells were then washed with ice-cold uptake buffer (15 mM Hepes, 100 mM glucose, and 150 mM KCl, pH 7.0) and then incubated in prewarmed uptake buffer containing 5 μg/ml ZnCl2 (PerkinElmer Life Sciences) and a shaking bath for 5 min. Cells were then washed by adding an equal volume of ice-cold uptake buffer supplemented with 1 mM EDTA (stop buffer). Cells were collected on nitrocellulose filters (Millipore; 0.45-μm pore size) and washed three times with 10 ml of stop buffer. Parallel experiments were conducted at 0 °C for cell surface Zn2+ 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 5650 counter. Zinc uptake was calculated using a standard curve and normalized to protein concentrations of cell lysates.

RESULTS

We have demonstrated recently that expression of the mouse ZIP4 cDNA stimulates zinc uptake in transiently transfected HEK293 cells (19). The initial focus of the present study was to identify the subcellular distribution of the mZIP protein. A HA epitope tag was fused to the carboxyl terminus of mZIP4 to allow detection of the mZIP4-HA protein using anti-HA antibodies (Fig. 1A). The HEK293 cell line was transfected with the mZIP4-HA plasmid construct, and several independent populations of cells expressing mZIP4-HA were isolated by selection with puromycin to generate HEK/mZIP4-HA cells. In each population, ~80% of the cells stably expressed the mZIP4-HA protein as determined by immunofluorescence microscopy using anti-HA antibodies (data not shown). Western blot analysis using anti-HA antibodies revealed a specific protein in HEK/mZIP4-HA cells which was absent in HEK293 cells transfected with the pcDNA3.1 vector alone (Fig. 1B). The apparent molecular mass of 90 kDa was larger than the expected size of 70 kDa for mZIP4-HA. This discrepancy was likely the result of glycosylation of mZIP4-HA because the expected size was observed when HEK/mZIP4-HA cells were treated with the glycosylation inhibitor, tunicamycin (data not shown). We then investigated whether altered zinc availability could affect the abundance or apparent molecular mass of the mZIP4-HA protein. HEK/mZIP4-HA cells were cultured for 4 h under conditions made zinc-deficient by supplementing the medium with...
the membrane-permeable zinc chelator, TPEN. However, this treatment did not alter the abundance or electrophoretic mobility of the mZIP4-HA protein (Fig. 1B). The effect of excess zinc (100 μM) in the medium was also tested, and again no apparent changes in abundance or apparent molecular mass of the mZIP4-HA protein were observed (Fig. 1B).

We then explored the possibility that the intracellular distribution of mZIP4-HA is regulated by zinc. In HEK/mZIP4-HA cells cultured in basal medium, immunofluorescence microscopy using anti-HA antibodies revealed that mZIP4-HA was distributed in cytoplasmic vesicles that were concentrated in the perinuclear region (Fig. 1C). There was no signal detected in HEK293 cells transfected with the empty vector (data not shown). A striking result was obtained when TPEN was added to the medium for 1 h to generate a zinc-deficient condition. The distribution of mZIP4-HA was shifted toward the periphery of cells, and labeling of the protein at the plasma membrane was clearly apparent (Fig. 1D). This TPEN-induced relocalization of mZIP4-HA was suppressed when equal amounts of zinc and TPEN were added together to the medium (Fig. 1E), suggesting that the redistribution of mZIP4-HA using TPEN alone was likely because of zinc limitation. The redistribution of mZIP4-HA was also observed when HEK/mZIP4-HA cells were cultured in medium from which metals had been previously extracted using the metal-chelating resin, Chelex 100, and this effect was suppressed by replacing zinc (data not shown). These effects of TPEN and Chelex treatments were specific for mZIP4-HA because these treatments did not affect the location of several proteins known to cycle or reside within endocytic compartments, including the transferrin receptor, the copper transporter, hCtr1, the early endosome marker EEA1, and the ATP7A copper exporter (data not shown). These data suggest that the location of mZIP4-HA is zinc-responsive and that zinc limitation increases its abundance at the plasma membrane.

The observation that under basal medium conditions, the mZIP4-HA protein was concentrated in perinuclear vesicles, together with the redistribution to the plasma membrane upon zinc limitation, suggested that a significant fraction of the protein exists in the recycling endosomal compartment. Thus, we investigated whether mZIP4-HA colocalized with the transferrin receptor, a marker of recycling endosomes. In HEK/mZIP4-HA cells cultured in basal medium, the transferrin receptor was located in a perinuclear vesicular compartment (Fig. 2B, green), and there was considerable overlap with the location of mZIP4-HA protein (Fig. 2A, red), as indicated in the merged images (Fig. 2C, yellow). These findings suggested that mZIP4-HA is abundant in recycling endosomes and may cycle between this compartment and the plasma membrane. To investigate further whether mZIP4-HA cycles via the plasma membrane, we tested whether treating cells with an endocytic inhibitor would lead to an increase in the levels of mZIP4-HA at the plasma membrane. HEK/mZIP4-HA cells were incubated in medium containing the general endocytosis inhibitor, MCD, which inhibits both clathrin- and caveolae-mediated endocytic pathways (23). MCD treatment resulted in the accumulation of mZIP4-HA at the cell surface (Fig. 2F). This finding supported the hypothesis that mZIP4-HA cycles between endosomal compartments and the plasma membrane.

Our studies then focused on whether zinc limitation resulted in increased levels of mZIP4-HA at the plasma membrane. The extracellular location of the HA epitope at the carboxyl terminus of mZIP4 enabled us to label only the surface fraction of mZIP4-HA protein with anti-HA antibodies. This was achieved by using anti-HA antibodies to probe intact HEK/mZIP4-HA cells that were fixed, but not permeabilized. Weaker punctate staining on the surface of HEK/mZIP4-HA cells was observed when these cells were cultured in basal medium (Fig. 3A). The punctate nature of this staining suggested that mZIP4-HA may localize within distinct microdomains of the plasma membrane, as found for other surface proteins (24). Notably, in TPEN-treated HEK/mZIP4-HA cells there was a marked increase in the levels of surface-bound anti-HA antibodies (Fig. 3A). This surface staining was specific for mZIP4-HA because there was no labeling of HEK293 cells transfected with the vector alone (Fig. 3A). Together with earlier experiments with permeabi-
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**Fig. 2.** The mZIP4-HA protein partially colocalizes with the transferrin receptor and cycles via the plasma membrane. A–C, the location of mZIP4 overlaps with the transferrin receptor. HEK/mZIP4-HA cells were grown in basal medium and fixed, permeabilized, and stained with antibodies against the HA epitope (A; red) and transferrin receptor (B; green). Colocalization of mZIP4-HA and the transferrin receptor is indicated by yellow staining in the merged panel (C). D–F, accumulation of mZIP4-HA at the plasma membrane of cells treated with the endocytosis inhibitor, MCD. HEK/mZIP4-HA cells or HEK/vector cells were grown for 30 min in basal medium (D and E) or basal medium containing the endocytic inhibitor, MCD (F). Cells were then fixed, permeabilized, and stained with anti-HA antibodies followed by Alexa-488 secondary antibodies.

**Fig. 3.** The time course of mZIP4-HA accumulation at the plasma membrane of HEK/mZIP4-HA cells pretreated with either basal medium or elevated zinc (Fig. 4). However, a pretreatment of the HEK/mZIP4-HA cells with TPEN to increase the level of mZIP4 at the plasma membrane resulted in a significantly increased mZIP4-HA uptake activity. The addition of zinc to the TPEN-containing medium suppressed this increased uptake activity in the HEK/mZIP4-HA cells, suggesting that the increased zinc uptake after TPEN treatment was the result of zinc deficiency. Notably, the zinc uptake differed little, if any, in the HEK/vector cells pretreated with basal medium, TPEN, or TPEN plus zinc. This suggested that the TPEN-induced increase in zinc uptake activity in HEK/mZIP4-HA cells was dependent on mZIP4-HA expression. In control experiments performed in parallel, an increase in the level of mZIP4-HA at the plasma membrane similar to that shown in Fig. 3 was observed (data not shown). These data support the hypothesis that the increased levels of mZIP4-HA protein at the plasma membrane in zinc-deficient conditions are likely a homeostatic mechanism to stimulate increased zinc uptake.

In Fig. 3, the speed with which the addition of zinc to zinc-deficient cells reduced the levels of mZIP4-HA at the plasma membrane prompted us to test whether this was accomplished through increased endocytosis of the protein. The endocytosis of mZIP4-HA was assessed using a strategy similar to that used previously to measure the endocytosis of the copper transporter, hCtr1 (25). We surmised that if mZIP4-HA undergoes endocytosis from the plasma membrane, anti-HA antibodies added to the growth medium would bind to surface mZIP4-HA and be internalized. HEK/mZIP4-HA cells were exposed for 5 min to medium containing anti-HA antibodies, rapidly cooled on ice, and the surface-bound anti-HA antibodies were removed by washing cells with ice-cold acidic buffer (data not shown). The internalized anti-HA antibodies were then detected by immunofluorescence microscopy. When HEK/mZIP4-HA cells were incubated in basal medium, anti-HA antibodies were readily detected within vesicular compartments (Fig. 5A). There was no accumulation of anti-HA antibodies in HEK293 cells transfected with the empty vector (data not shown), suggesting that the internalization of anti-HA antibodies in HEK/mZIP4-HA cells involved endocytosis of an mZIP4-HA-antibody complex. Significantly, in TPEN-treated cells the endocytosis of mZIP4-HA was reduced markedly, as evidenced by the weaker vesicular staining of internalized anti-HA antibodies (Fig. 5A). This reduced uptake of anti-HA antibodies was suppressed by adding zinc to the TPEN-containing medium (Fig. 5A). Together with earlier data, these
findings suggested that zinc limitation increases levels of mZIP4-HA at the plasma membrane by reducing endocytosis of the protein.

To assess more rigorously the zinc-responsive endocytosis of mZIP4-HA, we measured levels of internalized anti-HA antibodies by immunoblotting experiments. As we had done earlier for immunofluorescence, HEK/mZIP4-HA cells were incubated in medium containing anti-HA antibodies for various times, cooled on ice to stop further endocytosis, and then the surface-bound anti-HA antibodies were removed by several washes with ice-cold acidic buffer. Cell lysates were prepared to allow detection of the internalized antibodies using Western blots. After 5 min in basal medium, anti-HA antibodies were strongly detected in HEK/mZIP4-HA cells (Fig. 5B, lane 1). In contrast, there was little if any internalization of anti-HA antibodies in the HEK/mZIP4-HA cells during a 5-min incubation in either TPEN-containing medium or medium that had been depleted of metals using Chelex 100 resin (Fig. 5B, lanes 2 and 4). Significantly, adding zinc to the TPEN- or Chelex-treated medium stimulated the internalization of the anti-HA antibodies over 5 min (Fig. 5B, lanes 3 and 5). It was notable that longer incubations of 10 and 30 min in TPEN- and Chelex-treated medium resulted in the uptake of anti-HA antibodies, indicating that these zinc-limiting conditions did not completely inhibit the endocytosis of mZIP4-HA (Fig. 5B, lanes 7–11). In control experiments, the surface binding of anti-HA to fixed cells in the presence of TPEN- or Chelex-treated medium was no different for basal medium or zinc-supplemented medium (data not shown). This indicated that the increased uptake of anti-HA antibodies by TPEN- or Chelex-treated cells was not caused by alterations in the affinity of the antibody for the mZIP4-HA protein. Together with the immunofluorescence data, these findings suggested that zinc depletion reduces the endocytosis of mZIP4-HA at the plasma membrane, which is stimulated upon a transition to elevated zinc.

To characterize further the zinc-stimulated endocytosis of mZIP4-HA, we tested the concentration dependence of this response. This was achieved by assessing the minimal zinc concentrations required to stimulate mZIP4-HA endocytosis in medium that had been depleted of metals using Chelex 100. HEK/mZIP4-HA cells were initially incubated for 3 h in Chelex-treated medium to increase surface levels of mZIP4-HA. Anti-HA antibodies were then added to the Chelex-treated medium together with zinc over a range of final concentrations. After a 5-min incubation at 37 °C, the uptake of anti-HA antibodies was determined by Western blotting. Zinc levels as low as 0.5 μM were sufficient to stimulate mZIP4-HA endocytosis, as evidenced by the higher levels of internalized anti-HA antibodies relative to Chelex-treated medium without added zinc (Fig. 6A). There was also a concentration-dependent increase in mZIP4-HA endocytosis that was saturated with 5 μM zinc (Fig. 6A). These data are consistent with the high rates of mZIP4-HA endocytosis in basal medium (Fig. 5B), which contains ~2 μM zinc. We then tested the metal specificity of zinc-stimulated endocytosis of mZIP4-HA. Once again, HEK/mZIP4-HA cells were initially incubated for 3 h in Chelex-treated medium to increase surface levels of mZIP4-HA. The uptake of anti-HA antibodies by these cells was then assessed following the addition of either zinc, copper, iron, nickel, cobalt, or cadmium at a final concentration of either 1 or 20 μM. As shown in Fig. 6B,
Zinc was the only metal observed to stimulate mZIP4 endocytosis at a concentration of 1 μM. However, 20 μM manganese, cobalt, or cadmium was able to stimulate mZIP4 endocytosis (Fig. 6B), although this stimulation was not as great as that observed with 1 μM zinc (Fig. 6B). These data suggested that although mZIP4-HA endocytosis is responsive to high levels of manganese, cobalt, and cadmium, it is most responsive to physiological levels of zinc.

We then addressed whether the zinc-responsive localization of mZIP4 occurred in other cell types. HeLa cells were stably transfected with the mZIP4-HA construct and the effect of zinc depletion was investigated by immunofluorescence microscopy. As was the case in HEK293 cells, mZIP4-HA was predominantly located in cytoplasmic vesicles in HeLa cells cultured in basal medium (Fig. 7A). In TPEN-treated HeLa cells, mZIP4-HA was redistributed to the plasma membrane (Fig. 7B), and this effect was suppressed by the addition of zinc (Fig. 7C). The same zinc-dependent distribution of mZIP4-HA was observed in other cell types, such as Chinese hamster ovary cells, HT29 cells (human colon), and human fibroblasts (data not shown). These findings suggest that the trafficking machinery involved in zinc-responsive localization of mZIP4 exists in a range of different cell types.

To understand the broader relevance of our studies, we investigated whether the localization of human hZIP4 protein was also zinc-responsive. The mouse ZIP4 protein shares 76% similarity with the hZIP4, and both proteins are likely to be functionally equivalent. The HA epitope was fused to the carboxyl terminus of the hZIP4 cDNA and then subcloned into the pcDNA3.1 expression vector to generate the hZIP4-HA plasmid. HEK293 cells were stably transfected with the hZIP4-HA plasmid, and the localization of hZIP4-HA protein was assessed with and without TPEN treatment. As found for mZIP4-HA, hZIP4-HA was located predominantly in vesicles when cells were cultured in basal medium (Fig. 7D). The addition of TPEN to the medium caused the hZIP4-HA protein to accumulate at the plasma membrane (Fig. 7E), and this TPEN effect was prevented by adding zinc (Fig. 7F). Together with earlier data, these findings suggested that distribution of mouse and human ZIP4 protein is subject to regulation by zinc availability and that the trafficking machinery involved in this process is likely to exist in a variety of cell types.
The regulation of zinc uptake by mammalian cells is not well understood. The demonstration that the hZIP4 gene is mutated in patients with AE provided the first indication of its importance in mammalian zinc homeostasis. This has prompted recent studies into whether mZIP4 expression is regulated by zinc availability. Such studies have demonstrated that dietary zinc deficiency increases mZIP4 mRNA levels in the intestines and embryonic yolk sac of mice (19). Conversely, increased zinc availability by either dietary zinc supplementation or parenteral injection lowers the mZIP4 mRNA levels in these organs. This reduction in mRNA is also accompanied by a decrease in mZIP4 protein levels at the apical membrane of enterocytes. These findings suggest that mZIP4 mRNA levels are regulated by zinc availability and that such a mechanism is likely to be an important control point for regulating the entry of zinc into various cells that express the protein (19). The aim of this study was to investigate directly whether post-translational regulation of the mZIP4 protein by zinc is an additional level of homeostatic regulation in mammalian cells.

Analysis of the location of mZIP4-HA in HEK293 cells grown in basal medium indicated that the protein was distributed predominantly in perinuclear vesicles that overlapped considerably with recycling endosomes containing the transferrin receptor. By utilizing an exofacial HA tag at the carboxyl terminus of mZIP4, mZIP4-HA was shown to specifically internalize exogenous anti-HA antibodies supplied to the medium of living cells. Moreover, the endocytic inhibitor, MCD, caused the accumulation mZIP4-HA at the plasma membrane. Taken together, these findings indicated that the mZIP4-HA protein cycles between vesicular compartments and the plasma membrane. One of the most significant results of our study was the finding that the treatment of cells with the zinc chelator TPEN increased mZIP4-HA levels at the plasma membrane. Western blots indicated that this change in mZIP4-HA localization did not involve alterations in the level or apparent molecular mass of the protein. Indeed the rapidity of this relocalization, together with its occurrence in the presence of cycloheximide, was consistent with it being a post-translational response. The increased levels of mZIP4-HA at the plasma membrane in response to TPEN treatment were not caused by an adverse pharmacological effect because the same result was obtained when cells were exposed to medium from which zinc was previously extracted using Chelex 100 resin. Moreover, the shift of mZIP4-HA to the plasma membrane after TPEN or Chelex treatments was fully suppressed by the addition of zinc, suggesting that reduced zinc availability was responsible for this relocalization. The effect of TPEN and Chelex treatments was specific for mZIP4-HA, as we found no effect of these treatments on the locations of a variety of proteins known to cycle or reside within endocytic compartments, including the transferrin receptor, the copper transporter, hCtr1, the early endosome marker EEA1, and the ATP7A copper exporter (data not shown).

Our studies suggested that reduced endocytosis was associated with the increased surface levels of mZIP4 under zinc-limiting conditions. It is unclear at this stage whether zinc limitation also increases exocytosis of mZIP4 to the plasma membrane. A rapid dose-dependent stimulation of mZIP4-HA endocytosis was observed when zinc was supplied to Chelex-treated medium. This process was stimulated by zinc concentrations in the low micromolar range, indicating that this process is remarkably sensitive to physiologically relevant zinc concentrations. Other metals, such as manganese, cadmium, and cobalt, were also found to stimulate mZIP4 endocytosis, although at higher concentrations than for zinc.

What is biological significance of the increased surface levels of mZIP4 under zinc-limiting conditions? In zinc-deficient HEK/mZIP4-HA cells, the increased levels of mZIP4-HA at the plasma membrane were associated with increased zinc uptake activity. These findings suggested that the retention of mZIP4 at the plasma membrane is likely to be a homeostatic response that serves to increase zinc uptake under low zinc conditions. Indeed, this same response in vivo may contribute to the well recognized phenomenon of increased intestinal zinc uptake during periods of zinc deficiency (26–30). Our finding that the endocytosis of mZIP4 is increased upon transition to zinc repletion suggests that such a response may also serve to remove mZIP4 from the plasma membrane and prevent the overaccumulation of zinc. Although mZIP4 levels at the plasma membrane were reduced by zinc repletion, our studies indicated that the protein continues to cycle via the plasma membrane under these conditions. The basis for this cycling of mZIP4 is unclear, but an appealing hypothesis is that such a process may allow extracellular zinc levels to be constantly monitored by the protein, thereby permitting the rapid accumulation of mZIP4 at the plasma membrane if zinc becomes limiting. The mechanism by which zinc is “sensed” to induce endocytosis is unknown. One possibility is that extracellular zinc is sensed by the histidine-rich amino-terminal region, which then triggers conformational changes in mZIP4 which increase endocytosis, possibly by stimulating interactions between an endocytic targeting motif and the trafficking machinery. Alternatively, the endocytosis of mZIP4 may respond to intracellular zinc levels. These intracellular zinc levels may be sensed either through direct binding of zinc to mZIP4 or another protein that then acts as an intermediary to stimulate mZIP4 endocytosis.

Our recent studies indicated that the mZIP4 protein accumulates at the apical surface of intestinal enterocytes and visceral endoderm cells in zinc-deficient mice (19). When zinc was provided to these animals, either via the diet or parenterally, the mZIP4 protein was absent from the apical surface of these cells. Because zinc repletion also resulted in a marked reduction in mZIP4 mRNA levels, it was unclear whether the extent to which this loss of mZIP4 from the plasma membrane was the result of increased endocytosis or reduced mZIP4 expression. In the current study, mZIP4 was expressed in cultured cells from the constitutive cytomegalovirus promoter. This allowed us to examine mZIP4 protein trafficking without the confounding effects of transcriptional control by zinc. Together with the in vivo studies, our findings suggest that zinc uptake via mZIP4 is regulated by zinc availability both at the

**FIG. 7.** Analysis of zinc-responsive localization of mZIP4 and hZIP4 in HeLa and HEK293 cells. HeLa cells stably expressing the mZIP4-HA protein (A–C) and HEK293 cells stably expressing the hZIP4 in HeLa and HEK293 cells. (D–F) were exposed for 1 h to basal medium, 10 μM TPEN, or 10 μM TPEN plus 10 μM Zn²⁺ prior to immunofluorescence analysis using anti-HA antibodies as seen in Fig. 1.
mRNA level and at a post-translational level through zinc-responsive endocytosis of the mZIP4 protein. These two systems of mZIP4 regulation may provide the cell with both fast and slow responses to fluctuations in zinc availability and permit the “fine-tuning” of zinc uptake activity.

Our studies demonstrated that the localization of the human hZIP4 protein is also controlled by zinc availability. Importantly, this finding highlights the possibility that certain mutations in patients with AE may prevent retention of hZIP4 at the plasma membrane under zinc-deficient conditions and thus impair the ability to absorb zinc from low zinc diets. We are currently investigating the effects of a range of patient mutations on hZIP4 localization and endocytosis. The finding that the location of both human and mouse ZIP4 proteins are zinc-dependent indicates that regulated trafficking is likely to exist for other mammalian ZIP4 homologs. Our unpublished preliminary studies have confirmed that zinc-regulated trafficking exists for other mammalian members of the ZIP family. Together with previous findings that the ZRT1 protein in yeast undergoes zinc-stimulated endocytosis (31), these findings suggest that such a process probably evolved early during evolution and is likely well conserved among members of the ZIP family. Indeed, a general theme is emerging whereby metal ion homeostasis in mammalian cells is regulated by metal-responsive trafficking of transporters. Excessive zinc was recently shown to alter the location of the zinc exporters Znt4 and Znt6 from the Golgi complex (32). Moreover, the locations of the human copper exporters, ATP7A and ATP7B, and the copper importer, hCtr1, are regulated by copper availability (25, 33, 34). It will be of interest to determine whether common regulatory mechanisms underlie the trafficking of each of these metal transporters.

In summary, we have identified a novel post-translational mechanism for regulating high affinity zinc uptake in mammalian cells involving zinc-stimulated endocytosis of the mZIP4 transporter. We are currently exploring the mechanism of zinc sensing, the sorting signals involved in mediating endocytosis, and the vesicular compartments through which mZIP4 traffics. These studies will provide a better understanding of cellular and organismal zinc homeostasis and the etiology of AE.

Acknowledgments—We thank Drs. Elizabeth Rogers and Sherri Sachdev for critically reviewing the manuscript.

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Zn^{2+}-stimulated Endocytosis of the mZIP4 Zinc Transporter Regulates Its Location at the Plasma Membrane

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J. Biol. Chem. 2004, 279:4523-4530.
doi: 10.1074/jbc.M310799200 originally published online November 11, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M310799200

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