Zinc-stimulated Endocytosis Controls Activity of the Mouse ZIP1 and ZIP3 Zinc Uptake Transporters*

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The mouse mZip1 and mZip3 zinc transporters have been implicated in zinc acquisition by the cells of many tissues. This hypothesis raised the question of whether activity of these proteins is regulated to maintain zinc homeostasis. Neither mZIP1 nor mZIP3 mRNA levels are highly regulated by zinc status. Therefore, we investigated whether zinc controls the activity of these proteins post-translationally by altering their subcellular distribution. When expressed in transfected cells grown in zinc-replete medium, both mZip1 and mZip3 were largely present in intracellular organelles. However, these proteins were found to rapidly transit between the plasma membrane and intracellular compartments in zinc-replete cells. Zinc deficiency increased plasma membrane levels of mZip1 and mZip3 by decreasing their rates of endocytosis. Greater zinc deficiency was required to alter mZip3 distribution than was needed to affect mZip1. Increased surface levels correlated with increased zinc uptake activity. Taken together, these results suggest that post-translational control of mZip1 and mZip3 localization plays a role in zinc homeostasis. Moreover, our results indicate that zinc-responsive endocytosis is a conserved mechanism controlling activity of many mammalian zinc uptake transporters.

Zinc is an essential nutrient because of the many important roles it plays in biology. Therefore, cells require efficient uptake mechanisms to obtain zinc from their extracellular environment. Zinc is also potentially toxic if overaccumulated so homeostatic regulatory mechanisms are required to control the activity of zinc uptake systems and prevent zinc overaccumulation. Zinc uptake is mediated in many organisms by members of the ZIP1 (Zrt-, Irt-like protein) family (1–4). First identified in Arabidopsis, members of the ZIP family have been shown to be involved in metal acquisition in bacteria, yeast, and plants (5–11). Members of the ZIP family show considerable diversity and cluster into four distinct subfamilies based on their amino acid sequence similarities (2).

The human genome encodes 14 ZIP proteins and their corresponding genes are designated SLC39A1–SLC39A14 (12). The importance of mammalian ZIP proteins in zinc acquisition is becoming increasingly clear. For example, SLC39A4 encodes the hZip4 protein recently implicated in the uptake of zinc from the diet; SLC39A4 is mutated in patients with the heritable disease acrodermatitis enteropathica (AE) (13, 14). If untreated, AE patients become zinc-deficient because of a decreased ability to utilize dietary zinc. More recent studies have indicated that the orthologous mouse protein mZip4 is responsible for dietary zinc uptake by intestinal enterocytes (15, 16). hZip4 and mZip4 are members of the so-called LIV-1 subfamily of ZIP proteins (2, 4). Among the other mammalian ZIP-encoding genes are SLC39A1 and SLC39A3, encoding hZip1 and hZip3, and the orthologous mouse genes. These proteins belong to a different ZIP subfamily and share only limited sequence similarity to mammalian Zip4. Nonetheless, human and mouse Zip1 proteins have been shown to be active for zinc uptake and specific for zinc as their substrate (17–19). Mouse Zip3 has also been shown to be a zinc-specific transporter (19) although activity of the human protein has not yet been examined. ZIP1 is abundantly expressed in a wide variety of tissues (18–20). ZIP3 is also widely expressed in many tissue types (Unigene EST data base) although it is most abundant in testes (19). These results suggest that Zip1 and Zip3 are involved in zinc acquisition by many cells of the body.

Regulation of ZIP protein activity has been found to occur at both transcriptional and post-translational levels. In yeast, transcription of the ZRT1 and ZRT2 genes is induced by zinc limitation thereby increasing the activity of these transporters by as much as 80-fold (6, 7). In addition to this transcriptional control, Zrt1 activity is regulated post-translationally. When zinc is resupplied to zinc-deficient cells expressing high levels of Zrt1, the transporter is rapidly inactivated (21). This inactivation occurs because zinc stimulates the rate of endocytosis of the transporter and its removal from the plasma membrane. In mammals, regulation of some zinc uptake transporters occurs at the level of mRNA abundance possibly via transcriptional control. For example, SLC39A2 (hZIP2) mRNA levels rise almost 30-fold in cultured THP-1 monocytes made zinc-deficient by treatment with TPEN, a membrane-permeable zinc chelator (22). Similarly, mice fed a zinc-deficient diet accumulate ~10-fold more mZIP4 mRNA in intestinal enterocytes than is found in zinc-replete animals (15). We have recently found that mZip4 activity is regulated at a post-translational level as well. As was the case for yeast Zrt1, zinc stimulates endocytosis of the mouse and human Zip4 protein (23). It is therefore likely that regulation at the level of mRNA abundance combined

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‡ The abbreviations used are: ZIP, Zrt-, Irt-like protein; HA, hemagglutinin antigen; MCD, methyl β-cyclodextrin; TPEN, N,N,N′,N′-tetraakis(2-pyridyl-methyl)methylenediamine; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; HEK, human embryonic kidney.

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2 www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene.
with post-translational control regulate the influx of dietary zinc via Zip4.

In contrast to ZIP2 and ZIP4, expression of mammalian ZIP1 and ZIP3 is not highly regulated at the transcript level (19, 22, 24, 25). This observation raised the question of how cells expressing these transporters regulate zinc uptake. To address this question, we examined whether mouse Zip1 and Zip3 are regulated post-translationally by controlling their localization in the cell. In this study, we show that the intracellular distribution of these proteins is regulated by zinc at least in part by controlling their rate of endocytosis. These studies support the notion that post-translational regulation of ZIP zinc transporter localization plays a major role in zinc homeostasis in a wide range of cell types. Moreover, given the divergence of mammalian ZIP transporters now known to be regulated in this fashion, post-translational control of the zinc uptake transporter localization appears to be a general mechanism of zinc homeostasis in mammals.

MATERIALS AND METHODS

Plasmid Constructions—These studies used alleles of mZip1, mZip3, and mZip4 that were tagged at their C termini with one copy of the hemagglutinin (HA) antigen epitope. Construction of these plasmids is described elsewhere (15, 19). Each protein was expressed from the cytomegalovirus promoter and shown previously to be functional for zinc transport (15, 19, 23).

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 minimal essential medium non-essential amino acids (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). HEK293 (~2 × 106) cells were seeded in 25-mm2 flasks and transfected with plasmid DNAs using LipofectAMINE 2000 (Invitrogen). Stably transfected HEK293 cell lines were selected with 2.0 μg/ml G418 (Sigma) 48 h after transfection. Approximately 80% of cells in each population expressed the transgene as shown by immunofluorescence microscopy (data not shown).

65Zn Uptake Assays—Stably transfected HEK293 cells were used for 65Zn uptake assays as described previously (15). 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) 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). Parallel experiments were conducted at 0 °C to estimate 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 5650 γ-counter. In a parallel experiment, cells were washed three times with ice-cold uptake buffer and then lysed in PBS buffer plus 0.1% SDS, 1% Triton X-100. Protein levels were then determined using the Bio-Rad DC protein assay. Zinc accumulation was calculated and normalized to protein concentrations of cell lysates.

Immunoblotting—Transfected cells were harvested, washed three times with ice-cold PBS, scraped into PBS, and collected by centrifugation. After three additional washes in ice-cold PBS, the cells were lysed by sonication in buffer containing 62 mM Tris-Cl, pH 6.8, 2% SDS, 5 mM dithiothreitol, 1 mM EDTA, and protease inhibitors (Roche Applied Science). Loading buffer was added to the protein extracts and then incubated at 37 °C for 30 min. (Higher incubation temperatures were found to cause aggregation of these proteins.) Extract volumes equivalent to 20-μg protein extracts were separated using a 4–20% gradient SDS-PAGE Ready Gels (Bio-Rad), transferred to nitrocellulose membranes, and probed using an anti-HA polyclonal antibody (1:1000, Sigma) followed by an anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP; Pierce). As a loading control, the membranes were stripped of antibodies by incubating at 60 °C for 30 min in stripping buffer (62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol, and 2% (w/v) SDS), washed in TBST, and reprobed with a 1:40,000 dilution of mouse anti-tubulin (Sigma) primary antibody and 1:10,000 dilution of HRP-conjugated anti-mouse IgG (Pierce) as secondary antibody.

For immunoblot analysis of mZip-HA surface levels, the cells were cultured in 6-well trays. In some experiments, ZnCl2, or N,N,N′,N′-tetakis (2-pyrrolidinyl-methyl) ethylenediamine (TPEN) was added to the medium at the indicated concentrations and times. Cells were washed three times with PBS on ice, 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 SDS buffer solution containing 62 mM Tris-Cl (pH 6.8), 2% SDS, 5 mM dithiothreitol, and protease inhibitors (Roche Applied Science). Lysates containing the solubilized anti-HA antibodies that were bound to the mZip-HA protein at the plasma membrane were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the antibodies detected using anti-rabbit antibodies (1:10,000) by chemiluminescence (Roche Applied Science). The protein detected in the mZip-HA lanes co-migrated with purified anti-HA antibody (data not shown).

Endocytosis of mZip-HA proteins was determined by assaying the uptake of anti-HA antibodies added to the culture media of live cells. Cells were grown on 6-well plates in basal medium or in basal medium supplemented with 1 h with 10 μM zinc and/or 10 μM TPEN. The cells were chilled on ice for 10 min and then rewarmed and incubated in basal, zinc-, or TPEN-supplemented medium containing 5 μg/ml anti-HA antibodies for 5 min. During this time, antibodies bound to the HA epitope were internalized by endocytosis. Cells were transferred to ice to prevent further endocytosis, washed three times with ice-cold PBS, and surface-bound antibodies were removed by five washes with ice-cold acidic buffer (100 mM glycine, 20 mM magnesium acetate, 50 mM potassium chloride, pH 2.2). This treatment is sufficient to remove all detectable surface-bound antibodies (16, 23). After two additional washes with ice-cold PBS, the cells were collected, lysed, and analyzed for anti-HA antibodies by immunoblotting as described above.

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 ice-cold PBS, fixed in 3.7% paraformaldehyde in a shaking 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 then washed five times in PBS followed by incubation for 1 h with the anti-rabbit IgG 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. Permeabilized cells were incubated with 2 μg/ml Alexa 555-conjugated anti-HA antibodies for 5 min. During this time, antibodies bound to the HA epitope were endocytosed and internalized. The antibodies were then detected using anti-rabbit HRP antibody conjugated to Alexa 594 (Molecular Probes). The cells were then washed in PBS, and analyzed for anti-HA antibodies by immunoblotting as described above.

RESULTS

Expression and Subcellular Distribution of mZip1-HA and mZip3-HA Proteins in Transfected HEK293 Cells—Previous studies established that the mouse Zip1 and Zip3 proteins stimulate zinc uptake when expressed at high levels in transiently transfected HEK293 cells. To allow assessment of the subcellular distribution of these proteins, a hemagglutinin antigen (HA) epitope tag was fused to their C termini for detection of the mZip1-HA and mZip3-HA proteins. The tagged proteins were shown previously to be functional for 65Zn uptake (19). HEK293 cells were transfected with mZip1-HA and mZip3-HA plasmids expressing the gene from the cytomegalovirus promoter. Populations of cells stably expressing mZip1-HA or mZip3-HA were obtained following selection with puromycin and designated as HEK/mZip1-HA or HEK/mZip3-HA cell lines. In each population, ~80% of the cells expressed the HA-tagged protein as determined by immunofluorescence microscopy (data not shown). Immunoblot analysis using an anti-HA antibody detected a single protein of ~35 kDa molecular mass in both transfected cell populations consistent with the
predicted molecular masses of mZip1-HA and mZip3-HA (35.2 and 34.9 kDa, respectively) (Fig. 1A). No bands were detected in HEK293 cells transfected with the pcDNA3.1 vector alone. As a control for these and subsequent experiments, we also included a cell line stably expressing mZip4-HA. This cell line accumulated mZip4 protein to similar levels as observed in the HEK/mZip1-HA and HEK/mZip3-HA lines. We showed previously that mZip4-HA is N-glycosylated; inhibition of N-glycosylation in vivo or enzymatic removal of N-glycosyl groups in vitro reduced the apparent molecular mass of mZip4-HA (16). Similar treatments had no effect on mZip1-HA and mZip3-HA mobility indicating that these proteins are not glycosylated (data not shown).

The subcellular distribution of mZip1-HA, mZip3-HA, and mZip4-HA was assessed by immunofluorescence confocal microscopy. 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 mZip1-HA, mZip3-HA, and mZip4-HA showed abundant staining (Fig. 1B). A large proportion of each protein was distributed in intracellular vesicles scattered throughout the cytoplasm. These vesicles were especially concentrated in the perinuclear region, which co-localized with a marker protein, p230, of the trans-Golgi network (Fig. 1B, red). The merged images are shown in the right hand panel of Fig. 1B, and the overlap of mZip-HA (green) and p230 (red) is shown in yellow. Similar results were obtained when these proteins were expressed in CHO cells (data not shown).

To assess surface levels of these proteins in cells grown in basal medium, immunofluorescence studies were performed using cells that were fixed but not permeabilized. The C-terminal epitope tag of each protein is predicted to be extracellular and therefore exposed to antibody binding in non-permeabilized cells. No fluorescence was detected following surface labeling of vector-only transfectants (Fig. 2A). In contrast, fluorescence staining was clearly observed on the surface of mZip1-HA and mZip3-HA cells grown in basal medium. The punctate nature of this staining suggested that these proteins localize to lipid rafts or other discrete microdomains of the plasma membrane as has been found for other surface proteins (26). Notably, despite similar levels of total protein accumulation (Fig. 1A), surface levels of mZip4-HA protein appeared to be much higher than either mZip1-HA or mZip3-HA. As an alternative approach to assess surface levels, we used an immunoblotting technique used previously to assay surface levels of mZip4 (16, 23). This method uses immunoblots to assess the levels of anti-HA antibodies bound to the surface of non-permeabilized HEK/mZip-HA cells. Cells were grown in basal medium, fixed with paraformaldehyde, and the intact cells were then probed with anti-HA antibodies to label mZip-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. The results of this experiment indicated that surface levels of mZip4-HA are significantly higher than mZip1-HA or mZip3-HA (Fig. 2B). The absence of detectable surface antibody binding by vector-only transfectants indicated that nonspecific antibody binding was not occurring.

**mZip1-HA and mZip3-HA Transit between the Plasma Membrane and Endocytic Compartments**—We previously showed that mZip4-HA transits between the plasma membrane and intracellular compartments in cells grown in basal medium. To determine if mZip1-HA and mZip3-HA also cycle via the plasma membrane, we first examined the effects of the general endocytosis inhibitor, methyl β-cyclodextrin (MCD; 5 mM) (27). MCD inhibits both clathrin-dependent and caveolae-dependent endocytosis by extracting cholesterol from the plasma membrane. If mZip1-HA and mZip3-HA cycled via the plasma membrane, we predicted that MCD treatment would increase the level of protein accumulated on the cell surface. The effects of MCD on the distribution of total mZip-HA protein in permeabilized cells is shown in Fig. 3A. For each protein, MCD treatment for 1 h resulted in an apparent migration from the trans-Golgi network region to more dispersed cytoplasmic vesicles. Furthermore, an increase in fluorescence observed at the cell periphery suggested increased surface levels of each protein. This conclusion was confirmed when we examined the effects of MCD treatment on surface mZip-HA levels in non-
permeabilized cells (Fig. 3B). Increased surface levels upon MCD treatment were also found when surface levels were examined by immunoblotting of surface-bound anti-HA antibody as described in Fig. 2B. Moreover, these studies indicated that the change in surface levels occurs very rapidly. Surface levels of mZip1-HA and mZip4-HA increased to near or fully maximal levels after only 2 min of MCD treatment (Fig. 3C). Increased surface mZip3-HA levels were also detectable after 2–5 min of treatment but only reached maximum levels after 20 min of MCD treatment. Nonetheless, these results suggest that mZip1 and mZip3, like mZip4, rapidly cycle between the plasma membrane and endocytic compartments. The slower response of mZip3-HA to MCD suggests that this protein may transit more slowly than do mZip1-HA and mZip4-HA. It should be noted that the immunoblots for mZip1-HA and mZip3-HA are much longer exposures than the mZip4-HA blot shown to compensate for their lower surface levels (Fig. 2). Treating cells with other inhibitors of endocytosis, i.e. sucrose, chlorpromazine, K+ depletion, resulted in similar increases in mZip1-HA and mZip3-HA surface accumulation suggesting that the effects observed with MCD are not due to perturbations of cellular processes other than endocytosis (data not shown).

The extracellular location of the HA epitope at the C terminus of each Zip protein also allowed us to examine this cycling by assaying internalization of anti-HA antibodies from the growth medium by living cells. We found previously that when mZip4-HA undergoes endocytosis from the plasma membrane, anti-HA antibodies added to the medium can bind to surface mZip4-HA and be internalized. Thus, endocytosis of the protein correlates with the amount of anti-HA antibody internalized. We used this same method to assess endocytosis of mZip1-HA and mZip3-HA. HEK/mZip-HA cells were exposed for 5–30 min to medium containing anti-HA antibodies, chilled to stop further endocytosis, and then surface-bound antibodies were removed by washing with cold acidic buffer. This treatment was shown to be sufficient to remove all surface-bound antibodies (Fig. 4, lane 1 versus 2). The cells were then lysed and analyzed by immunoblotting to detect internalized anti-HA antibodies. No internalized antibodies were detected for vector-only transfectants even after 30 min of antibody treatment (Fig. 4, lane 3). In contrast, mZip1-HA, mZip3-HA, and mZip4-HA cells accumulated antibody over the 30-min time period (Fig. 4, lanes 4–6). These results supported our conclusion that despite their largely intracellular steady state distribution, mZip1-HA and mZip3-HA cycle rapidly to and from the plasma membrane.

mZip1-HA and mZip3-HA Distribution Is Altered by Zinc Status—Our previous studies of yeast Zrt1 and mammalian Zip4 demonstrated that zinc status controls the subcellular distribution of these proteins. In low zinc, Zrt1 and Zip4 accumulate on the cell surface to mediate zinc uptake. In high zinc, the protein is removed from the cell surface and uptake activity decreases. To assess the effects of zinc deficiency on mZip1-HA and mZip3-HA, we first examined surface levels of these proteins by immunofluorescence microscopy of non-permeabilized cells. As shown previously, 1 h of incubation in medium pretreated with Chelex 100 to remove labile zinc resulted in increased mZip4-HA surface levels (Fig. 5A). Similarly, surface detection of mZip1-HA also increased following exposure to zinc-deficient Chelex-treated medium. Consistent with these results, causing zinc deficiency by treating HEK/mZip1-HA and HEK/mZip4-HA cells with TPEN also resulted in increased surface levels. This effect was blocked by simultaneous incubation with TPEN plus equimolar levels of zinc.

In contrast, incubation of mZip3-HA in Chelex-treated medium did not alter surface levels whereas incubation with TPEN increased surface levels dramatically. These results suggested that the distribution of mZip3-HA is also zinc responsive but may require greater zinc limitation, as is afforded by TPEN treatment, to occur. Experiments described later in this report supported this hypothesis. Immunoblots of total protein extracts from these various cell lines, and treatment conditions indicated no changes in protein levels had occurred (Fig. 5B). Therefore, the changes in distribution observed are not due to effects on protein abundance.

Zinc-responsive protein trafficking was further supported by complementary experiments assessing the level of surface mZip-HA proteins by immunoblotting. As shown in Fig. 6A, incubation of HEK/mZip1-HA and HEK/mZip4-HA in Chelex-treated medium resulted in increased surface levels of these two proteins. While surface levels of mZip4-HA rapidly increased over 5 min and reached its new steady state abundance within 15 min, no detectable increase in mZip1-HA surface levels was apparent until after 15 min. Consistent with the immunofluorescence microscopy result in Fig. 5A, surface levels of mZip3-HA remained constant for 60 min (Fig. 6A) and for 24 h (data not shown) incubation in Chelex-treated medium. As shown in Fig. 6B, surface levels of all three proteins increased.
in response to TPEN treatment. Again, mZip4-HA responded quickly, i.e. within 5 min, whereas mZip1-HA and mZip3-HA responded more slowly. Surface accumulation of mZip1-HA, mZip3-HA, and mZip4-HA was blocked by incubation in TPEN/H11001 equimolar zinc.

The failure of mZip3-HA to respond to Chelex-treated medium suggested that this protein required greater zinc limitation than did mZip1-HA or mZip4-HA. This hypothesis was supported when we examined surface mZip-HA levels in cells treated over a range of TPEN concentrations. HEK/mZip1-HA, HEK/mZip3-HA, and HEK/mZip4-HA cells were treated with 0–20 μM TPEN for 60 min, and surface levels were then assayed by immunoblotting. As little as 1 μM TPEN was sufficient to trigger mZip1-HA and mZip4-HA surface accumulation whereas 5–10 μM TPEN was required to increase surface levels of mZip3-HA (Fig. 7A). Similarly, when cells were treated with medium in which increasing zinc concentrations were added to a fixed concentration of TPEN (10 μM), more zinc was required to prevent increased surface accumulation of mZip1-HA and mZip4-HA than was required for mZip3-HA (Fig. 7B). These results suggest that mZip3-HA relocalization responds only to

vector-only control lane indicates that detection is mZip-HA-specific. The mZip1-HA and mZip3-HA blots were exposed for longer times to allow comparison with mZip4-HA levels. The same blots were stripped and reprobed with anti-tubulin to confirm equal loading.

**FIG. 3.** mZip1-HA and mZip3-HA transit via the plasma membrane. A and B, immunofluorescence confocal microscopy analysis of the distribution and surface level of mZip1-HA, mZip3-HA, and mZip4-HA in stably transfected HEK293 cells. Cells were cultured in basal medium or basal medium plus 5 mM MCD for 30 min, and then fixed, permeabilized (A) or not permeabilized (B), and analyzed for mZip-HA distribution as described in the legends to Figs. 1 and 2. C, time course of mZip1-HA, mZip3-HA, and mZip4-HA proteins accumulated on the plasma membrane following MCD treatment. Cells were incubated in basal medium plus 5 mM MCD for the indicated times. Cells were fixed, probed with rabbit anti-HA antibody, and then collected, lysed, and proteins separated by SDS-PAGE. The surface-bound anti-HA antibodies were detected using HRP-conjugated anti-rabbit IgG antibody. The absence of surface-bound anti-HA antibodies in the

**FIG. 4.** Endocytosis of mZip1-HA, mZip3-HA, and mZip4-HA proteins in stably transfected HEK293 cells. Endocytosis of these proteins was established by assaying the internalization of anti-HA antibody at 37 °C. Live cells were incubated in basal medium plus rabbit anti-HA antibody (5 μg/ml) for 5–30 min (lanes 3–6). Surface-bound anti-HA antibody was removed by washing in an acidic buffer and intracellular antibody proteins were detected by immunoblotting using anti-rabbit IgG antibody conjugated to HRP. The efficacy of the acidic wash step in removing surface-bound antibodies is shown in lanes 1 and 2. The HEK293/mZips-HA cells were labeled on ice with anti-HA antibody, washed with either PBS (lane 1) or acidic buffer (lane 2), and surface-bound antibodies were detected by immunoblotting. The results showed that the acidic buffer removed all of the surface-bound anti-HA antibody. The mZip1-HA and mZip3-HA blots were exposed for longer times to allow comparison with mZip4-HA levels. The same blots were stripped and reprobed with anti-tubulin to confirm equal loading.

The failure of mZip3-HA to respond to Chelex-treated medium suggested that this protein required greater zinc limitation than did mZip1-HA or mZip4-HA. This hypothesis was supported when we examined surface mZip-HA levels in cells treated over a range of TPEN concentrations. HEK/mZip1-HA, HEK/mZip3-HA, and HEK/mZip4-HA cells were treated with 0–20 μM TPEN for 60 min, and surface levels were then assayed by immunoblotting. As little as 1 μM TPEN was sufficient to trigger mZip1-HA and mZip4-HA surface accumulation whereas 5–10 μM TPEN was required to increase surface levels of mZip3-HA (Fig. 7A). Similarly, when cells were treated with medium in which increasing zinc concentrations were added to a fixed concentration of TPEN (10 μM), more zinc was required to prevent increased surface accumulation of mZip1-HA and mZip4-HA than was required for mZip3-HA (Fig. 7B). These results suggest that mZip3-HA relocalization responds only to...
severe zinc deficiency whereas mZip1-HA and mZip4-HA require only moderately limiting conditions.

As was found previously for mZip4 (23), increased surface levels of mZip1-HA and mZip3-HA under zinc deficiency could be the result of decreased rates of endocytosis. To test this hypothesis, we examined antibody internalization in zinc-replete and deficient cells as described in Fig. 4. HEK293 cells expressing mZip1-HA, mZip3-HA, or mZip4-HA were incubated for 1 h in basal medium, basal plus 10 μM TPEN, or basal plus 10 μM TPEN and 10 μM ZnCl₂. Anti-HA antibodies were then added to the growth medium and the cells were allowed to internalize these antibodies for 10 min. The cells were then chilled, stripped of surface-bound antibodies, and assayed for internalized antibodies by immunoblotting. No antibody internalization was detected in vector-only cells (Fig. 8). For mZip1-HA, mZip3-HA, and mZip4-HA, antibody internalization was then added to the growth medium and the cells were allowed to internalize these antibodies for 10 min. The cells were then chilled, stripped of surface-bound antibodies, and assayed for internalized antibodies by immunoblotting. No antibody internalization was detected in vector-only cells (Fig. 8).

Accumulation of mZip1-HA and mZip3-HA on the Cell Surface Increases Zinc Uptake Activity—The zinc-responsive accumulation of mZip1-HA and mZip3-HA on the cell surface may be a homeostatic mechanism to control zinc uptake activity in response to zinc status/availability as was found previously for mZip4 (23). To test this hypothesis, HEK/mZip1-HA and HEK/mZip3-HA cells were pretreated (1 h) in either basal medium, basal plus 10 μM TPEN, or basal plus 10 μM TPEN and 10 μM ZnCl₂. Anti-HA antibodies were then added to the growth medium and the cells were allowed to internalize these antibodies for 10 min. The cells were then chilled, stripped of surface-bound antibodies, and assayed for internalized antibodies by immunoblotting. For mZip1-HA and mZip3-HA, antibody internalization was detected in vector-only cells (Fig. 8). For mZip1-HA, mZip3-HA, and mZip4-HA, antibody internalization was higher in untreated cells relative to cells exposed to the endocytosis inhibitor MCD. Similarly, TPEN treatment also reduced the rate of internalization whereas TPEN + zinc treatment had no effect. These results indicate that like mZip4 (23), the endocytosis rate of mZip1-HA and mZip3-HA decreases under zinc deficiency and explains, at least in part, the increase in surface levels under these conditions.

Accumulation of mZip1-HA and mZip3-HA on the Cell Surface Increases Zinc Uptake Activity—The zinc-responsive accumulation of mZip1-HA and mZip3-HA on the cell surface may be a homeostatic mechanism to control zinc uptake activity in response to zinc status/availability as was found previously for mZip4 (23). To test this hypothesis, HEK/mZip1-HA and HEK/mZip3-HA cells were pretreated (1 h) in either basal medium, zinc-deficient medium (basal + TPEN), or zinc-deficient medium supplemented with zinc (basal + TPEN + Zn), washed, and then assayed for zinc uptake activity over 5 min in buffer containing ⁶⁵Zn. These treatments had no effect on the uptake activity of vector-only HEK293 transfectants (Fig. 9). Zinc uptake activity was markedly increased by pretreating the cells expressing the mZip-HA proteins with TPEN. Adding zinc back to the TPEN-treated cells suppressed this increase in zinc uptake activity. Consistent with the results of incubating cells in Chelex-treated medium on surface levels of mZip1-HA and
mZip3-HA (Figs. 5 and 6), $^{65}$Zn uptake activity of mZip1-HA-expressing cells increased following this treatment, whereas no such increase was observed with mZip3-HA-expressing cells. These data support the hypothesis that the increased levels of mZip-HA proteins at the cell surface in zinc-deficient conditions is likely to be a homeostatic mechanism to stimulate zinc uptake activity.

**DISCUSSION**

Regulating zinc uptake transporter activity is an important aspect of zinc homeostasis in bacteria, yeast, and plants and its relevance to mammalian zinc metabolism is becoming increasingly clear. Mouse Zip4 is highly regulated by zinc status (15). One component of this regulation occurs at the mRNA level; mZIP4 mRNA levels are high in zinc-limited animals and low when zinc replete. Regulation of mZip4 activity was also found to occur at a post-translational level. When expressed in cultured cells, the distribution of mZip4 changes in response to zinc status (23). In zinc-limited cells, the protein is localized to the cell surface. Upon repletion, Zip4 is redistributed to intra-
cellular organelles via zinc-stimulated endocytosis. The level of Zip4 on the plasma membrane serves to regulate the zinc uptake activity of the protein. Therefore, given the role of Zip4 in dietary zinc absorption, we propose that these transcriptional and post-translational mechanisms of controlling Zip4 activity provide for zinc homeostasis at the organism level.

The identification of mammalian Zip1 and Zip3 has made it possible to analyze the regulation of mammalian zinc transporters of a different ZIP subfamily than that to which Zip4 belongs. Mouse and human Zip1 and mZip3 are expressed in many tissues. This observation, combined with the ability of these proteins to confer zinc-specific uptake activity in vitro, suggested that Zip1 and Zip3 are involved in providing zinc to a variety of cell types. Consistent with this hypothesis, inhibiting expression of hZip1 in K562 and PC-3 cells using antisense RNA dramatically reduced zinc uptake activity (18, 28). These results suggested that Zip1 is the major zinc uptake transporter in these cells. If true that Zip1 and mZip3 are important for zinc delivery to many types of cells, their regulation by zinc status could be a critical component of zinc homeostasis at the cellular level.

In contrast to mZip4, little regulation of mZip1 or mZip3 mRNA levels by zinc status has been observed (19, 22, 24). These observations led us to investigate whether the activity of these proteins was regulated at a post-translational level. When cells were grown in zinc-replete basal medium, mZip1 and mZip3 were largely distributed to intracellular organelles and very little resided on the cell surface. However, this steady state distribution is maintained by a dynamic cycling of the proteins to the plasma membrane and rapid endocytosis. One way in which this was demonstrated was by treating cells with MCD, an endocytosis inhibitor, which resulted in accumulation of the proteins on the cell surface. Given that MCD impairs both clathrin- and caveolae-dependent endocytosis (27), the specific pathway of endocytosis taken by these proteins is not yet established. Remarkably, when these cells were made zinc-deficient, mZip1 and mZip3 increased in cell surface abundance. This effect was the result of decreased endocytosis in zinc-limited cells. We propose that this is a homeostatic regulatory mechanism to increase zinc uptake during deficiency, and this conclusion was supported by the results of 65Zn uptake experiments.

While effects of zinc on endocytosis rates were demonstrated, our studies do not preclude changes in exocytosis rates also occurring in response to zinc. Another major question is whether the distribution of endogenous mZip1 and/or mZip3 protein, as opposed to the protein expressed heterologously in a transfected cell, changes in response to zinc status. Although trafficking of endogenous mZip1 has not yet been examined, a recent study by Kelleher and Lonnerdal (28) observed a zinc-responsive redistribution of endogenous Zip3 protein in cultured mouse mammary tumor cells. Without zinc, endogenous mZip3 was localized on the cell surface. Following zinc treatment, the protein was more abundant in intracellular compartments. We can now explain these results at a mechanistic level. Our results may also explain a paradox in the literature regarding the normal location of mammalian Zip1 protein. Human Zip1 was localized to the plasma membrane in non-adherent cells such as K562 erythroleukemia cells but was largely intracellular in adherent cells such as COS-7 and PC-3 despite culturing in the same medium (18, 29) (data not shown). One intriguing explanation for this difference in localization is that perhaps K562 cells lack the machinery required for zinc-stimulated endocytosis. Alternatively, zinc metabolism may differ between cell types such that K562 cells are zinc-deficient even when grown in basal medium.

The mechanisms of zinc sensing that controls mZip1, mZip3, or mZip4 endocytosis are currently unknown. For example, our studies do not distinguish between sensing of intracellular or extracellular pools of zinc. TPEN is a membrane permeable chelator and therefore will perturb both intracellular and extracellular pools of zinc. Although the effects of incubation in Chelex-treated medium may initially suggest that sensing of extracellular zinc is involved, it must be recognized that intracellular pools of labile zinc are likely to be very small (30). Thus, incubation in Chelex-treated medium may be sufficient to rapidly deplete intracellular zinc if steady replenishment of those supplies fails to occur for even short periods.

It is also notable that the mZip proteins do not respond to similar degrees of zinc deficiency. The first hint of this difference was the observation that mZip1 and mZip4 were altered in distribution by incubation in Chelex-treated medium whereas mZip3 was unaffected. Only under the more severe conditions of TPEN treatment did surface levels of mZip3 increase. These data argued that stimulation of mZip3 endocytosis requires more zinc than does mZip1 or mZip4. This difference may reflect different zinc requirements of the respective tissues in which they are expressed. Alternatively, they may reflect a two-tiered system of zinc uptake in cells where both proteins are expressed. As these cells became increasing zinc-deficient, mZip1 would first localize to the surface while mZip3 would be mobilized under conditions of greater limitation. mZip1 and mZip3 have similar apparent $K_m$ values for zinc (19) so this regulation would likely control the capacity rather than the affinity of the cellular zinc uptake systems.

In summary, we have established in this report that the subcellular distributions of mZip1 and mZip3, and thereby their activities, are zinc regulated. Given that mZip1 and mZip3 mRNA levels change little in response to zinc status, we propose that this post-translational control is a major element of zinc homeostasis in the many cells in which these proteins are expressed. Furthermore, given that mZip1 and mZip3 are very divergent from mZip4, our results suggest that this post-translational control of zinc uptake activity is a conserved feature of mammalian ZIP proteins.

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