ZIP7 gene (Slc39a7) encodes a zinc transporter involved in zinc homeostasis of the Golgi apparatus

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Running Title: ZIP7 negates zinc accumulation in the Golgi apparatus

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Summary

It has been suggested that ZIP7 (Kc4, Slc39a7) belongs to the ZIP family of zinc transporters. Transient expression of the V5-tagged human ZIP7 fusion protein in CHO cells led to elevation of the cytoplasmic zinc level. However, the precise function of ZIP7 in cellular zinc homeostasis is not clear. Here we report that the ZIP7 gene is ubiquitously expressed in human and mouse tissues. The endogenous ZIP7 was associated with the Golgi apparatus and was capable of transporting zinc from the Golgi apparatus into the cytoplasm of the cell. Moreover, by using the yeast mutant strain Δzrt3 that was defective in release of stored zinc from vacuoles, we found that ZIP7 was able to decrease the level of accumulated zinc and in the meantime to increase the nuclear/cytoplasmic labile zinc level in the ZIP7 expressing Δzrt3 mutant. We showed that the protein expression of ZIP7 was repressed under zinc-rich condition, whereas there were no effects of zinc on ZIP7 gene expression and intracellular localization. Neither did zinc deficiency affect the intracellular distribution of ZIP7 in mammalian cells. Our study demonstrates that ZIP7 is a functional zinc transporter that acts by transporting zinc from the Golgi apparatus to the cytoplasm of the cell.
Zinc plays multiple roles in life processes. It is an essential co-factor for many enzymes and a structural element for many zinc-finger or ring-finger proteins. Therefore, when zinc is deficient, a variety of biochemical processes of the human body become dysfunctional resulting in retarded growth, poor cognitive function, abnormal neuro-sensory function, skin rash, delayed wound healing, hair loss, poor appetite, frequent infections, severe diarrhea, and male hypogonadism (1-3). If not treated, zinc deficiency can be lethal (3).

Zinc homeostasis is maintained by a diverse array of zinc transporters through zinc uptake, intracellular sequestration, restoration, and export (4, 5). Uptake of zinc from the lumen of the small intestine or from the blood circulation of the body is achieved by the members of the ZIP family (ZRT1 and IRT1-like protein) (6). The ZIP proteins are predicted to have eight transmembrane domains with an intracellular histidine-rich loop between transmembrane domains 3 and 4. The loop region of the ZIP protein may play important role in zinc-binding as mutations of these histidine residues in the loop region destroyed zinc transport activity of these ZIP proteins (6). So far, fourteen mammalian members of the ZIP family have been identified through mouse and human genome analyses (http://www.ncbi.nlm.nih.gov). Conversely, zinc efflux is accomplished by the members of the ZnT proteins (zinc transporter) (5). The ZnT proteins are predicted to have six transmembrane domains with a histidine-rich loop between transmembrane domains 4 and 5. This histidine-rich loop of the ZnT proteins may play similar function as the one in the ZIP proteins.

Within the ZnT family, the ZnT1 protein is involved in zinc efflux from the cell while other ZnT proteins (ZnT2-7) are engaged in subcellular zinc sequestration when zinc is abundant (5). The outcome of the ZnT protein function is to reduce the cytoplasmic zinc concentrations to avoid zinc toxicity when zinc is in excess. On the other hand, the ZIP members are essential for
an increase of the cytoplasmic zinc concentrations by enhancement of zinc uptake or release of stored zinc from subcellular compartments to the cytoplasm of the cell when zinc is deficient (6). Indeed, yeast ZRT1 and ZRT2 as well as mammalian ZIP1-5 proteins have been demonstrated to function in zinc uptake (7-15). Although a yeast zinc transporter, ZRT3, was reported to mediate the release of the intracellular compartmentalized zinc into the cytoplasm of the yeast cell (16), the mammalian counterpart is still not known. To identify a possible mammalian zinc transporter(s) that functions in transporting zinc from subcellular compartments to the cytoplasm of the cell, we performed a prediction analysis for subcellular localization of fourteen mammalian members of the ZIP family based on their amino acid sequences using the online PSORT II (http://www.psort.org) program. Among the fourteen mammalian members of the ZIP family, ZIP7 and ZIP8 were predicted to have more than a 50% probability to be localized on the membranes of intracellular organelles. In addition, the likelihoods that ZIP7 and ZIP8 resided on the plasma membrane of the cell were less than 20%.

The mouse ZIP7 (Ke4) gene was discovered while characterizing genes in the major histocompatibility complex on chromosome 17 (17). Human ZIP7 (HKE4), the human homologue, was mapped to the HLA class II region on chromosome 6 (18). The mouse ZIP7 protein has been shown to suppress the iar1 mutant phenotype when it was expressed in the Arabidopsis iar1 mutant plant (19). The IAR1 gene in Arabidopsis encodes a protein with similarity to the members of the ZIP family. Null mutations in the iar1 gene of Arabidopsis led to suppression of the inhibitory effects of several IAA (indole-3-acetic acid)-amino acid conjugates. It was speculated that the IAR1 protein is a metal transporter to carry metal ions (zinc and/or copper) out of intracellular compartments to the cytoplasm of the plant cell (19). The human ZIP7-V5 recombinant protein was detected intracellularly in the transient transfected
CHO (Chinese-hamster ovarian) cells. Moreover, expression of the ZIP7-V5 fusion protein in CHO cells led to an increase of the intracellular free zinc ions as measured with Newport Green, a zinc-specific fluorescent dye (20).

The ZIP8 gene (*LIV-1*) was isolated in an effort to identify the oestrogen-regulated genes in a human breast cancer cell line, ZR-75-1 (21). The mRNA expression of ZIP8 was up-regulated about 4-fold in the presence of 10^{-8} M oestradiol in the culture medium (21). Immunofluorescent microscopy study of recombinant human ZIP8 indicated that ZIP8 was localized to the plasma membrane of the CHO cells (22) which is in disagreement with the prediction of its cellular localization. Presence of an ubiquitin binding site in the ZIP8 protein may explain its predicted localization (22).

Here we describe the functional characterization of the endogenous ZIP7 protein in mammalian cells. Our study demonstrates that the ZIP7 mRNA was abundantly expressed in both human and mouse tissues. The endogenous ZIP7 protein was localized in the Golgi apparatus and the ZIP7 protein transported intracellular zinc from the Golgi apparatus to the cytoplasm of the cell. Overexpression of mouse ZIP7 alleviated compartmental zinc accumulation and resulted in increased labile zinc pool in yeast zrt3 mutant cells (16). In addition, the protein expression of ZIP7 was repressed by zinc and the gene expression of ZIP7 and the intracellular localization of ZIP7 were not affected by zinc availability.

**MATERIALS AND METHODS**

*Northern blot analysis.* The human multiple tissue northern blot was purchased from Clontech, BD Biosciences. The mouse multiple tissue northern blot was prepared using 2 μg of poly (A)^+^ RNA isolated from liver, kidney, spleen, heart, brain, small intestine, and lung of a
C57BL/6J mouse using a FastTrack 2.0 mRNA isolation system (Invitrogen). The blots were probed with the ^32^P-labeled full-length ORF (open reading frame) cDNA fragments purified from either the human or the mouse ZIP7 gene (EST clones BG823375 and BG342480). The hybridization was performed in the ExpressHyb hybridization solution (Clontech, BD Biosciences) at 65°C for 2 hours. The blots were rinsed twice with 2X SSC/0.1% (w/v) SDS solution and then washed twice with 1X SSC/0.1% (w/v) SDS solution at 50°C for 15 minutes. Finally, the blots were washed twice with 0.1X SSC/0.1% (w/v) SDS solution at 50°C for 10 minutes. The blots were exposed to films with intensifying screens at -80°C for overnight.

Western Blot Analysis. The mouse liver and brain tissues isolated from a C57BL/6J mouse were washed with 1X PBS, pH 7.4 and homogenized in a lysis buffer containing 1X PBS, pH 7.4, 1% Nonidet P40, 0.1% SDS, and 0.5% Sodium Deoxycholate. One mini proteinase inhibitor tablet (Roche) and 56 μl of 100 mM PMSF was added into 10 ml of the lysis buffer just before use. The homogenized tissue was then heated at 100°C for 5 minutes and centrifuged at 4°C for 10 minutes. The supernatant was collected and quantified using the Bio-Rad protein assay reagents. 200 μg protein extracts from liver and brain were separated on a 4-20% Tris-HCl gel (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad).

RWPE1 cells were cultured in a Keratinocyte-Serum Free medium supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract (Invitrogen) for 24 hours at 37°C. Cells were then treated with 0, or 75 μM ZnSO4 for another 24 hours at 37°C. Cells were harvested and cell lysates were prepared as previously described (23). 100 μg protein extracts were separated on a 4-20% Tris-HCl gel (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad).
The bacterial lysates containing the GST-ZIP7 fusion protein or the GST protein alone were prepared according to the manufacture’s instructions (Amersham Biosciences). 1 μg of the bacterial lysates was loaded to each well of a 4-20% Tris-HCl gel (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad).

The ZIP7 protein was detected as described previously (23) using an affinity-purified anti-ZIP7 antibody (1:400 dilution) followed by a peroxidase-conjugated secondary antibody (1:2,500-1:20,000) (PIERCE). ZIP7 was visualized by using a SuperSignal west femto kit (PIERCE) and an Alpha Innotech Gel Documentation System (Alpha Innotech). The GST-ZIP7 fusion protein was visualized by an ECL kit (Amersham Biosciences) and the blot was subsequently exposed to a film (Kodak).

**Antibodies.** Rabbit anti-mouse ZIP7 antibody was raised against a synthetic peptide from amino acids of mouse ZIP7 (RRGGNTGPRDGPVKPQS) and affinity-purified (PIERCE). The result from a BLAST search of the SWISSPORT database indicated that this peptide is unique. The monoclonal anti-Myc antibody was purchased from Stressgen. The Alexa 488- or 594-conjugated goat anti-rabbit or anti-mouse antibodies were purchased from Molecular Probes. The peroxidase-conjugated goat anti-rabbit antibody was purchased from PIERCE.

**Cell culture and generation of stable cell lines.** WI-38 (human lung fibroblast cells) and MCF-7 (human mammary gland epithelial cells) were cultured in a high glucose DMEM medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin (basal medium) (Invitrogen). RWPE1 (normal human prostate epithelial cells) was cultured in a Keratinocyte-Serum Free medium supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract (basal medium) (Invitrogen). K-562 (human erythroleukemia cells) was cultured in an Iscove's modified Dulbecco's medium.
containing 10% FBS, 4 mM L-glutamine and 1.5 mg/ml sodium bicarbonate. CHO/FRT/ZnT7 cells were cultured in 1:1 DMEM:F12 (Ham) medium with 15 mM HEPES, 2.5 mM L-glutamine, 2 mg/ml pyridoxine hydrochloride, and 10% fetal bovine serum (FBS) (Invitrogen) (24). The mouse ZIP7 expressing cell line and the control cell line were generated by transfecting pcDNA3.1/ZIP7 or pcDNA3.1 (Invitrogen) into the ZnT7 expressing CHO cells using a LipofectAMINE plus kit (Invitrogen). The stable cell lines were selected and maintained in 1:1 DMEM:F12 medium containing 0.1 mg/ml of hygromycin B and 0.6 mg/ml of G418 (Invitrogen).

**Plasmids.** The EST clone, BG342480, containing a full length of the mouse ZIP7 open reading frame (ORF) was purchased from ResGen (Invitrogen). The ZIP7 ORF sequence was PCR amplified and cloned into the HindIII and XbaI sites of a yeast expression vector, pYES2 (Invitrogen). The resulting plasmid pY-ZIP7 was confirmed by sequencing and used for transformation of yeast mutant stains. The mammalian ZIP7 expressing plasmid, pHM6/ZIP7, was constructed by insertion of the ZIP7 ORF cDNA sequence into the HindIII and EcoRI sites of pHM6 vector (Roche). The plasmid was confirmed by sequencing and used for generation of a stable ZnT7/ZIP7 co-expressing CHO cell line. The yeast ZRE-lacZ reporter plasmid used in this study was kindly given by Dr. David Eide at University of Wisconsin (16). The bacterial GST-ZIP7 fusion protein expressing plasmid, pGEX/ZIP7, was constructed by insertion of the ZIP7 ORF cDNA sequence into the EccRI and XhoI sites of pGEX-4T-3 vector (Amersham Biosciences).

**Immunofluorescent Microscopy.** Immunofluorescent analysis was performed as previously described (23). WI-38, RWPE1, MCF-7, K-562, CHO/FRT/ZnT7, and CHO/FRT/ZnT7/ZIP7 cells were cultured in slide chambers for 48 hours, fixed with 4% paraformaldehyde, and
permeabilized with 0.4% saponin (Sigma). Where indicated, MCF-7 cells were treated with Brefeldin A (5 μg/ml) for the indicated time prior to the fixation. In the study of the effect of zinc on the cellular localization of the ZIP7 protein, WI-38 and RWPE1 cells were treated with 0, or 75 μM ZnSO₄ in the serum-free DMEM (WI-38) or Keratinocyte-Serum Free medium without supplements (RWPE1) for 2 hours prior to the fixation. In the study of the effect of intracellular zinc deficiency on the cellular distribution of ZIP7, WI-38 and RWPE1 cells were treated with 0, 10 μM TPEN \([N,N',N',N''\text{-tetrakis(2-pyridylmethyl)ethylenediamine}, \text{Sigma}]\) or 10 μM TPEN plus 75 μM ZnSO₄ for one hour before fixation (32). The cells were then stained with the polyclonal anti-ZIP7 (1:100 dilution) or monoclonal anti-Myc antibody (1:100 dilution for the ZIP7-Myc fusion protein) followed by Alexa 488- and 594-conjugated goat anti-rabbit or anti-mouse antibody (1:250 dilution), respectively. Photomicrographs were obtained by a Nikon Eclipse 800 microscope with a digital camera.

**Zinquin staining.** Zinquin staining was performed using the pcDNA5/ZnT7 and pcDNA5/ZnT7/pHM6/ZIP7 stably transfected CHO cells. Cells were grown in slide chambers for 48 hours and then treated with 75 μM ZnSO₄ for 3 hours in the DMEM/F12 medium containing 10% chelex-treated FBS (Bio-Rad), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin (Invitrogen). After ZnSO₄ treatment, cells were rinsed 3 times with 1X PBS (pH7.4) and then incubated in the fresh medium containing 5 μg/ml Zinquin ethyl ester (Dojindo) for 2 hours. Cells were washed with 1X PBS (pH7.4), fixed with 4% paraformaldehyde, and permeabilized with 0.4% saponin (Sigma). The ZnT7 and ZIP7 proteins were detected by anti-Myc and anti-ZIP7 antibodies, respectively. Alexa 488- (green color) or 594- (red color) conjugated goat anti-rabbit or anti-mouse antibody (1:250 dilution) were used to visualize the
proteins. The blue fluorescence of ZnQ staining was visualized and photographed using a digital Nikon Eclipse 800 microscope with a C-9051 filter (Nikon).

Yeast stains and culture conditions. The yeast mutants of zinc homeostasis were from Dr. David Eide at University of Wisconsin (Δzrt1), Dr. D. Conklin at Cold Spring Harbor Laboratory (Δzrt3), and ResGen, Invitrogen (Δzrc1 and Δmsc2). Yeast cells were grown in a synthetic defined medium (SD) supplemented with auxotrophic requirements containing either 2% glucose or 2% galactose/1% raffinose.

Measurement of β-galactosidase activity and cell-associated zinc contents. Yeast cells were harvested six hours after growth in the inducible SD medium containing 2% galactose/1% raffinose. The β-galactosidase activity was measured as described previously (25). The total protein contents were determined by the Bio-Rad Protein Assay. The cell numbers were determined by measuring the absorbance of yeast cell suspensions at OD600 nm (A_{600}) and converting to cell number based on a standard curve. The cell-associated zinc was measured by a Vista AX Simultaneous ICP-AES (Varian) using a nitric acid digestion method (26).

Total RNA isolation and cDNA synthesis. RWPE1 cells were grown in 100-mm plates for 24 hours and then treated with either 0 or 75 µM ZnSO₄ for 24 hours before harvesting. The total RNA was purified by a micro total RNA purification kit (Invitrogen). The cDNA was synthesized from 3 µg of total RNA using the SuperScript Choice system (Invitrogen).

Quantitative RT-PCR analysis. cDNA was diluted 2-fold and 2 µl of cDNA was added to a quantitative PCR mixture containing corresponding primer pair and a FAM-labeled TaqMan probe (Applied Biosystems). The quantitative PCR reactions were performed on a PRISM® ABI 7900HT Sequence Detection System (Applied Biosystems) in triplicate and the expression of β-actin (BACT) was used for normalization. The relative changes of gene expression in response
to the ZnSO₄ treatment was calculated using relative quantification as follows: \( \Delta \Delta Ct = \Delta Ct_q - \Delta Ct_{cb} \); where \( Ct \) = the cycle number at which amplification rises above the background threshold, \( \Delta Ct \) = the change in \( Ct \) between two test samples, for example, zinc untreated and treated samples; \( q \) = the target gene, and \( cb \) = the calibrator gene (the calibrator used in this study was BACT). Gene expression is then calculated as \( 2^{-\Delta \Delta Ct} \) (Applied Biosystems).

RESULTS

Expression of ZIP7 in mouse and human tissues. The cDNA and amino acid sequences of human ZIP7 (HKE4) and the comparison of the ZIP7 protein sequence to the other ZIP family members were reported previously by K.M. Taylor et. al. (20). To investigate the role of ZIP7 in mammalian cellular zinc homeostasis, we first examined the mRNA expression level of ZIP7 in mouse and human tissues by Northern blot analysis. As shown in Fig. 1, the mouse ZIP7 cDNA probe hybridized to a band at approximately 2.4 kb in the mRNA samples isolated from liver, kidney, spleen, heart, brain, small intestine, and lung. However, two similar intensive bands of ZIP7 at approximately 2.2 kb and 2.5 kb were detected in all human tissues examined (Fig. 1). The two different ZIP7 transcripts may be generated by an alternative splicing event that occurred in the 5'-UTR region of ZIP7 as two groups of EST clones of ZIP7 differing in 302 bp in their 5'-UTR region have been found in the GenBank EST database. The expression of ZIP7 was abundant in mouse liver and human heart, skeletal muscle, and placenta (Fig. 1). In addition, the mouse and human ZIP7 mRNAs were also detected in many cDNA libraries including embryo, mammary gland, ovary, uterus, cervix, testis, prostate, tongue, larynx, stomach, pancreas, bladder, eye, pituitary, bone, bone marrow, skin, and peripheral nervous system.
(UniGene Clusters Mm .18556 and Hs .278721). Taking together, the ZIP7 mRNA is ubiquitously expressed in mouse and human tissues.

In an effort to detect the endogenous ZIP7 protein, a polyclonal antibody against a synthetic peptide corresponding to the amino acids 296-312 of the mouse ZIP7 protein was raised in rabbit. The selected peptide sequence of the mouse ZIP7 protein was assured to be unique among the ZIP proteins. The resulting rabbit antiserum was affinity-purified with the same peptide used for raising the antibody. 200 µg of protein extracts from liver and brain were analyzed using anti-ZIP7 antibody by western blot assay. As shown in Fig. 2a, a protein band migrating at approximately 56 kDa was detected in the mouse tissues of liver and brain. These protein bands were not seen when preimmune serum were applied (data not shown). The apparent molecular mass of ~56 kDa is consistent with the calculated molecular mass of mouse ZIP7 (~51 kDa).

The specificity of the newly synthesized anti-ZIP7 antibody was validated by a western blot analysis using protein extracts from the bacteria expressing either the GST-ZIP7 fusion protein or the GST protein alone. A protein band (~88 kDa) in agreement with the predicted molecular mass of the GST-ZIP7 fusion protein (~81 kDa) was detected by the anti-ZIP7 antibody in the bacterial lysate containing the GST-ZIP7 fusion protein whereas no protein band was detected in the bacterial lysate containing the GST protein alone (Fig.2b). Taking together, the results indicate that the newly synthesized anti-ZIP7 antibody specifically reacts with the ZIP7 protein.

Localization of ZIP7 to the Golgi apparatus in mammalian cells. Intracellular zinc homeostasis is maintained by the physiological processes that include zinc uptake, subcellular organelle zinc sequestration and restoration, and zinc export. The members of the ZIP family have been demonstrated to be involved in zinc uptake and in the release of stored zinc into the cytoplasm of cells when zinc is deficient. In yeast, ZRT1 and ZRT2 function as zinc uptake
proteins while ZRT3 functions as a zinc transporter to release stored zinc into the cytoplasm of the yeast cell. In mammalian cells, the ZIP1-5 proteins have been reported to function as zinc uptake proteins. However, the potential mammalian counterpart(s) of the yeast ZRT3 has not been identified. Previous studies from others demonstrated that the ZIP7-V5 fusion protein resided intracellularly in the transiently transfected CHO cells (20). In order to determine the precise subcellular localization of the endogenous ZIP7 in mammalian cells, human cells including human lung fibroblasts (WI-38), human prostate epithelial cells (RWPE1), human erythroleukemia cells (K-562), and human mammary gland epithelial cells (MCF-7) were examined using immunofluorescent microscopy analysis. As shown in Fig. 3a, the majority of the anti-ZIP7 antibody-stained fluorescence clustered at the perinuclear regions of WI-38, RWPE1, K-562, and MCF-7 cells. This subcellular localization of ZIP7 resembles that of GM130 (cis-Golgi matrix protein), a Golgi marker (27). These results suggested that ZIP7 was localized to the Golgi apparatus. To confirm the localization of ZIP7 in the Golgi apparatus, we treated the cultured MCF-7 with Brefeldin A (BFA), a fungal macrocyclic lactone known to disrupt the Golgi apparatus, prior to the immunofluorescent staining. As shown in Figure 3b, the perinuclear staining of ZIP7 in the MCF-7 cells (panel A) diffused into the cytoplasm and formed a network staining pattern after 30 minute treatment (panel B). Removal of BFA after 30 minute treatment followed by the incubation of cells in the fresh medium for an hour restored the normal localization of ZIP7 (panel C), strongly suggesting that ZIP7 is associated with Golgi apparatus.

Negation of zinc accumulation in the Golgi apparatus by ZIP7. The similarity of ZIP7 to the known zinc-uptake proteins and its cellular localization made ZIP7 a compelling candidate as a zinc transporter functioning in zinc delivery from the Golgi apparatus to the cytoplasm of cells.
To test this hypothesis, we took advantage of a ZnT7-Myc expressing CHO cell line that we previously generated for studying the function of ZnT7 (24). Our previous study demonstrated that ZnT7 was localized in the Golgi apparatus and was able to transport zinc ions from the cytoplasm to the Golgi apparatus in the ZnT7 expressing CHO cells. We reasoned that if ZIP7 was able to negate the function of ZnT7, the accumulation of zinc in the Golgi apparatus of the ZnT7/ZIP7 co-expressing CHO cells would be decreased when compared to that of the ZnT7 expressing CHO cells. To visualize the effect of ZIP7 on ZnT7-mediated zinc accumulation, CHO cells expressing ZnT7 only or ZnT7/ZIP7 were co-cultured in a slide chamber for 48 hours and treated with 75 μM ZnSO₄ for 3 hours followed by Zinquin treatment for 2 hours before Immunofluorescent staining. These cells were then co-stained with mouse anti-Myc and rabbit anti-ZIP7 antibodies. The ZnT7 protein was visualized by an Alexa 594-conjugated anti-mouse antibody (red fluorescence) (Fig. 4, panel A) while the ZIP7 protein was recognized by an Alexa 488-conjugated anti-rabbit antibody (green fluorescence) (Fig. 4, panel B). Zinquin staining (blue fluorescence) was displayed in Fig. 4, panel C. The images demonstrated in Fig. 4 were captured from the same view using different color filters. Consistent with previous observations (24), cells expressing ZnT7 only was able to accumulate zinc ions in the Golgi apparatus evidenced by the bright-blue fluorescence around the nuclei in the ZnT7 expressing CHO cells (Fig. 4, panel A and C) after 3 hours of 75 μM ZnSO₄ treatment. However, little to no blue fluorescence was observed in the ZnT7/ZIP7 co-expressing CHO cells (Fig. 4, panel A-C).

Taken together, the results suggest that ZIP7 is a zinc transporter that mediates the transport of zinc from the Golgi apparatus to the cytoplasm of the cell.

Effects of overexpression of mouse ZIP7 on yeast zinc homeostasis. The yeast Zrt3 gene encodes a protein that transports zinc ions from the vacuole to the cytoplasm of the cell (16). The
Zrt3 gene is not essential for yeast as the zrt3 mutant is viable. However, null mutation of the Zrt3 gene results in a low labile nuclear/cytoplasmic zinc pool in the yeast cell accompanied by a high zinc accumulation in the vacuole (16). The great degree of functional conservation between mammalian ZIP7 and yeast ZRT3 promoted us to examine the effects of ZIP7 expression on yeast zinc homeostasis of the zrt3 mutant cells. We first examined the effects of the ZIP7 expression in the yeast zrt3 mutant cells on the labile zinc pool by indirectly measuring the activity of a zinc responsive element (ZRE) binding protein, Zap1p (28). The activity of Zap1p can be quantified by using a ZRE-lacZ reporter in which a β-galactosidase gene is under the control of the ZREs (16). When the nuclear/cytoplasmic labile zinc pool is low, the transcriptional activity of Zap1p is up-regulated resulting in a higher β-galactosidase activity. The zrt3 mutant cells bearing both ZRE-lacZ reporter plasmid and ZIP7 expressing plasmid or a vector control were grown in a standard minimal medium (SD) overnight and then grown in an inducible minimal medium containing 2% galactose/1% raffinose for six hours. The cells were then harvested and the β-galactosidase activity was determined. As shown in Fig. 5a, a higher β-galactosidase activity was observed in the control zrt3 mutant cells and the enzyme activity was suppressed by adding 100 μM ZnCl2 into the culture medium. The expression of ZIP7 in the zrt3 mutant cells resulted in 95% decrease of β-galactosidase activity compared to that in the control yeast zrt3 mutant cells. Addition of zinc into the culture medium did not further suppress the expression of the ZRE-lacZ gene in the ZIP7 expressing zrt3 mutant cells (Fig. 5a).

We next determined the effect of ZIP7 overexpression on zinc accumulation in the zrt3 mutant cells. We found that the control zrt3 mutant cells cultured in the medium with or without extra zinc had accumulated higher levels of zinc compared to that in the ZIP7 expressing zrt3 mutant cells (Fig. 5b). It was also noted that a greater reduction of cell-associated zinc was
observed in the ZIP7 expressing *zrt3* mutant cells when these cells were cultured in the medium containing 100 μM ZnCl₂. Taken together, these results are consistent with a role for ZIP7 in release of stored zinc into the cytoplasm of the cell.

Finally, we examined the effect of overexpression of ZIP7 in the *zrt3* mutant on cell growth. We found that the ZIP7 expressing Δ*zrt3* cells grew slower than the control in the inducible minimal medium (Fig. 6). Interestingly, the growth of the ZIP7 expressing Δ*zrt3* cells was completely inhibited by 1 mM zinc while the growth of the control cells was not affected. The growth inhibition of the ZIP7 expressing Δ*zrt3* cells in the presence of 1 mM zinc did not result from the fortuitous effect of overexpression of the ZIP7 protein as overexpression of ZIP7 in the *zrt1*, *zrc1*, and *msc2* mutant cells (7, 29-31) had no effects on growth of these cells cultured in the medium with or without zinc added (Fig. 6).

*Regulation of ZIP7 by zinc.* Mammalian ZIP proteins are known to be regulated at transcriptional and post-translational levels by zinc (13, 32-34). To study the regulation of ZIP7 expression by zinc, we examined the gene and protein expression of ZIP7 in RWPE1 cells. In this experiment, human prostate cells were grown in the basal medium to 70% confluence and then treated with 0 or 75 μM ZnSO₄ for 24 hours before harvest. The gene expression levels of ZIP7 were determined using real-time quantitative RT-PCR analysis with TaqMan probes specific to the human ZIP7 gene (Applied Biosystems). The gene expression of ZNT1 was also monitored as it is known that its transcription is up-regulated by zinc. As shown in Fig. 7a, the mRNA expression levels of ZIP7 were similar between zinc untreated and treated RWPE1 cells. However, the expression of ZNT1 mRNA was up-regulated about 13-folds in response to the zinc treatment. The protein expression levels of ZIP7 were determined by western blot analysis. As shown in Fig 7b, the level of ZIP7 showed a dramatic decrease in RWPE1 cells treated with
75 μM ZnSO₄ for 24 hours compared to those in the mock-treated cells. In addition, the human ZIP7 protein in prostate epithelial cells was detected with higher molecular mass (~79 kDa) compared to the mouse ZIP7 protein in the brain tissue (~56 kDa), suggesting that the ZIP7 protein may be post-translationally modified in RWPE1 cells. Tissue specific post-translational modifications were previously seen in other zinc transporter proteins, such as, ZnT6 and ZnT7 (23, 24).

To test whether the subcellular localization of ZIP7 was affected by zinc, we compared the localization of ZIP7 in human lung fibroblast cells (WI-38) and human prostate cells (RWPE1) treated with 0 or 75 μM ZnSO₄ in the serum-free DMEM (WI-38) or Keratinocyte-Serum Free medium without supplements (RWPE1) for 2 hours before cell staining. The zinc contents for the serum-free DMEM and Keratinocyte-Serum Free medium without supplements were about 0.5 μM determined by a Vista AX Simultaneous ICP-AES (Varian). Consistent with our earlier observations (Fig. 3), the majority of the ZIP7 protein exhibited perinuclear staining patterns in the zinc-treated and -untreated WI-38 and RWPE1 cells (Fig. 7c). Next, we examined the effect of zinc deficiency on the intracellular distributions of ZIP7 in WI-38 and RWPE1 cells. WI-38 and RWPE1 cells were cultured in the basal media for 24 hours. 10 μM TPEN, a known membrane-permeable zinc chelator, or 10 μM TPEN plus 75 μM ZnSO₄ was then added the culture medium for 1 hour at 37°C before immunofluorescent microscopy assay (32). Unlike the other ZIP proteins (13, 32), the zinc limiting condition induced by TPEN treatment did not alter the intracellular distribution of ZIP7 in WI-38 and RWPE1 cells (Fig. 7d). Again, no changes in ZIP7 intracellular distributions were observed in the WI-38 and RWPE1 cells under zinc repletion condition (Fig. 7d). Taken together, zinc may regulate the expression of ZIP7 only through a decrease in protein accumulation under zinc excess condition in mammalian cells.
DISCUSSION

In this study, we functionally characterized the mammalian ZIP7 protein. We showed that the expression of the ZIP7 gene was ubiquitous in both human and mouse tissues. The apparent molecular weight of the endogenous ZIP7 protein in mouse liver and brain was ~56 kDa which is consistent with the calculated molecular mass of ZIP7. The human ZIP7 protein may be post-translationally modified as its apparent molecular weight was greater than the calculated molecular mass in human prostate cells. The ZIP proteins have been implicated to play role in raising the cytoplasmic zinc by transporting zinc out of the intracellular compartments. We showed that the endogenous ZIP7 protein was localized in the Golgi apparatus of mammalian lung fibroblasts, human erythroleukemia cells, mammary epithelia, and prostate epithelia. The ZIP7 protein was able to negate the zinc accumulation in the Golgi apparatus caused by overexpression of ZnT7 in CHO cells. Moreover, overexpression of ZIP7 in the yeast zrt3 mutant that is defective in release of stored zinc from vacuoles to the cytoplasm led to an increase of the nuclear/cytoplasmic labile zinc pool and at the same time leading to a decrease of the total cell-associated zinc content. The effects of ZIP7 on the zrt3 mutant was specific as there was no effect on the nuclear/cytoplasmic labile zinc pool when the ZIP7 protein was expressed in other yeast mutants of zinc homeostasis including Δzrt1, Δzrc1, and Δmsc2 (data not shown). These findings strongly indicate that ZIP7 is a zinc transporter involved in translocation of zinc from the Golgi apparatus into the cytoplasm of the cell. Although the zinc transporter (ZRT3) functioning in the release of compartmentalized zinc into the cytoplasm of the cell has been described in yeast, this is the first description of such a transporter in the mammalian cell.
Our study also indicates that zinc is important for the normal function of the Golgi apparatus. Constitutive overexpression of ZIP7 in mammalian cells, such as CHO cells, disturbed cell function as it was evidenced by gradual loss of the ZIP7 expressing cells and increase of non-ZIP7 expressing cells under the normal culture conditions in the stable ZIP7 expressing CHO cells and the ZnT7/ZIP7 co-expressing CHO cells (data not shown). In addition, although overexpression of ZIP7 alleviated the zinc accumulation phenotype of the yeast zrt3 mutant, it made the zrt3 mutant zinc sensitive. We speculate that in the ZIP7 expressing zrt3 mutant cells, the compartmentalized zinc was constitutively exported into the cytoplasm. The depletion of zinc from the intracellular compartments including the Golgi apparatus and vacuoles and the down-regulation of the zrc1 gene caused by the increased cytoplasmic zinc concentration made the ZIP7 expressing zrt3 mutant cells sensitive to the environment zinc (29, 30). Moreover, no effect of ZIP7 on the growth of zrt1, zrc1, and msc2 mutant cells was observed, supporting that the higher labile zinc pool and/or depletion of zinc from intracellular organelles may be the causes for the slow growth of the ZIP7 expressing zrt3 mutant cells.

Studies have indicated that the expression of the ZIP proteins responds to intracellular zinc concentrations (13, 32-34). For example, the expression of ZIP4 is down-regulated under zinc excess condition at transcriptional and post-translational levels (32, 33). Our study indicates that the expression of the ZIP7 protein was repressed 24 hours after addition of zinc ions into the culture medium (Fig. 7b) which is consistent with regulatory patterns for the ZIP proteins by zinc. However, this protein level change was not apparent following 1-2 hours of zinc treatment (Fig. 7c and d). In addition, the mRNA expression of ZIP7 did not change under either zinc limiting or excess condition (Fig. 7a) which is similar to that of ZIP5 (33). Furthermore, the subcellular localization of the ZIP7 protein was not regulated by the intracellular zinc availability (Fig. 7c
and d), suggesting that ZIP7 may be a constant resident of the Golgi apparatus. The observed patterns of regulation and localization of ZIP7 strongly support that ZIP7 plays a role in mobilizing zinc from the Golgi apparatus to the cytoplasm under zinc limiting condition.

Over the past a few years, numerous discoveries at the molecular level have given us an insight into the mechanism of how the mammalian cell maintains zinc homeostasis under different conditions. It has been demonstrated that in mammalian cells zinc is brought in by zinc uptake proteins including ZIP1, ZIP2, ZIP3, ZIP4, and ZIP5 when the level of cytoplasmic zinc decreases (9, 11, 13-15). The available cytoplasmic zinc can then be used as co-factors for many metalloprotiens. In the meantime, zinc is transported into the specialized vesicular compartments for protein synthesis, protein trafficking, neuronal signal transmission, secretion, and storage. These processes are accomplished by a series of ZnT protein including ZnT2, ZnT3, ZnT4, ZnT5, ZnT6, and ZnT7 (23, 24, 35-38). The zinc efflux protein, ZnT1, exports zinc out of the cell when the cytoplasmic zinc concentration rises (39, 40). Little is known about zinc release from storage into the cytoplasm for use when zinc is limited. The discovery that ZIP7 is involved in zinc export from the Golgi apparatus into the cytoplasm of the cell has advanced our knowledge of mammalian zinc homeostasis.

FOOTNOTE

This work was supported by the United States Department of Agriculture (CRIS-5306-53000-008-00D). 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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The abbreviations used are: ZnT, zinc transporter; ZIP, ZRT1 and IRT1-like protein; Zrt, zinc-regulated transporter; zrc, zinc resistance conferring; Msc2, meiotic sister-chromatid recombination; TGN, trans Golgi network; ORF, open reading frame; EST, expressed sequence tag; BFA, brefeldin A. FBS, fetal bovine serum; CHO, Chinese hamster ovary; WI-38, human lung fibroblast cells.; MCF-7, human mammary gland epithelial cells; RWPE1, normal human prostate epithelial cells; FBS, fetal bovine serum; PBS, phosphate buffered saline; ZRE, zinc-responsive element.

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FIGURE LEGEND

Figure 1. Expression of ZIP7 in human and mouse tissues. Northern blot membranes were hybridized with human or mouse ZIP7 cDNA corresponding to the ORF sequences. 1 μg or 2 μg of poly (A)+ RNA from human and mouse tissues, respectively, were loaded in each lane. The position of the RNA size marker is indicated.
Figure 2. Expression of the ZIP7 protein.  

a. Detection of ZIP7 in mouse tissues. Western blot containing 200 \( \mu \)g of protein extracts isolated from mouse liver and brain (C57BL/6J) was probed with a rabbit anti-ZIP7 antibody followed by a peroxidase-conjugated secondary antibody. ZIP7 was visualized using a SuperSignal west femto kit (PIERCE).

b. Detection of the GST-ZIP7 fusion protein. Western blots containing 1 \( \mu \)g of bacterial lysates was probed with the anti-ZIP7 antibody followed by a peroxidase-conjugated secondary antibody. GST-ZIP7 was detected by an ECL kit (Amersham Biosciences) and visualized by exposing the blot to a film (Kodak). The protein markers (Bio-Rad) are shown.

Figure 3. Subcellular localization of ZIP7.  

a. Detection of the ZIP7 protein. The endogenous ZIP7 protein in WI-38, RWPE1, K-562, and MCF-7 cells was detected by an affinity-purified anti-ZIP7 antibody (1:100 dilution) followed by a Alexa 488-conjugated goat anti-rabbit antibody (1:250 dilution) (Molecular Probes).

b. The effect of BFA on the subcellular localizations of the endogenous ZIP7 protein in MCF-7. The MCF-7 cells were grown for 48 hours and then incubated with 5 \( \mu \)g/ml BFA for 0 or 30 min at 37\(^{\circ}\)C (panel A and B). As indicated, the BFA treated MCF-7 cells were further incubated with fresh medium at 37\(^{\circ}\)C for 60 min (panel C). The perinuclear ZIP7 staining (panel A) dispersed into punctuate structures (panel B) after 30 min of BFA treatment. The BFA effect was reversed after removal of BFA in the culture medium (panel C). An Alexa 488 conjugated goat anti-rabbit antibody was used as the secondary antibody for the photomicrographs.

Figure 4. Zinquin fluorescent staining of ZnT7-Myc/ZIP7 co-expressing CHO cells. The ZnT7-Myc expression and ZnT7-Myc/ZIP7 co-expressing CHO cells were co-cultured at 37\(^{\circ}\)C for 48
hours before zinc treatment. Cells were then incubated in the medium containing 75 μM ZnSO₄ for 3 hours followed by incubation of cells in the fresh medium containing 5 μg/ml Zinquin for 2 hours. Cells were washed, fixed, and permeabilized. The ZnT7-Myc fusion protein was detected by a monoclonal anti-Myc antibody (1:100 dilution) (Stressgen) (panel A). The solid arrows indicate the ZnT7 only expressing CHO cells. The ZIP7 protein was detected by an affinity-purified anti-ZIP7 antibody (panel B). The open arrows indicate the ZnT7/ZIP7 co-expressing CHO cells. The accumulated zinc ions were detected by zinquin staining (panel C). An Alexa 594 conjugated goat anti-mouse (red fluorescent image) or an Alexa 488-conjugated anti-rabbit (green fluorescent image) antibody was used as the secondary antibodies for the photomicrographs. The fluorescent images were captured by Nikon Eclipse 800 microscope with the color filters designated for red, green, and blue fluorescence.

Figure 5. Effects of overexpression of mouse ZIP7 in the yeast zrt3 mutant. The zrt3 mutant cells transformed with either an empty vector (pYES2) or a ZIP7 expressing plasmid, pY/ZIP7, were further transformed with the pDg2 ZRE-lacZ reporter plasmid. The yeast cells were grown in SD medium overnight and then washed and grown in the inducible medium containing 0 or 100 μM ZnCl₂ for 6 hours. Cells were harvested and assayed for β-galactosidase activity (a) and total cell-associated zinc (b). Results of β-galactosidase activity were the mean ± S.D. of four independent experiments. Results of zinc accumulation assay were the mean ± S.E. of two independent experiments.
Figure 6. Effect of overexpression of mouse ZIP7 on the growth of yeast mutants of zinc transporters. zrt1, zrt3, zrc1, and msc2 mutant cells were transformed with either an empty vector, pYES, or a ZIP7 expressing plasmid, pY-ZIP7. Yeast cell were grown on the inducible synthetic defined-ura medium (2% galactose and 1% raffinose) containing either 0 or 1.0 mM ZnCl₂ at 30°C for 3 days.

Figure 7. Regulatory effects of zinc on ZIP7 expression and cellular localization. a. Regulation of ZIP7 gene expression. RWPE1 cells were grown for 24 hours and treated with 0 or 75 μM ZnSO₄ for 24 hours. Cells were harvested and total RNA was isolated. The gene expression of ZIP7 and ZNT1 were measured by quantitative RT-PCR with TaqMan probes. The gene expression was normalized to the expression of β-actin in each sample. The changes in response to the zinc treatment were calculated relative to the expression for each gene in 0 μM ZnSO₄-treated RWPE1 cells using the comparative C_T method (Applied Biosystems). Values are the means ±SD. b. Regulation of ZIP7 protein levels. RWPE1 cells were grown for 24 hours and treated with 0 or 75 μM ZnSO₄ for 24 hours. Cell lysates were then prepared and analyzed by western blot assay. The human ZIP7 proteins were detected by a polyclonal anti-ZIP7 antibody and visualized by a peroxidase-conjugated anti-rabbit antibody followed by application of a SuperSignal west femto kit (PIERCE). The mouse brain lysate was used as a positive control. Protein markers are shown. c. Regulation of cellular localization of ZIP7 by zinc. WI-38 and RWPE1 cells were grown for 24 hours and exposed to basal medium containing 0, 10 μM ZnSO₄, or 75 μM ZnSO₄ in the serum-free DMEM (WI-38) or Keratinocyte-Serum Free medium without supplements (RWPE1) for 2 hours before fixation. d. Regulation of cellular localization of ZIP7 in zinc-limited cells. WI-38 and RWPE1 cells were grown for 24 hours and exposed to basal medium containing 0, 10 μM
TPEN, or 10 μM TPEN plus 75 μM ZnSO₄ for 1 hour before fixation. The human ZIP7 proteins were detected by a polyclonal anti-ZIP7 antibody and visualized by an Alexa 488-conjugated anti-rabbit antibody.
REFERENCES


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Figure 1
Figure 2

- **a.**
  - Size Markers
  - Brain
  - Liver
  - kDa.
    - 120
    - 100
    - 56
    - 38

- **b.**
  - GST-ZIP7
  - GST
  - kDa.
    - 91
    - 51.4
    - 34.7

- GST-ZIP7
- ZIP7

Figure 2
Figure 3
Proteins detected

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<th>ZnQ</th>
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Figure 4
Figure 5
Figure 6

0 1.0 mM ZnCl$_2$
a. Figure 7a

b. Figure 7b

Figure 7 a&b
c. WI-38 RWPE1

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d. WI-38 RWPE1

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<td>10 µM TPEN + 75 µM ZnSO4</td>
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Figure 7 c&d
ZIP7 gene (Slc39a7) encodes a zinc transporter involved in zinc homeostasis of the golgi apparatus

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