Functional Characterization of a Novel Mammalian Zinc Transporter, ZnT6*

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We describe ZnT6, a new member of the CDF (cation diffusion facilitator) family of heavy metal transporters. The human ZNT6 gene was mapped at 2p21–22, while the mouse Znt6 was localized to chromosome 17. Overexpression of ZnT6 in both wild-type yeast and mutants that are deficient in cytoplasmic zinc causes growth inhibition, but this inhibition is abolished in mutant cells with high cytoplasmic zinc. ZnT6 may function in transporting the cytoplasmic zinc into the Golgi apparatus as well as the vesicular compartment, as evidenced by its overlapping intracellular localization with TGN38 and transferrin receptor in the normal rat kidney cells. We also demonstrate that the intracellular distributions of ZnT6 as well as ZnT4 are regulated by zinc in the normal rat kidney cells. The results from this report, combined with those from other studies, suggest that the intracellular zinc homeostasis is mediated by many ZnT proteins, which act in tissue-, cell-, and organelle-specific manners.

Zinc is an essential trace element required for the structural stability of a variety of proteins involved in transcription and protein trafficking as well as for the catalytic activity of metalloenzymes such as pancreatic carboxypeptidases, alkaline phosphatase, various dehydrogenases, and superoxide dismutase (1). In mammals, zinc is absorbed through the brush border of small intestinal mucosa from diet and transported through blood to the tissues and cells where zinc is needed (2).

Two families of zinc transporter proteins, ZnT1 (zinc transporter) and ZIP (ZRT1/-zinc-regulated transporter) and IRT1 (iron-regulated transporter)-like protein have been identified in mammals, (3–8). The ZnT proteins, which are the members of the CDF family (cation diffusion facilitator), appear to function either by transporting zinc out of cells or by sequestering zinc into intracellular compartments, (3–6, 9, 10). In contrast, the ZIP proteins appear to function in uptake of zinc into the cytoplasm (7, 8, 11).

Four ZnT proteins, ZnT1–ZnT4, have been identified. In general, the ZnT proteins are predicted to have similar protein structures with features including six transmembrane domains and a histidine-rich cytoplasmic loop between transmembrane domain IV and V. ZnT1 is expressed in many tissues, and the protein resides on the plasma membrane. Overexpression of ZnT1 in zinc-sensitive cells confers zinc resistance probably by direct export of zinc out cells (3). ZnT2 and ZnT3 are more similar to each other than they are to ZnT1. They are located on the vesicular membranes and involved in transporting zinc from the cytoplasm into vesicles. The expression of ZnT2 has been demonstrated in tissues including small intestine, kidney, seminal vesicles, testis, and placenta. However, the expression of ZnT3 was detected only in brain and testis (4, 5). ZnT4 is more related to ZnT2 and ZnT3 than ZnT1, and it appears to be expressed ubiquitously with abundant expression in brain, mammary gland, and small intestine. ZnT4 confers zinc resistance to Δzrc1, a yeast strain that has a defect in a vacuolar zinc transporter (ZRC1) (zinc resistance conferring), suggesting that it is involved in sequestration of cytoplasmic zinc into vacuoles when expressed in yeast (6). In addition, ZnT4 is deficient in the lethal milk mouse mutant in which pups of any genotype suckled on homozygous lethal milk mothers die of zinc deficiency before weaning (12, 13). The zinc level in the milk of homozygous lethal milk animals is about 50% that of normal animals (14, 15) demonstrating that ZnT4 plays a crucial role in depositing the cytoplasmic zinc into the secretory vesicles in the lactating mammary glands.

Yeast mutants of zinc metabolism have provided useful tools to analyze the function of mammalian zinc transporters. Recent studies have been shown that at least five zinc transporters of Saccharomyces cerevisiae are involved in yeast zinc homeostasis. The ZRT1 and ZRT2 genes encode the high and low affinity zinc importers, respectively (16, 17). The ZRC1 and ZRT3 genes encode the zinc transporters responsible for zinc movement from the cytoplasm to vacuoles and from vacuoles to the cytoplasm, respectively (18, 19). And the Msc2 (meiotic sister-chromatid recombination) gene encodes a zinc transporter that may act in transporting zinc out of the endoplasmic reticulum/nucleus (20).

Here we describe the identification and characterization of a sixth member of the ZnT family, which we designate as ZnT6. We provide evidence that ZnT6 functions as a zinc transporter responsible for relocating the cytoplasmic zinc into the trans Golgi network (TGN) as well as the vesicular compartment. We also show that the intracellular localizations of ZnT6 and ZnT4 in the normal rat kidney (NRK) cells are regulated by zinc.
Characterization of the Mammalian ZnT6 Zinc Transporter

MATERIALS AND METHODS

Cell Line and DNAs—NRK cells were cultured in Dulbecco’s modified eagle medium (DMEM) with 4.5 g/liter glucose, 25 mM HEPES, and 10% fetal bovine serum (FBS) (Invitrogen). CHO cells were maintained in 1:1 DMEM:F12 (Ham) medium with 15 mM HEPES, 2.5 mM l-glutamine, 2 mg/ml pyridoxine hydrochloride, and 10% FBS (Invitrogen). The pcDNA5/FRT or pcDNA5/ZnT6-Myc-transfected CHO cells were cultured in the same medium as described above plus 100 μg/ml of hygromycin B (Invitrogen). Expressed sequence tag (EST) clones were purchased from ResGen (Invitrogen).

Yeast Strains and Culture Conditions—The yeast null-mutant of Δzrt1 was a gift from Dr. David Eide at University of Missouri-Columbia, Columbia, MO. The yeast deletion strains Δznt3 and Δmsc2 were purchased from ResGen (Invitrogen). Δzrc1 was kindly provided by Dr. Conklin at University of Missouri—Columbia (Cold Spring Harbor Laboratories). Wild-type yeast BY4741 (MATa his3/D1 leu2D0 met15D0 ura3D0) was purchased from ResGen. Yeast strains were grown in synthetic defined medium supplemented with auxotrophic requirements and either 2% glucose or 2% galactose and 1% raffinose.

Plasmid Construction—The entire open reading frame (ORF) sequence of mouse ZnT6 was PCR-amplified using primers with a HindIII site incorporated immediately before the methionine codon and an XbaI site immediately after the stop codon. The mouse EST clone AA386648 was used as the template for the PCR amplification. The plasmid pY-ZnT6 was generated by digestion and insertion of the amplified sequence into the HindIII and XbaI sites of a yeast expression vector, pYES2 (Invitrogen). The expression plasmid, pcDNA5/ZnT6-Myc, which was used to transfect CHO cells, was made by several cloning steps. The ORF sequence, lacking a stop codon, was cloned into the HindIII site of the yeast expression vector, pYES2, and after digestion and insertion of the amplified sequence, the pcDNA5/ZnT6-Myc was sequenced and transfected into CHO cells alone with pOG44 (Flp isolator). The pcDNA5/ZnT6-Myc-transfected CHO cells were cultured in the same medium as described above plus 100 μg/ml of hygromycin B (Invitrogen). Expressed sequence tag (EST) clones were purchased from ResGen (Invitrogen).

Northern blot analysis was performed essentially as described (21). NRK cells were cultured in slide chambers for 24 or 48 h, fixed with 4% paraformaldehyde, and permeabilized with 0.4% saponin (Sigma). Where indicated, the cells were treated with ZnSO4 for 2 h prior to fixation. The cells were subsequently stained with an affinity-purified anti-ZnT6 (1:200 dilution) or anti-ZnT4 (1:50 dilution) antibody followed by an Alexa 488-conjugated goat anti-rabbit antibody (1:250 dilution). In the brefeldin A treatment and colocalization studies, the NRK cells were co-transfected with an anti-ZnT6 and an anti-TGN38 (1:200 dilution) antibodies followed by an Alexa 488-conjugated goat anti-rabbit antibody and an Alexa 594-conjugated goat anti-mouse antibody (1:250 dilution), respectively. Photomicrographs were obtained by Nikon Eclipse 800 microscope with a digital camera.

RESULTS

Identification of ZnT6—Search of the EST data bases with the amino acid sequence of mouse ZnT4 uncovered several mouse and human EST clones predicted to encode a protein similar to the members of the ZnT family. The mouse EST clone AA386648 was purchased from ResGen (Invitrogen) and fully sequenced. This clone contains a single open reading frame encoding a 460-amino acid protein with a calculated molecular mass of 51 kDa. Based on the predicted amino acid sequence similarity to the members of the ZnT family (32%, 33%, and 35% amino acid identities to rat ZnT2, mouse ZnT3, and mouse ZnT4, respectively), we designated this novel gene as ZnT6 (Slc30a6) and the gene product as ZnT6. ZnT6 shares many features described previously for the ZnT family, including six predicted transmembrane domains, cytoplasmic N- and C-terminal domains as well as a loop region between transmembrane domain IV and V (Fig. 1). However, in contrast to other ZnT proteins, the ZnT6 loop lacks multiple histidine residues, the potential ligands for zinc, while retaining serine residues, a finding reminiscent of CZCD, a prokaryotic transporter of zinc, cadmium, and cobalt (22) (Fig. 1). Another distinguishing feature of ZnT6 is its longer C terminus.

The human ZN7 gene encodes the same size protein (460 amino acids) as that of the mouse and has 92% amino acid identity to ZnT6. Data base analysis revealed that two BAC clones (accession nos. AL121653 and AL121658) from human chromosome 2p21–22 contain the ZnT6 gene. The ZN76 gene has at least 14 exons, and the genomic sequence spans about 56 kb. The locus of the mouse ZnT6 sequence is on chromosome 17, a region with homology by synteny (The Mouse Genome Sequencing Consortium). No human or mouse disease of suspected zinc metabolism maps to these loci.

By Northern blot analysis, two ZnT6 transcripts of 2.4- and 1.5-kb size were detected in the mouse liver, kidney, intestine, brain, and heart. The data suggest that ZnT6 may be expressed in at least these organs.
1.7-kb are revealed in mouse tissues, with relatively abundant expression in liver and brain (Fig. 2a). The expression of the 1.7-kb transcript is higher than that of the 2.4-kb in all tissues examined. The two sizes are reflected in two groups of EST clones, derived from mammary gland, skin, and embryo and differing only in their 3' untranslated region. Comparison to the genomic sequence showed that two different polyadenylation signals are utilized. The Western blot analysis confirmed that a single ZnT6 protein of the predicted size (51 kDa) is produced in mouse brain and a much fainter signal is detected in small intestine and kidney (Fig. 2b), which are consistent with the levels of transcription observed on the Northern blot (Fig. 2a). A single protein band of slightly higher molecular mass (55 kDa) was detected in lung, indicating that ZnT6 may be posttranslationally modified in lung tissue (Fig. 2b). No ZnT6 protein band was detected in liver and heart on the Western blot although high level of the ZnT6 mRNA was found in liver on the Northern blot (Fig. 2). The discrepancy between the levels of ZnT6 mRNA and protein expression in lung (less mRNA detected but more protein expressed) and liver (abundant mRNA detected but no protein detected) suggests that a posttranscriptional mechanism may play a role in tissue-specific expression of the ZnT6 protein.

The specificity of the affinity-purified polyclonal anti-ZnT6 antibody was assessed by Western blots of the protein extract from a mouse brain probed with a preimmune serum, and the total protein extract from the CHO cells that were stably transfected with a plasmid expressing ZnT6-Myc fusion protein probed with a monoclonal anti-Myc antibody (Stressgen). No protein of the predicted size (51 kDa) was detected in brain when the preimmune serum was used (Fig. 2b). A protein band of the predicted size (55 kDa) was detected in the CHO cells expressing ZnT6-Myc fusion protein, whereas no protein was detected in the CHO cells transfected with vector alone (Fig. 2b). Taking together, the results indicate that the affinity-purified anti-ZnT6 antibody specifically reacts with the endogenous ZnT6 protein in the mouse tissues.

ZnT6 Acts as a Functional Zinc Transporter—We asked whether ZnT6 has the ability to transport zinc indirectly by looking at the effect of ZnT6 expression in a series of yeast mutants that are defective in zinc metabolism. First, we tested whether ZnT6 can relieve the sensitivity to zinc found in the yeast mutant zrc1, in which zinc accumulates in the cytoplasm due to a defect in a vacuolar zinc transporter ZRC1 (Fig. 1). Whereas ZnT4 was previously shown to rescue the growth of the yeast mutant in high zinc medium (6), ZnT6 failed to complement the zrc1 defect under similar conditions (data not shown). This finding suggests that ZnT6 may not be involved in the transport of the cytoplasmic zinc into the vacuoles, which largely reduces the cytoplasmic zinc concentration in cells.

We then asked whether expression of ZnT6 might exacerbate the cytoplasmic zinc deficiency seen in three yeast mutants:

![Alignment of the amino acid sequences of eight members of the CDF family. Mouse ZnT6 was aligned with mouse ZnT1, ZnT3, ZnT4, rat ZnT2, yeast ZRC1, and COT1 (cobalt ion transporter) (S. cerevisiae) and bacterial Czcd (Alcaligenes eutrophus CH34). The amino acid residues of ZnT6 that are conserved in other proteins are shown in red. The six predicted transmembrane domains are underlined and numbered.](http://www.jbc.org/)

**FIG. 1.** Alignment of the amino acid sequences of eight members of the CDF family. Mouse ZnT6 was aligned with mouse ZnT1, ZnT3, ZnT4, rat ZnT2, yeast ZRC1, and COT1 (cobalt ion transporter) (S. cerevisiae) and bacterial Czcd (Alcaligenes eutrophus CH34). The amino acid residues of ZnT6 that are conserved in other proteins are shown in red. The six predicted transmembrane domains are underlined and numbered.
Δzrt1, which is defective in the yeast high affinity zinc uptake protein, and Δzrt3 and Δmcs2, which have defects in relocation of zinc from the vacuoles and endoplasmic reticulum/nucleus to the cytoplasm, respectively (16, 19, 20). In each mutant, overexpression of ZnT6 resulted in poor to no growth in the inducible media, whereas the mutants transformed with vector alone grew well (Fig. 3a). Furthermore, the inhibitory effect of ZnT6 on yeast growth was observed in the wild-type yeast strain (BY4741) (Fig. 3b). These findings are consistent with the hypothesis that ZnT6 transports zinc out of the cytoplasm into either an intracellular pool or out of the cell, leading to a severe diminution in the cytoplasmic zinc and concomitant growth retardation. Indeed, under inducible media, ZnT6 did not inhibit the growth of Δzrc1 mutant cells (Fig. 3a).

To further test this hypothesis, we asked whether addition of zinc might counter the inhibitory effect of ZnT6 on cell growth. Looking at both wild-type and Δzrt1 strains, growth inhibition can be partially alleviated by adding 1.0 mM ZnCl2 in the inducible media (Fig. 3b and data not shown). However, higher zinc concentrations were no more effective (data not shown). The effect of zinc was specific: addition of iron (0.35 mM FeSO4), copper (0.1 mM CuSO4), cobalt (0.5 mM CoSO4), or manganese (1.0 mM MnCl2) did not help to restore growth in either wild-type or Δzrt1 strains expressing ZnT6 (data not shown).

Immunofluorescence Analysis of ZnT6 in NRK Cells—As each of the previously described ZnT proteins appears to serve a specific cellular zinc transport need, we asked by immunofluorescence analysis whether ZnT6 lies in a particular subcellular compartment in mammalian cells. In the cultured rat NRK cells, ZnT6 displayed a perinuclear location as well as a punctate/tubular pattern throughout the cytoplasm of the cells and underlying the plasma membrane. Localization of ZnT6 was compared with that of TGN38, a known TGN protein and to the TfR, a plasma membrane protein that recycles to the TGN shortly after internalization via recycling endosomes. As shown in Fig. 4, A, D, and G, the perinuclear staining of ZnT6 is completely coincident with that of TGN38, strongly suggesting that ZnT6 is associated with TGN. The tubular/punctuate staining of ZnT6 partially overlaps with that of transferrin receptor, whereas the perinuclear staining of both proteins completely overlaps, indicating that ZnT6 may reside in the vesicles in addition to the recycling endosomes in the NRK cells (data not shown).

Fig. 4, B–I confirms that ZnT6 localizes to the TGN, as an addition of a fungal macrocyclic lactone, brefeldin A (BFA), known to disrupt the Golgi compartment, also disrupts ZnT6 with the same time course as that of TGN38. After 30 min of BFA treatment, the fluorescence perinuclear staining of ZnT6 completely disappears and the signal has diffused into the cytoplasm (Fig. 4B). Meanwhile, BFA treatment causes the fluorescence signal of TGN38 to diffuse into the cytoplasm and form a strong juxtanuclear spot (Fig. 4E). Incubation of BFA-treated cells in BFA-free media for 60 min resulted in nearly complete restoration of the normal localization of both ZnT6 and TGN38 (Fig 4, C, F, and D). These BFA-induced changes and the recovery of the changes by the fresh medium are
characteristic features for the proteins that associate with Golgi compartment.

The Intracellular Location of ZnT6 Is Regulated by Zinc—We next asked whether the expression or distribution of ZnT6 was influenced by zinc. No changes in mRNA or protein abundance were elicited in the NRK cells cultured in the medium containing 200 μM ZnSO₄ for 24 h (data not shown). However, by immunofluorescence analysis, we detected zinc-induced trafficking of ZnT6 at 2 and 24 h after ZnSO₄ treatment in the NRK cells. The panels on the left (A–D) in Fig. 5 show the staining for ZnT6 in the NRK cells exposed to various ZnSO₄ concentrations. After 2 h, the perinuclear capping started to diffuse into the cytoplasm and the punctate/tubular signals were enhanced and distributed toward the periphery of the cells with the addition of an increased amount of zinc (Fig. 5, B–D). We were unable to detect a clear delineation of the plasma membrane staining with the increased zinc concentration in the media.

To investigate whether this zinc-induced protein trafficking is a common phenomenon for the ZnT proteins, we studied the localization of ZnT4 under the same conditions. ZnT4 has been shown to reside in the TGN as well as in the endosomal compartment (Fig. 5E) (10). As shown in Fig. 5, although ZnT4 does redistribute from its perinuclear location to the cytoplasm, it is less sensitive to zinc than ZnT6, with dramatic effects seen only at 200 μM ZnSO₄. Our data demonstrate that although the punctate staining patterns differ between ZnT6 and ZnT4, indicating they may reside in two different vesicular compartments, both proteins are localized in the TGN (Fig. 5, A and E and data not shown), and their intracellular localizations are regulated by extracellular zinc concentration.

DISCUSSION

In this paper, we have functionally characterized the sixth member of the ZnT family. We conclude that ZnT6 is a zinc transporter that translocates the cytoplasmic zinc into the TGN as well as the vesicular compartments based on the evidences from our studies. First, ZnT6 is closely related to several previously characterized ZnT members from mammals, yeast, and bacteria (Fig. 1). Second, ZnT6 was able to suppress the

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growth of several yeast mutants that are defective in accumulation of the cytoplasmic zinc. Third, the higher cytoplasmic zinc content was able to alleviate ZnT6-transformed Δzrt1 from growth inhibition by ZnT6, and the extracellular zinc was able to partially rescue the growth inhibition by ZnT6 in wild-type, Δzrt1, and Δzrt1/Δzrt2 yeast strains (Fig. 3. and data not shown). Last, ZnT6 is localized predominantly in the TGN compartment in NRK cells in the normal culture condition. However, its distribution from the TGN to the vesicular compartment in NRK cells is regulated by extracellular zinc ions in the culture medium.

The zinc-induced redistribution of ZnT proteins is reminiscent of the findlings with a copper export protein described for the Menkes disease protein (MNK), whose relocation from the TGN to the plasma membrane is induced by elevated copper, which in turn exports excess intracellular copper (23). The zinc-induced protein trafficking of ZnT4 is consistent with a functional role of ZnT4 in the lactating mammary gland as zinc is deficient in the milk of homozygous lethal milk mothers. ZnT4 may transport zinc into the TGN vesicles, and zinc may leave the cells through the secretory pathway. We speculate that ZnT6 functions in two major aspects of intracellular zinc homeostasis. First, ZnT6 may be involved in transporting the cytoplasmic zinc into the TGN vesicles, where it may be incorporated into zinc-requiring proteins. In this situation, the function of ZnT6 overlaps with that of ZnT4. The partial overlap of cellular localization of ZnT6 and ZnT4 may explain the lack of phenotypes in the other organs in the lethal milk mutant. Second, ZnT6 may play a role in zinc export based on its intracellular distribution, its zinc-induced intracellular redistribution, and its ability to diminish the cytoplasmic zinc when overexpressed in yeast cells. ZnT6 may cycle between the TGN and the plasma membrane both in basal and elevated cytoplasmic zinc concentration. Perhaps we did not detect a clear delineation of the plasma membrane staining because ZnT6 may be constantly recycled. Alternatively, ZnT6 may transport zinc into the TGN and the cytoplasmic vesicles, which could leave the cell through the secretory pathway.

A key aspect of the function of ZnT6 in regulating the cytoplasmic zinc levels in cells may be its zinc-induced relocation from the TGN to the cytoplasmic vesicles and then removal of zinc from cells either by the export function of ZnT6 on plasma membrane or by the secretory pathway because the expression of ZnT6 is not regulated by zinc at the transcriptional level or at the translational level in vitro. The molecular basis for the internalization of ZnT6 from the plasma membrane might be the di-leucine motifs at amino acid positions 363 and 364 as well as 401 and 402 of ZnT6 (23, 24). The C-terminal di-leucine motif has been demonstrated to mediate copper-induced trafficking of the MNK from the TGN to the plasma membrane, followed by return to the TGN via clathrin-coated vesicles. Further mutagenesis studies are required to demonstrate the importance of these motifs in the intracellular distribution and zinc-induced redistribution of ZnT6 (23, 25).

The multiple histidine residues in the loop region between transmembrane IV and V in the ZnT proteins have been hypothesized to act for a zinc binding domain. Our preliminary data indicate that the multiple serine residues in the loop region of ZnT6, which lacks multiple histidine residues in that region, are critical for the function of ZnT6 in vivo. The serine residues may coordinate the zinc binding with the histidine residue at the C-terminal end of the ZnT proteins (His-300 in ZnT6) to facilitate zinc cross-membrane trafficking. Further studies are under way to elucidate the mechanism of how zinc transporters relay zinc across membranes and where the energy comes from.

The presence of translationally inactive ZnT6 mRNA in liver is reminiscent of the expression pattern of ZnT3 (5). ZnT3 mRNA is abundantly expressed in adult testis of mice, as detected by Northern blot analysis and reverse transcription-PCR, while the ZnT3 protein is undetectable by Western blot analysis. It has been shown that the ZnT3 transcripts in testis are predominantly associated with monoribosomes, suggesting that this mRNA may not be translated efficiently (5). A modified ZnT6 protein with slightly higher molecular mass was observed in lung. In addition, the amount of the ZnT6 protein detected in lung is substantial as compared with its low level of transcripts. Many potential protein kinase C and CK2 phosphorylation and N-glycosylation sites are predicted in the ZnT6 protein using the ProfileScan Server (hits.isb-sib.ch). Phosphorylation and/or glycosylation of ZnT6 may increase the stability of the ZnT6 protein in lung. The functional difference between the native ZnT6 and modified ZnT6 proteins in intracellular zinc transportation remains unknown.

It has become clear from our studies and others that zinc is transported into and out of a variety of intracellular compart-
ments, and there appear to be dedicated zinc transporters for each event. The findings of the present study extend our understanding of what zinc transporters do in cells, how the expression of zinc transporters regulate, and how the transporters get to their various compartments.

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Addendum—As this manuscript is under review a paper describing the finding of a fifth member of the ZnT family, ZnT5, was published (Kambe, T., Narita, H., Yamaguchi-Iwai, Y., Hirose, J., Amano, T., Sugiura, N., Sasaki, R., Mori, K., Iwanaga, T., and Nagao, M. (2002) J. Biol. Chem. 277, 19049–19055).

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