Vesicular zinc transporters (ZnTs) play a critical role in regulating Zn\(^{2+}\) homeostasis in various cellular compartments and are linked to major diseases ranging from Alzheimer disease to diabetes. Despite their importance, the intracellular localization of ZnTs poses a major challenge for establishing the mechanisms by which they function and the identity of their ion binding sites. Here, we combine fluorescence-based functional analysis and structural modeling aimed at elucidating these functional aspects. Expression of ZnT5 was followed by both accelerated removal of Zn\(^{2+}\) from the cytoplasm and its increased vesicular sequestration. Further, activity of this zinc transport was coupled to alkalinization of the trans-Golgi network. Finally, structural modeling of ZnT5, based on the x-ray structure of the bacterial metal transporter YiiP, identified four residues that can potentially form the zinc binding site on ZnT5. Consistent with this model, replacement of these residues, Asp\(^{599}\) and His\(^{451}\), with alanine was sufficient to block Zn\(^{2+}\) transport. These findings indicate, for the first time, that Zn\(^{2+}\) transport mediated by a mammalian ZnT is catalyzed by H\(^+\)/Zn\(^{2+}\) exchange and identify the zinc binding site of ZnT proteins essential for zinc transport.

Zn\(^{2+}\) ions are essential for a very large number and variety of cellular functions (1) but are also potentially toxic. Zinc homeostasis is therefore dynamically maintained by a variety of transporters and other proteins distributed in distinct cellular compartments. Zinc transport is mediated by two major protein families: the Zip family, which mediates Zn\(^{2+}\) influx, and the ZnTs, which are primarily linked to Zn\(^{2+}\) sequestration into intracellular compartments and are, thereby, involved in lowering cytoplasmic Zn\(^{2+}\) concentrations (2).

The ZnT family of proteins consists of 10 known members that play versatile roles in many physiological and pathophysiological aspects of zinc homeostasis (3). For example, sequestration of zinc into synaptic vesicles is mediated by ZnT3, with changes in ZnT3 expression being linked to gender-specific susceptibility to Alzheimer disease (4). Another example, zinc deficiency in maternal milk has been linked to mutation in ZnT4 in mice (5). Finally, ZnT8 mediates zinc transport into insulin secretory vesicles and is a marker for the genetic polymorphism associated with type 2 diabetes in humans (6).

In the present work, we have focused on ZnT5, since it exemplifies both the complexity and physiological importance of ZnT transport activity. ZnT5 was previously shown to localize to the Golgi apparatus and to mediate the Zn\(^{2+}\) transport that is essential for proper folding of Zn\(^{2+}\) binding proteins within this compartment (7, 8). The phenotypes of ZnT5 knock-out mice underscore the importance of this transporter in multiple contexts. Among the most striking are decreased bone density, reduced weight, and fatal, male-specific, cardiac bradycardia and hypothyroidia (9). ZnT5 has been also shown to mediate Zn\(^{2+}\) uptake (10). It has also been suggested that ZnT5 subunits assemble with those of ZnT6 and that this hetero-oligomerization is required for the activation of the Zn\(^{2+}\)–dependent alkaline phosphatases (11). Oligomerization is also essential for functional complementation of yeast Zn\(^{2+}\) transporters (12). It is unclear, however, whether such hetero-oligomerization is essential for ZnT5 catalytic activity or merely up-regulates Zn\(^{2+}\) transport mediated by ZnT5. The manner in which mammalian ZnTs, among them ZnT5, mediate Zn\(^{2+}\) transport into organelles, however, is not known.

Clues about the functional mechanism of mammalian ZnTs arise from studies on their bacterial, yeast, and plant homologues. The bacterial Zn\(^{2+}\) transporter, ZitB, was shown to catalyze H\(^+\)/Zn\(^{2+}\) exchange when reconstituted into proteoliposomes (13). The recently determined structure of the YiiP protein suggested that the catalytic Zn\(^{2+}\) binding site contains four residues that are spatially arranged to coordinate Zn\(^{2+}\) (14). Studies performed on yeast and plant ZnT homologues have demonstrated that Zn\(^{2+}\) transport is dependent on the electrochemical gradient generated by the vacuolar ATPase (15, 16). Notably, however, no direct H\(^+\)/Zn\(^{2+}\) exchange has yet been demonstrated for these transporters, presumably because of leakiness of the purified vesicles to H\(^+\). Thus, whether the yeast or plant transporters directly mediate H\(^+\)/Zn\(^{2+}\) exchange or are merely utilizing the vesicular H\(^+\) gradient for an alternate mode of transport is still unclear.

Focusing on ZnT5 as our model protein, the primary aims of the current study were to 1) elucidate the functional mechanism by which ZnTs mediate Zn\(^{2+}\) sequestration and 2) ident-
Mechanisms of Zinc Transport by the ZnTs

tify the catalytic domains and residues that form the Zn\(^{2+}\) binding site in mammalian ZnTs.

**EXPERIMENTAL PROCEDURES**

*Cell Cultures and Plasmid Transfection*—HEK293-T cells (human embryonic kidney cell line) were cultured in Dulbecco’s modified Eagle’s medium (17). Briefly, plasmid transfection was performed using the CaPO\(_4\) precipitation protocol in cultures of 50–70% confluence in either 60- or 100-mm dishes (17). The hZnT5 and ZnT6 plasmids were used for transfection (18, 19).

*Generation of Mutants*—Site-directed mutagenesis was performed on the hZnT5 double-stranded plasmid (containing the hZnT5 gene; accession number AF461760), using the QuikChange site-directed mutagenesis kit (Stratagene) according to protocols provided by the manufacturer. The following primers were utilized: D599A, CTACATGTTTTGGCAGCTACTCTTGGCGAGAATGGTGGCAAGAGATCTCTTGGCAGCATTG; D599E, CTACATGTTTTGGCAGCTACTCTTGGCGAGAATGGTGGCAAGAGATCTCTTGGCAGCATTG; HA1, GATCTCGGATGGATCTGGCATGCTTTTTGACTGC. The oligonucleotide sequences used for mutation screening encompassed a 391-bp fragment for Asp599 and a 398-bp fragment for His451, which contains the mutations. A cassette encompassing the entire hZnT5 ORF or containing the mutations was subsequently transferred into the original plasmid.

*Immunocytochemistry*—Transfected HEK293-T cells were fixed using 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The cells were then preincubated with a blocking solution containing 4% normal goat serum, 1% bovine serum albumin, and 0.5% Triton X-100 (20) and incubated for 3 h at room temperature with either monoclonal anti-ZnT5 (18) or monoclonal anti-HA antibodies for the detection of ZnT6, as previously described (11, 18). Antibodies were diluted 1:300 and 1:50, respectively. Cells were subsequently incubated for 1 h with Cy3-conjugated AffiniPure goat anti-mouse IgG H+L (Jackson Immunoreagents) diluted 1:300.

*Fluorescent Imaging of Ion Transport*—Zn\(^{2+}\) transport was determined in cells loaded with either 3.5 \(\mu\)M Fura-2 AM (TEFLabs), 0.5 \(\mu\)M FluoZin-3 AM, or 5 \(\mu\)M Newport Green AM (Invitrogen), using an imaging system as described previously (21). To verify that the fluorescence changes were related to intracellular Zn\(^{2+}\), the cell-permeable heavy metal chelator \(N_2N_2N_4N_4\)-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN; 20 \(\mu\)M) was used. Zn\(^{2+}\) sequestration into the intracellular compartments was determined by staining cells with ZinPyr-1 (5 \(\mu\)M), a Zn\(^{2+}\)-sensitive dye that preferentially accumulates within the trans-Golgi compartment (TGN) (22). Changes in vesicular pH were determined in HEK-293T cells expressing pHluorins, which are targeted to the trans-Golgi region (23). TGN-pHluorin plasmid was kindly provided by Terry Machen (University of California, Berkeley) (24). For this purpose, cells were co-transfected with 0.25 \(\mu\)g of the pHluorin plasmid in a 60-mm culture dish. Emission images of cells expressing pHluorin were collected through a 510-nm long pass filter during sequential excitation at 410 and 470 nm.

Analysis of the fluorescent signals was performed by normalizing the fluorescence (or the ratio of the fluorescent signal) to an initial base line averaged over the first 10 s acquired in each experiment and was averaged over at least 30 cells. For the pHluorin measurements, the Golgi pH was calibrated using a high K\(^+\) Ringer’s solution set to pH values of 6–8 in the presence of nigericin, as previously described (23). Initial rates of H\(^+\) or Zn\(^{2+}\) transport were determined using this paradigm by calculating either \(d[\text{pH}]/dt\) or \(d[Zn^{2+}]/dt\), respectively. The initial rates were normalized as indicated in each experiment, and their averages ± S.E. are shown in the *bar graphs*.

Changes in intracellular pH were triggered using the ammonium prepulse paradigm, as described previously (25). Briefly, cells were superfused with Ringer’s solution containing NH\(_4\)Cl (30 mM, replacing the equivalent 30 mM NaCl), which was subsequently replaced with NH\(_4\)Cl-free solution, thus triggering intracellular acidification.

*Immunoblot Analysis*—Cells were extracted using 300 \(\mu\)l of boiling denaturative lysis buffer (1% SDS, 10 mM Tris-HCl, pH 8)/100-mm plate and transferred to ice. A protease inhibitor mixture (Boehringer Complete protease inhibitor mixture; Roche Applied Science) was then added, and protein concentrations were determined using the modified Lowry procedure (26) and stored at −80 °C. SDS-PAGE and immunoblot analyses were performed as previously described (27), using the ZnT5 and the HA antibodies at a dilution of 1:2000 and 1:1000, respectively.

*Three-dimensional Structural Modeling of ZnT5*—The ZnT5 sequence was sent to the 3D-Jury metaserver (28), which suggested the bacterial Zn\(^{2+}\) transporter, YiiP (14), as a high confidence homologue. The alignment proposed by the server was further edited manually to accommodate experimental results, which

---

**FIGURE 1. Expression and intracellular distribution of ZnT5 and ZnT6.**

*a*—HEK293-T cells were transfected with hZnT5 or HA-tagged ZnT6 plasmids. Immunoblot analysis of cell lysates (50 \(\mu\)g) was performed using either anti-hZnT5 or anti-HA monoclonal antibodies, respectively. *b*—HEK293-T cells transfected with hZnT5 or HA-ZnT6 (labeled in red, lower left panel) were co-transfected with pHluorins (targeted to the TGN, labeled in green, shown in the upper left panel). Cells were imaged using a confocal microscope (magnification \(\times100\)). Colocalization of pHluorins and ZnT5 or ZnT6 is shown in the corresponding bottom right images by overlapping the images obtained with each stain with the bright field image (shown in the upper right panel).
indicate a cytoplasmic position for the histidine-rich loop (residues 543–590). It should be emphasized that this manipulation did not involve any of the key residues discussed in this paper. The final model was generated by the homology model program of the MESHI package (29).

Statistical Analysis of Data—Data analysis was performed using the SPSS software (version 14.0; SPSS Inc., Chicago, IL). All results shown are the means ± S.E. of at least three individual experiments (n = 3) with averaged responses of 40–100 cells in each experiment. t test p values of < 0.05 were considered significant following Levene’s test for equality of variances. Significance of the results is indicated as follows: relative to control (*) or relative to ZnT5 (#). *, p < 0.05; **, p < 0.01.

RESULTS

Cytoplasmic Zn2\(^{2+}\) Removal Rates in Cells Expressing ZnTs—To determine the expression levels and cellular localization of heterologously expressed ZnT5 and ZnT6, we conducted Western blot and immunocytochemical analyses of HEK293-T cells transfected with the indicated plasmids. Transfection of HEK293-T cells was followed by increased expression of ZnT5 and ZnT6 polypeptides at their expected molecular masses (18, 30) of 55 and 47 kDa, respectively (Fig. 1a). We also found that both ZnT5 and ZnT6 migrated in a doublet form, which is consistent with expression of mature and immature forms following overexpression in HEK293 cells (31). To determine the cellular distribution of ZnT5 and ZnT6, cells transfected with either plasmid were co-transfected with pHluorins (23). As shown in Fig. 1b, an overlap was observed between the expression pattern of ZnT5 or ZnT6 and pHluorins, indicating, in agreement with previous studies (18, 30), that heterologously expressed ZnTs localize to the trans-Golgi compartment of HEK293-T cells. Because the above and previous results (18, 30) show that ZnT5 and ZnT6 are expressed in intracellular compartments, our experimental paradigm, aimed at monitoring Zn2\(^{2+}\) sequestration by ZnT5, consisted of loading cells with cytoplasmic Zn2\(^{2+}\) and recording its subsequent transport by these proteins. We hypothesized...
Mechanisms of Zinc Transport by the ZnTs

that ZnT5 and ZnT6 activity will result in a decrease in cytoplasmic [Zn²⁺] levels concomitant with a reciprocal increase in its level in intracellular compartments. HEK293-T cells, expressing either ZnT5 or ZnT6 or co-expressing ZnT5 and ZnT6 (ZnT5+6) were preloaded with Fura-2, which is a ratiometric dye sensitive to Zn²⁺ (32, 33). Loading of cells with Zn²⁺ was subsequently achieved by superfusion of Ringer’s solution containing Zn²⁺ (200 μM) in the presence of the Zn²⁺ ionophore, pyrithione (2 μM), as indicated by the rise in Fura-2 fluorescence (Fig. 2a). Cells were subsequently washed with Ringer’s solution without Zn²⁺ or pyrithione. No rapid change in fluorescence was observed in the control cells following perfusion with pyrithione-free Ringer’s solution, indicating that Zn²⁺ ions were trapped within the cells. Finally, application of the cell-permeable Zn²⁺ chelator, TPEN (20 μM), was followed by reduction of the fluorescent signal back to baseline, indicating that the Fura-2 signal is indeed related to changes in [Zn²⁺]], (Fig. 2a). We then compared the rate of cytoplasmic fluorescent signal decrease during the wash period (marked with a bar in Fig. 2a) in cells expressing ZnT5, ZnT6, or ZnT5+6 versus control cells (transfected with an empty vector). The averaged normalized rate of cytoplasmic Zn²⁺ transport, compared with control cells, was about 3-fold higher (316 ± 70% of control, p = 0.001) in cells co-expressing ZnT5+6 or 2-fold fold higher (182 ± 26% of control, p = 0.04) in cells expressing ZnT5 alone. In contrast, no significant change in fluorescence was observed in ZnT6-expressing cells following preloading with Zn²⁺, suggesting that ZnT6 by itself is not involved in reducing the cytoplasmic Zn²⁺ level. Because Zn²⁺ transport mediated by ZnT6 was indistinguishable from that of control cells, we have subsequently focused on monitoring the activity of ZnT5 and ZnT5+6. To further verify that the change in cytoplasmic fluorescent signal was related to Zn²⁺, we performed the same protocol using the specific Zn²⁺-sensitive fluorescent dye, Newport Green. As shown in Fig. 2c, and consistent with the data presented in Fig. 2b, the rate of cytoplasmic Zn²⁺ removal in cells co-transfected with ZnT5+6 was enhanced by ~4-fold (307 ± 70%, p = 0.02) compared with controls. Taken together, these results suggest that expression of ZnT5, but not of ZnT6, is followed by accelerated removal of cytoplasmic Zn²⁺. Co-expression of ZnT5 and ZnT6, moreover, markedly enhanced the rate of cytoplasmic Zn²⁺ removal.

We next sought to determine whether cytoplasmic Zn²⁺ removal, mediated by ZnT5+6, is associated with vesicular sequestration of Zn²⁺ into the Golgi network. Cells were superfused with Zn²⁺, using the same experimental paradigm described above and subsequently stained with the Zn²⁺-sensitive dye, Zinpyr-1, that preferentially accumulates within the trans-Golgi apparatus or Golgi-associated vesicles (22). As shown in Fig. 3, the vesicular Zn²⁺ accumulation was about 3.5-fold (337 ± 59% of control, p = 0.001) higher in cells expressing ZnT5+6 compared with control cells (transfected with the vector alone) and 3-fold (290 ± 58% of control, p = 0.021) higher in the ZnT5-expressing cells. Application of TPEN was again followed by a reduction of the fluorescent signal to levels monitored in control cells (p = 0.368). These results, taken together with the vesicular targeting of ZnT5 and ZnT6 and the enhanced cytoplasmic removal of Zn²⁺, support the conclusion that expression of ZnT5 or co-expression of ZnT5+6 leads to enhanced cytoplasmic Zn²⁺ removal and its accumulation in Golgi vesicles.

Rates of Vesicular H⁺ Transport in ZnT-expressing Cells—If the driving force for vesicular Zn²⁺ transport is the H⁺ gradient, then changes in vesicular H⁺ gradient would be expected to affect cytoplasmic Zn²⁺ removal. Cells were subjected to NH₄Cl prepulse, which was reported to activate the V-type ATPase activity (34), and subsequently rates of ion transport were monitored. We initially asked if enhanced Zn²⁺ transport mediated by ZnT5+6 would be monitored by this experimental paradigm. HEK293-T cells were loaded with Fura-2 and treated with Zn²⁺ pyrithione, following the NH₄Cl prepulse. The rate of cytoplasmic Zn²⁺ removal was subsequently compared in ZnT5 or ZnT5+6-expressing cells versus controls (Fig. 4a). The rate of cytoplasmic Zn²⁺ removal, following this procedure,
Mechanisms of Zinc Transport by the ZnTs

was enhanced by about 2-fold (184 ± 24% of control, p = 0.018) in ZnT5-expressing cells and by 3-fold (314 ± 90% of control, p = 0.002) following expression of both ZnT5 and ZnT6. Rates of Zn2+ transport were similar to those obtained in the absence of the NH4Cl prepulse. This may be related to the fact that the NH4Cl prepulse preceded Zn2+ loading, and due to the leaky nature of this compartment the resulting increase in the pH gradient was at least partially dissipated. Nevertheless, the increase in Zn2+ efflux rates in cells expressing ZnT5 or ZnT5+6 versus control was maintained using this protocol. To determine the role of the vesicular pH gradient, the V-type H\(^+\) pump inhibitor, bafilomycin (100 nM), was applied to cells co-expressing ZnT5+6, and rates of cytoplasmic Zn2+ removal were monitored. Using this experimental paradigm, in HEK293-T cells expressing the Golgi-targeted pH sensor, pHluorin, we have found that bafilomycin enhanced the alkalinization rate in the Golgi (see Fig. 4b, inset), indicating that the V-type ATPase was inhibited by bafilomycin. In cells loaded with Fura-2, a reduction in the rates of Zn2+ transport of ∼2-fold (46 ± 8% of control, p = 0.024) was observed in the presence of bafilomycin, indicating that the vesicular pH gradi-
Mechanisms of Zinc Transport by the ZnTs

The ZnT5 transporter, is a potential, reliable homologue to the C-terminal region of ZnT5, which encompasses about 50% of ZnT5 (residues 411–732 were analyzed). The overall sequence homology between the ZnT5 construct and YiiP is rather low (i.e., 15%), although the pattern of putative transmembranal helices is conserved, as are several key membrane-embedded residues. Among these, most notable are three of the four charged residues that coordinate the binding of Zn\(^{2+}\) ions in the bacterial transporter. Asp\(^{45}\) of the bacterial binding site is replaced in ZnT5 by a histidine residue, which has higher selectivity for Zn\(^{2+}\). Based on the YiiP structure, a model for the C-terminal domain of ZnT5 was constructed (Fig. 6a). This homology-based ZnT5 model suggested the putative binding site for Zn\(^{2+}\) (Fig. 6b), supporting the notion that this ZnT5 fragment is the catalytic domain. Thus, the working hypothesis that guided our subsequent experiments was that residues His\(^{451}\) and Asp\(^{455}\) in helix 11 and His\(^{595}\) and Asp\(^{599}\) in helix 14 are involved in Zn\(^{2+}\) coordination as they are on the bacterial YiiP protein.

If these residues compose the binding site, then replacement of even one of the residues would be expected to interfere with this process and hence to inhibit ZnT5-mediated Zn\(^{2+}\) transport. To test this hypothesis, the Asp\(^{599}\) residue was mutated to either Ala (D599A) or Glu (D599E). Expression analysis of the WT and mutants showed that the amount of WT plasmid required to achieve expression levels similar to that of the mutants was about 2-fold lower for the ZnT5 D599E mutant (Fig. 7a). The amount of plasmid used for the expression of the D599E mutant was adjusted accordingly for the functional analysis. Cellular distribution of mutant and WT ZnT5 was determined using confocal microscopy on cells co-expressing pHluorins in a manner to similar that described in the legend to Fig. 1. As shown in Fig. 7b, the cellular distribution of WT ZnT5 and the Asp\(^{599}\) mutations was similar and overlapped with the distribution of pHluorins, indicating that the mutants are also targeted to the trans-Golgi.

We next assessed whether the mutations described above affect Zn\(^{2+}\) transport, by monitoring the rate of cytoplasmic Zn\(^{2+}\) transport, using the paradigm shown in Fig. 2a, in cells loaded with FluoZin-3. As shown in Fig. 7c, cytoplasmic Zn\(^{2+}\) removal was blocked in cells transfected with the D599A ZnT5 mutant (21 ± 14% of the WT ZnT5, \(p = 0.01\)). When Asp\(^{599}\) was replaced with glutamate, however, a similar rate of Zn\(^{2+}\) transport activity was observed (119 ± 29% of the WT ZnT5). We then asked if substitution of another residue, which, according to our model, coordinates Zn\(^{2+}\), also leads to inhibition of cytoplasmic Zn\(^{2+}\) transport. As shown in Fig. 7d, although the

FIGURE 5. Zinc-dependent alkalinization of the Golgi in cells expressing ZnT proteins. a, cells co-expressing pHluorins, targeted to the TGN, and the indicated ZnTs were superfused with Zn\(^{2+}\)-free Ringer’s solution. Averaged normalized rates of vesicular pHluorin fluorescence change are shown at the right (mean ± S.E., \(n = 10\)). b, rates of vesicular pH in cells co-expressing either ZnT5+6, ZnT5 alone, or ZnT6 alone were determined by measuring the rate at the time period following Zn\(^{2+}\) loading. Averaged normalized rates are shown on the right (mean ± S.E., \(n = 8\)). c, representative traces of cytoplasmic Zn\(^{2+}\) imaged with Fura-2 (as in Fig. 2a) and vesicular pH imaged with pHluorins (as in b), following loading with Zn\(^{2+}\) (mean ± S.E., \(n = 7\)) are shown. Note the reciprocal change in Zn\(^{2+}\) and pH, indicating that the transport of these ions is coupled (\(p < 0.05\); **) \(p < 0.01\).
Mechanisms of Zinc Transport by the ZnTs

expression level of the ZnT5 H451A mutant is similar to that of WT ZnT5, cytoplasmic Zn2+ transport in cells expressing this mutant was blocked (20 ± 15% of the WT ZnT5, p = 0.01). Finally, we asked if these residues are also responsible for the sequestration of Zn2+ into the Golgi, by monitoring the change in vesicular Zn2+. Cells were superfused with a Zn2+-containing Ringer’s solution and were stained with Zinpyr-1, as described in the legend to Fig. 3. Consistent with the Zn2+ efflux results, vesicular Zn2+ sequestration was blocked in cells expressing the D599A and H451A mutants and was indistinguishable from that monitored in cells transfected with vector only that expressed the D599A and H451A mutants and was indistinguishable from that monitored in cells transfected with vector alone (Fig. 7e). These results suggest that Asp599 and His451 are critical residues of the Zn2+ binding site, as predicted by the YiPy-based homology model.

DISCUSSION

A major challenge in analyzing the activity of ZnTs is their intracellular localization, which limits direct access for the manipulating ion gradients. To overcome this, we have devised an experimental paradigm based on intracellular trapping of Zn2+ using the ionophore, pyrithione. This approach enabled us to image changes in ion concentrations (Zn2+ and H+) in the cytoplasmic and Golgi compartments. Focusing on ZnT5 and ZnT6, we demonstrated, for the first time, that vesicular ZnTs catalyze H+/Zn2+ exchange. This conclusion is based on the following findings. 1) Vesicular ZnTs are H+/Zn2+ exchangers, which a primary pump mediates the Zn2+ transport and in favor of a secondary active mechanism underlying the activity of ZnT proteins. This finding is also consistent with previous studies suggesting that sequestration of vesicular Zn2+ transport, presumably mediated by ZnT3, is attenuated in the presence of bafilomycin (35). 3) Vesicular H+ efflux from the trans-Golgi network is accelerated in the presence of cytoplasmic Zn2+ only in cells expressing ZnT5. It may also be argued that the Zn2+-dependent alkalinization of the Golgi observed by us is mediated by the modulation of the vesicular H+ channel. The latter is a major conductive pathway for H+ in the Golgi, and Zn2+ is a low affinity inhibitor of this channel (36). This scenario is unlikely, however, based upon the following. 1) Zn2+-dependent alkalinization was found only in ZnT-expressing cells. Zinc-dependent alkalinization was not found in control cells or even in cells expressing only ZnT6, linking the alkalinization to the expression of ZnT5 rather than to loading of cells with Zn2+. 2) Because Zn2+ is an inhibitor of the channel, loading the cells with this ion is expected to block the H+ conductive pathway and to reduce the H+ leak. This would result in enhanced acidification of the Golgi. We found, in contrast, that loading of Zn2+ into cells expressing ZnT5 leads to alkalinization of the Golgi. Thus, our results are not consistent with a model in which modulation of the H+ conductive pathway occurs but agree well with one in which H+/Zn2+ exchange is mediated by the ZnT.

The pH dependence of vesicular ZnTs has potentially far reaching physiological implications, since it predicts that a large vesicular H+ electrochemical gradient will be followed by enhanced Zn2+ sequestration. An intriguing example is the glucose-dependent acidification of insulin secretory vesicles (37). Although the exact physiological role of this acidification is unknown, it has been suggested that it may enhance the stabilization of insulin hexamers (38). The pH dependence of Zn2+ transport mediated by ZnT5, as described here, provides a plausible mechanism by which an increased H+ electrochemical gradient across the membrane of insulin secretory vesicles will trigger enhanced Zn2+ sequestration, followed by an accelerated rate of insulin hexamer formation.

If the vesicular ZnTs are H+/Zn2+ exchangers, then cytoplasmic pH may also affect Zn2+ sequestration/release from the vesicles. Consistent with this, it was demonstrated previously that acidification of astroglia led to a dramatic rise in cytoplasmic Zn2+, attributed to the enhanced dissociation of Zn2+ from metallothionines (39). Our results suggest that the
Mechanisms of Zinc Transport by the ZnTs

(a) Plasmid amount

- α-hZnT5
- α-actin

(b) ZnT5 D599A

(c) Vector D599A

(d) D599E

(e) ZnT5

(f) H451A

(g) ΔZnT5

(h) H451A

(i) [Zn^{2+}]_ves(%)
rise in cytoplasmic Zn\(^{2+}\) may also be the result of inhibition or even reversal of Zn\(^{2+}\) transport mediated by vesicular ZnTs.

The Functional Implications of the Oligomeric Structure of ZnT Proteins—ZnTs, particularly ZnT5 and ZnT6, are capable of forming hetero-oligomers. Whether this organization is catalytically essential or only helps enhance ZnT activity is unclear. In agreement with our study, heterologous expression of ZnT5 was sufficient to trigger Zn\(^{2+}\) transport in vesicles prepared from hZnT5-transfected HeLa cells (18). Endogenous ZnT6 expression could have contributed to ZnT5 activity; however, the simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. We also found that neither Zn\(^{2+}\) nor H\(^{+}\) transport is mediated by ZnT6, a finding consistent with a previous study suggesting that ZnT6 lacks the catalytic domain required for Zn\(^{2+}\) transport (19). In the chicken cell line, DT40, and in the yeast Zrg17/Msc2 Zn\(^{2+}\) transporter mutant, however, the simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. Alternatively, hetero-oligomerization may be required for either targeting of these transporters to distinct subcompartments of the Golgi (40) or for maintaining complex stability (41), issues that our assay could not resolve.

The Catalytic Domain of ZnTs—Little is known about the organization of the catalytic domain and particularly the residues composing the binding site for ZnTs. Among the domains previously investigated, most notable is a His-rich region—three of which are highly conserved—suggesting that ZnT6 lacks the catalytic domain required for Zn\(^{2+}\) transport. The simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. We also found that neither Zn\(^{2+}\) nor H\(^{+}\) transport is mediated by ZnT6, a finding consistent with a previous study suggesting that ZnT6 lacks the catalytic domain required for Zn\(^{2+}\) transport (19). In the chicken cell line, DT40, and in the yeast Zrg17/Msc2 Zn\(^{2+}\) transporter mutant, however, the simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. Alternatively, hetero-oligomerization may be required for either targeting of these transporters to distinct subcompartments of the Golgi (40) or for maintaining complex stability (41), issues that our assay could not resolve.

The simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. We also found that neither Zn\(^{2+}\) nor H\(^{+}\) transport is mediated by ZnT6, a finding consistent with a previous study suggesting that ZnT6 lacks the catalytic domain required for Zn\(^{2+}\) transport (19). In the chicken cell line, DT40, and in the yeast Zrg17/Msc2 Zn\(^{2+}\) transporter mutant, however, the simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. Alternatively, hetero-oligomerization may be required for either targeting of these transporters to distinct subcompartments of the Golgi (40) or for maintaining complex stability (41), issues that our assay could not resolve.

The simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. We also found that neither Zn\(^{2+}\) nor H\(^{+}\) transport is mediated by ZnT6, a finding consistent with a previous study suggesting that ZnT6 lacks the catalytic domain required for Zn\(^{2+}\) transport (19). In the chicken cell line, DT40, and in the yeast Zrg17/Msc2 Zn\(^{2+}\) transporter mutant, however, the simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. Alternatively, hetero-oligomerization may be required for either targeting of these transporters to distinct subcompartments of the Golgi (40) or for maintaining complex stability (41), issues that our assay could not resolve.

The simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. We also found that neither Zn\(^{2+}\) nor H\(^{+}\) transport is mediated by ZnT6, a finding consistent with a previous study suggesting that ZnT6 lacks the catalytic domain required for Zn\(^{2+}\) transport (19). In the chicken cell line, DT40, and in the yeast Zrg17/Msc2 Zn\(^{2+}\) transporter mutant, however, the simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. Alternatively, hetero-oligomerization may be required for either targeting of these transporters to distinct subcompartments of the Golgi (40) or for maintaining complex stability (41), issues that our assay could not resolve.

The simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. We also found that neither Zn\(^{2+}\) nor H\(^{+}\) transport is mediated by ZnT6, a finding consistent with a previous study suggesting that ZnT6 lacks the catalytic domain required for Zn\(^{2+}\) transport (19). In the chicken cell line, DT40, and in the yeast Zrg17/Msc2 Zn\(^{2+}\) transporter mutant, however, the simplest explanation is that ZnT5 can support Zn\(^{2+}\) transport. Alternatively, hetero-oligomerization may be required for either targeting of these transporters to distinct subcompartments of the Golgi (40) or for maintaining complex stability (41), issues that our assay could not resolve.
Mechanisms of Zinc Transport by the ZnTs

12. Ellis, C. D., Macdiarmid, C. W., and Eide, D. J. (2005) J. Biol. Chem. 280, 28811–28818
13. Chao, Y., and Fu, D. (2004) J. Biol. Chem. 279, 12043–12050
14. Lu, M., and Fu, D. (2007) Science 317, 1746–1748
15. MacDiarmid, C. W., Milanick, M. A., and Eide, D. J. (2002) J. Biol. Chem. 277, 39187–39194
16. Kawachi, M., Kobae, Y., Mimura, T., and Maeshima, M. (2008) J. Biol. Chem. 283, 19049–19055
17. Ohana, E., Segal, D., Palty, R., Ton-That, D., Moran, A., Sensi, S. L., Weiss, J. H., Hershfinkel, M., and Sekler, I. (2004) J. Biol. Chem. 279, 4278–4284
18. Kambe, T., Narita, H., Yamaguchi-Iwai, Y., Hirose, J., Amano, T., Sugiiura, N., Sasaki, R., Mori, K., Iwanaga, T., and Nagao, M. (2002) J. Biol. Chem. 277, 19049–19055
19. Suzuki, T., Ishihara, K., Migaki, H., Ishihara, K., Nagao, M., Yamaguchi-Iwai, Y., and Kambe, T. (2005) J. Biol. Chem. 280, 30956–30962
20. Nitzan, Y. B., Sekler, I., Hershfinkel, M., Moran, A., and Silverman, W. F. (2002) Brain Res. Dev. Brain Res. 137, 149–157
21. Hershfinkel, M., Moran, A., Grossman, N., and Sekler, I. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11749–11754
22. Burdette, S. C., Walkup, G. K., Spingler, B., Tsien, R. Y., and Lippard, S. J. (2001) J. Am. Chem. Soc. 123, 7831–7841
23. Machen, T. E., Leigh, M. J., Taylor, C., Kimura, T., Asano, S., and Moore, H. P. (2003) Am. J. Physiol. Cell Physiol. 285, C205–214
24. Miesenbo¨ck, G., De Angelis, D. A., and Rothman, J. E. (1998) Nature 394, 192–195
25. Azirol-Tamir, H., Sharir, H., Schwartz, B., and Hershfinkel, M. (2004) J. Biol. Chem. 279, 51804–51816
26. Markwell, M. A., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978) Anal. Biochem. 87, 206–210
27. Sekler, I., Moran, A., Hershfinkel, M., Dori, A., Margulis, A., Birenzweig, N., Nitzan, Y., and Silverman, W. F. (2002) J. Comp. Neurol. 447, 201–209
28. Ginalski, K., Elofsson, A., Fischer, D., and Rychlewski, L. (2003) Bioinformatics 19, 1015–1018
29. Kalisman, N., Levi, A., Maximova, T., Reshef, D., Zafriri-Lynn, S., Gleyzer, Y., and Keasar, C. (2005) Bioinformatics 21, 3931–3932
30. Huang, L., Kirschke, C. P., and Gitschier, J. (2002) J. Biol. Chem. 277, 26389–26395
31. Sekler, I., Lo, R. S., and Kopito, R. R. (1995) J. Biol. Chem. 270, 28751–28758
32. Atar, D., Backx, P. H., Appel, M. M., Gao, W. D., and Marban, E. (1995) J. Biol. Chem. 270, 2473–2477
33. Canzoniero, L. M., Sensi, S. L., and Choi, D. W. (1997) Neurobiol. Dis. 4, 275–279
34. Schapiro, F. B., and Grinstein, S. (2000) J. Biol. Chem. 275, 21025–21032
35. Love, R., Salazar, G., and Faundez, V. (2005) Brain Res. 1061, 1–12
36. Ramsey, I. S., Moran, M. M., Chong, J. A., and Clapham, D. E. (2006) Nature 440, 1213–1216
37. Stiernet, P., Guiot, Y., Gilon, P., and Henguin, J. C. (2006) J. Biol. Chem. 281, 22142–22151
38. Dunn, M. F. (2005) Biometals 18, 295–303
39. Sensi, S. L., Rockabrand, E., and Canzoniero, L. M. (2006) Biogerontology 7, 367–374
40. Jordan, B. A., Trapaidze, N., Gomes, I., Nivarthi, R., and Devi, L. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 343–348
41. Levi, V., Rossi, J. P., Castello, P. R., and González Flecha, F. L. (2002) Biophys. J. 82, 437–446
42. Wei, Y., and Fu, D. (2006) J. Biol. Chem. 281, 23492–23502
43. Mao, X., Kim, B. E., Wang, F., Eide, D. J., and Petris, M. J. (2007) J. Biol. Chem. 282, 6992–7000
44. Montanini, B., Blaudez, D., Jeandroz, S., Sanders, D., and Chalot, M. (2007) BMC Genomics 8, 107
45. Obara, K., Miyashita, N., Xu, C., Toyoshima, I., Sugita, Y., Inesi, G., and Toyoshima, C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 14489–14496