Two Different Zinc Transport Complexes of Cation Diffusion Facilitator Proteins Localized in the Secretory Pathway Operate to Activate Alkaline Phosphatases in Vertebrate Cells

Zinc is an essential component for the catalytic activity of numerous zinc-requiring enzymes. However, until recently little has been known about the molecules involved in the pathways required for supplying zinc to these enzymes. We showed recently (Suzuki, T., Ishihara, H., Migaki, H., Matsuura, W., Kohda, A., Okumura, K., Nagao, M., Yamaguchi-Iwaï, Y., and Kambe, T. (2005) J. Biol. Chem. 280, 637–643) that zinc transporters, ZnT5 and ZnT7, are required for the activation of zinc-requiring enzymes, alkaline phosphatases (ALPs), by transporting zinc into the lumens of the Golgi apparatus and the vesicular compartments where ALPs locate and converting apoALPs to holoALPs. ZnT6 is also located in the vesicular compartments like ZnT5 and ZnT7. However, the functions of ZnT6 and relationships among these three transporters have not been characterized yet. Here, we characterized the cellular function of ZnT6 together with ZnT5 and ZnT7 by gene-targeting studies using DT40 cells. ZnT6-deficient DT40 cells showed low ALP activity, suggesting that ZnT6 is required for the activation of zinc-requiring enzymes like ZnT5 and ZnT7. Combined disruptions of three transporter genes and re-expressions of transgenes revealed that ZnT5 and ZnT6 work in the same pathway, whereas ZnT7 acts alone. Furthermore, co-immunoprecipitation studies revealed that ZnT5 and ZnT6 formed hetero-oligomers, whereas ZnT7 formed homo-oligomers. Interestingly, the Ser-rich loop in ZnT6, a potential zinc-binding site, was dispensable for the zinc-supplying function of ZnT5/ZnT6 hetero-oligomers, suggesting that the His-rich loop in ZnT5 may be important for zinc binding and that the loop in ZnT6 may acquire another function in the hetero-oligomer formation. These results suggest that two different zinc transport complexes operate to activate ALPs.

The cation diffusion facilitator (CDF) family is a family of metal transport proteins found in diverse organisms from prokaryotes to eukaryotes (1–7). To date, eight CDF proteins designated ZnT1–8 (for zinc transporters 1–8) have been characterized in mammals (8–15). Members of this protein family have the same predicted membrane topology of six membrane-spanning domains and the cytoplasmic His-rich loop between membrane-spanning domains IV and V (2, 3, 6), with some exceptions such as ZnT5, which has a long N-terminal portion with extra membrane-spanning domains, and ZnT6, which lacks most of the histidine residues in a potential metal binding His-rich loop while retaining a Ser-rich loop (12, 13). Most CDF proteins have been characterized as zinc transporters that facilitate zinc efflux from the cytosol and mobilize the cytosolic zinc into intracellular organelles (1–7). In most cases the mobilization of zinc into the organelles is shown to contribute to zinc storage and/or detoxification during zinc excesses; in such cases the CDF proteins are localized to vacuoles and endosome/lysosome compartments (9, 11, 16–18).

There are a couple of CDF proteins localized in the secretory pathway, where zinc is important for the activity of many proteins. The Saccharomyces cerevisiae Msc2 protein, which is the major route of zinc entry into the endoplasmic reticulum, is required to maintain the proper function of the endoplasmic reticulum (19). A number of secretory, membrane-bound, or organelle-resident enzymes biosynthesized and become functional in the secretory pathway require zinc as an essential component for their catalytic activity; e.g. matrix metalloproteinases (20), angiotensin-converting enzyme (21), and alkaline phosphatases (ALPs) (22). We recently showed that vertebrate ZnT5 and ZnT7 proteins are required for zinc incorporation into ALPs in the Golgi apparatus and the vesicular compartments to convert apoALPs to holoALPs (23).

ZnT6 is also localized to the Golgi apparatus and is thought to be implicated in the delivery of zinc into the Golgi lumen in addition to ZnT5 and ZnT7 (13). However, the direct evidence of ZnT6 function in the secretory pathway has not, to date, been reported. In this study, we examined the cellular function of ZnT6 along with that of ZnT5 and ZnT7 by means of gene disruption studies in vertebrate cells and found that ZnT6 plays a role in the activation of tissue-nonspecific ALP (TNAP) and needs the co-expression of ZnT5 for its function, whereas neither ZnT5 nor ZnT6 needs to be co-expressed for ZnT7 to activate ALP in vertebrate cells. Furthermore, our biochemical analysis identified the formation of hetero-oligomeric complexes of ZnT5 and ZnT6 and the homo-oligomeric complexes of ZnT7.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AY986776.

The on-line version of this article (available at http://www.jbc.org) contains supplemental data in the form of a figure presenting experimental strategy and targeting constructs.

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The abbreviations used are: CDF, cation diffusion facilitator; ZnT, zinc transporter; hZnT, human ZnT; cZnT, chicken ZnT; ALP, alkaline phosphatase; TNAP, tissue-nonspecific ALP; HA, hemagglutinin.

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Materials and Methods

Plasmid Construction—Two targeting constructs designed to disrupt the chicken ZnT6 (cZnT6) gene were constructed. The ~13-kb cZnT6 gene was amplified with a pair of gene-specific primers using DT40 genomic DNA as a template by LA-Taq (TaKaRa). Each amplified DNA fragment was used to map the restriction sites. The long or short arm was PCR-amplified and subcloned downstream or upstream of the drug selection marker cassettes including drug-resistant genes (BsrR or HisR) flanked by mutant loxP sites (24). Plasmids that express human ZnT5 (hZnT5), chicken ZnT6 (cZnT6), human ZnT6 (hZnT6), or human ZnT7 (hZnT7) were constructed by inserting each cDNA into a pA-Puro vector and a PA-Zeocin vector (25). To construct pA-HA-hZnT6, we fused the DNA fragment encoding a hemagglutinin (HA) epitope (YPYDVPDYA) plus the start Met to the first ATG codon of hZnT6 cDNA (12) into pA-Puro and PA-Zeocin. To construct pA-hZnT7-FLAG or pA-hZnT7-HA, we fused the DNA fragment encoding the FLAG epitope or the HA epitope in-frame with the 3’-end of hZnT7 cDNA and inserted it into pA-Puro and PA-Zeocin. The pANMerCreMer plasmid encoding the tamoxifen-regulated chimeric Cre recombinase was a kind gift from Dr. Michael Reth (25). All plasmids were linearized with appropriate restriction enzymes prior to electroporation.

Cell Culture and Transfection—Chicken B lymphocyte-derived DT40 cells were maintained in RPMI 1640 (Nacalai Tesque) supplemented with 10% heat-inactivated fetal calf serum (BioWest), 1% chicken serum (Invitrogen), and 10 μg 2-mercaptoethanol (Sigma) at an initial cell density of 10^7 cells/mL. DT40 cells were carried out as described previously (23). For screening, we used 30 μg/mL blasticidin S (Calbiochem), 1 mg/mL histidinol (Sigma), 2.25–2.75 mg/mL hygromycin (Invitrogene), 0.25–0.35 mg/mL zeocin (Invitrogen), and 0.5 μg/mL puromycin (Sigma).

Generation of Mutant Cells—Our experimental strategy and the targeting constructs we used are shown in the supplemental data available in the online version of this article. ZnT5^-/- cells, ZnT5 cells, and ZnT5 ZnT7 ZnT7^-/- cells were established as described previously (23). To obtain ZnT5^-/- cells, we transfected the wild-type DT40 cells sequentially with ZnT6-BsrR and ZnT6-HisR targeting constructs. ZnT5^-/- cells were transfected with ZnT5^NeoR targeting construct to produce ZnT5^-/- ZnT6^-/- cells. To generate ZnT6^-/- ZnT7^-/- cells or ZnT5 ZnT6^-/- ZnT7^-/- cells we excised the drug selection marker cassettes in ZnT7^-/- cells or ZnT5 ZnT7^-/- cells according to methods described elsewhere (24). Briefly, these cells stably harboring the plasmid containing pANMerCreMer were cultured for 2 days in the presence of 200 μM 4-hydroxytamoxifen (Sigma), which translocates the MerCreMer protein into the nucleus and, thereby, the MerCreMer protein recombines DNA at mutant loxP sites. Excision of the drug selection marker cassettes was confirmed by Southern blotting or genomic PCR after limiting dilution. The cells for which the excision was confirmed were transfected sequentially with ZnT6-BsrR and ZnT6-HisR targeting constructs to obtain ZnT6^-/- ZnT7^-/- cells or ZnT5^-/- ZnT6^-/- ZnT7^-/- cells.

Southern Blotting—Twenty micrograms of genomic DNA prepared from DT40 cells were digested with appropriate restriction enzymes for 24 h. After digestion, DNA was electrophoresed on agarose gel and transferred to a nitrocellulose membrane filter in 0.4N NaOH. The membrane was hybridized to the radiolabeled DNA probes and exposed to an imaging plate (Fuji). Radioisopic images were obtained using a BAS 2500 bioimaging analyzer (Fuji).

RNA Preparation and Northern Blotting—Total RNA was extracted from DT40 cells using Sepasol I (Nacalai Tesque). The RNA (20 μg) was electrophoresed on agarose gel and transferred to a nitrocellulose membrane filter in 20× SSC. The membrane was hybridized to the radiolabeled DNA probes and exposed to an imaging plate (Fuji). Radioisopic images were obtained using a BAS 2500 bioimaging analyzer (Fuji).

Immunofluorescence Staining—Immunofluorescence analysis of FLAG-hZnT5 or HA-hZnT6 was performed as described previously (23). Briefly, DT40 cells were harvested, washed with phosphate-buffered saline, and incubated on coverslips coated with poly-L-lysine for 30 min following fixation with 4% paraformaldehyde at room temperature for 30 min and permeabilization with 0.1% TritonX-100 at room temperature for 10 min. The cells were immunostained with anti-FLAG antibody M5 (Sigma; 1: 400 dilution), followed by donkey anti-mouse IgG conjugated with Alexa 594 (Molecular Probes) as the secondary antibody, and with anti-HA antibody 3F10 (1: 250 dilution), followed by goat anti-rat IgG and rabbit anti-goat IgG conjugated with Alexa 488 (Molecular Probes) as the secondary and third antibodies. The stained cells were observed under a confocal laser-scanning microscope (Fluoview, Olympus).

Preparation of the Membrane Fraction—The membrane fraction was prepared as described previously with minor revisions (23). Briefly, DT40 cells (~2 × 10^7) were resuspended in 1 ml of cold homogenizing buffer and homogenized with 60 strokes of a 7-ml Dounce homogenizer. To remove the nucleus, we centrifuged the homogenate at 400 × g for 10 min at 4 °C. The post-nuclear supernatant was centrifuged at 20,400 × g for 60 min at 4 °C. The supernatant was centrifuged at 70 °C until use.

Immunoprecipitation Analysis—DT40 cells (2 × 10^7) were collected and washed with phosphate-buffered saline. The cells were then lysed with 1 ml of Nonidet P-40 lysis buffer (50 mM HEPES-HCl, pH 7.4, 100 mM NaCl, 1.5 mM MgCl_2, 1% (v/v) Nonidet P-40) containing protein inhibitors (Nacalai Tesque), followed by rotation for 2 h at 4 °C. The whole cell lysates were adjusted to 1 μg/ml with Nonidet P-40 lysis buffer. An aliquot of the lysates was precipitated with three volumes of acetone for 2 h at −20 °C and centrifuged at 20,400 × g for 15 min at 4 °C. The pellet was lysed with 5× Ling’s solubilizing buffer (150 mM sucrose, 50 mM Tris-HCl, pH 8.0, 20 mM dithiothreitol, 10% SDS, and 5
mM EDTA) and used as the input fraction of the immunoprecipitation. Other aliquots of the lysates were immunoprecipitated with monoclonal antibodies, anti-FLAG M2 (Sigma; 1:200 dilution), or anti-HA 3F10 (Roche Applied Science; 1:200 dilution) in the presence of 2% bovine serum albumin. After rotating for 1 h, 25 μl of protein G-Sepharose beads (Amersham Biosciences) was added and rotated for 2 h at 4°C. After centrifugation at 400 × g for 5 min, the pelleted beads containing the immunoprecipitates were washed three times with Nonidet P-40 lysis buffer and lysed in the 5× Ling’s solubilizing buffer. An equal volume of 2× urea buffer (8 M urea, 30 mM sucrose, 10 mM Tris-HCl, pH 8.0, 4 mM dithiothreitol, 2% SDS, and 1 mM EDTA) was added and incubated at 37 °C for 30 min before electrophoresis.

Immunoblot Analysis—Proteins were separated with electrophoresis through 8% SDS-polyacrylamide gels. After the transfer of proteins to nitrocellulose membrane (Hybond-ECL, Amersham), the blot was blocked with blocking solution (5% skim milk and 0.1% Tween 20 in phosphate-buffered saline) and then incubated with anti-hZnT5 (12) (1:2000 dilution), anti-FLAG M2 (1:2000 dilution), anti-HA 3F10 (1:1000 dilution), anti-HA 12CA5 (Roche Applied Science; 1:200 dilution), anti-HA 16B12 (BAbCO; 1:500 dilution) or anti-calnexin (Stressgen Biotechnologies; 1:5000 dilution) antibody in blocking solution. Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) were used at a 1:3000 dilution. A Super Signal chemiluminescent substrate (PIERCE) was used for detection. The fluoroblot was obtained using the LAS1000 Plus (Fuji).

RESULTS
Low TNAP Activity in ZnT6-deficient DT40 Cells—To investigate the cellular function of ZnT6, we generated ZnT6-deficient DT40 cells (ZnT6−/− cells) as described under “Materials and Methods.” Because ZnT6 has been shown to be localized to the subcellular compartments that are involved in the secretory pathway such as the Golgi apparatus, it may be employed in supplying zinc to zinc-requiring enzymes biosynthesized and matured through the secretory pathway, a role similar to that of ZnT5 and ZnT7 (23). To examine this possibility, we measured the TNAP activity in the ZnT6−/− cells. We previously established the assay by using the TNAP activity as a marker
Two Zinc Transport Complexes Operate to Activate TNAP

ZnT5 and ZnT6 Work in the Same Pathway, but ZnT7 Works Alone—Two CDF proteins, ZnT5, ZnT6, and ZnT7, seem to work on the activation of TNAP. We therefore investigated the contribution and relationship among these transporters to the expression of TNAP activity by means of the multiple gene disruptions of these transporters. First, we measured the TNAP activity using the total cellular protein prepared from single gene disruptant cell lines, namely ZnT5−/− cells, ZnT6−/− cells, and ZnT7−/− cells. The TNAP activity in ZnT7−/− cells was reduced only by 30%, whereas the activity in ZnT5−/− cells was reduced by 70%. The TNAP activity in ZnT6−/− cells was reduced by 80%, which was somewhat similar to the case of ZnT5−/− cells (Fig. 2A, upper section). Then, we assayed double gene disruptant cell lines, specifically ZnT5−/−ZnT6−/− cells, ZnT5−/−ZnT7−/− cells, and ZnT6−/−ZnT7−/− cells. The TNAP activity in ZnT6−/−ZnT7−/− cells was severely diminished to <1% of that in wild-type cells, as was the case in ZnT5−/−ZnT7−/− cells (Fig. 2A, upper section) (also see Ref. 23), whereas the activity in ZnT5−/−ZnT6−/− cells maintained ~30% of the TNAP activity in wild-type cells (Fig. 2A, upper section), which is not lower than that in the single gene disruptants, ZnT5−/− cells and ZnT6−/− cells, suggesting that ZnT5 and ZnT6 work in the same pathway supplying zinc to TNAP. In addition, the TNAP activity in ZnT5−/−ZnT6−/−ZnT7−/− cells was severely reduced (<1% of that in wild-type cells; Fig. 2A, upper section), implying that ZnT7 works in different pathways from those of ZnT5 and ZnT6 to supply zinc to TNAP. In all cell lines, the expression levels of TNAP mRNA were unaffected (Fig. 2A, lower section).

These mutant cells showed normal microscopic morphology and showed sensitivity to a high zinc concentration similar to that of the wild-type cells. Total cellular zinc in the mutant cells was comparable with that in wild-type cells in the range of 60–75 μg/g of lyophilized cells (Fig. 2B), which confirms that the cellular functions of ZnT5, ZnT6, and ZnT7 are for zinc supply into the lumens of the secretory compartments and not for storage and/or detoxification of zinc in an acidic endosomal/lysosomal compartment, as has been proposed for ZnT2 (9, 26).

Consistently, when we expressed ZnT5 and ZnT6 transgenes in ZnT5−/−ZnT6−/−ZnT7−/− cells (human ZnT5 N-terminally tagged with FLAG epitope (FLAG-hZnT5) and human ZnT6 N-terminally tagged with HA epitope (HA-hZnT6) were used in this setting), TNAP activity was restored only in the cells expressing both genes but not in the cells expressing either one of the two transporters (Fig. 3A). On the contrary, when we expressed the ZnT7 transgene in ZnT5−/−ZnT6−/−ZnT7−/− cells (human ZnT7 C-terminally tagged with FLAG epitope (hZnT7-FLAG)) as a control (lower section), the expression of ZnT7 alone is functional for the activation of TNAP in ZnT5−/−ZnT6−/−ZnT7−/− cells. TNAP activity was measured using total cellular protein prepared from wild-type (WT) cells, ZnT5−/−ZnT6−/−ZnT7−/− cells, and ZnT5−/−ZnT6−/−ZnT7−/− cells expressing hZnT7-FLAG (upper section). Expression of hZnT7-FLAG in these cells was confirmed by immunoblot analysis using membrane protein prepared from the indicated cells. Calnexin (CNX) is shown as a control (lower sections). Each value in the upper sections of panels A and B is the mean ± S.D. of triplicate experiments, and the immunoblots in the lower sections of panels A and B show representative results.

ZnT5 and ZnT6 Work in the Same Pathway, but ZnT7 Works Alone—Three CDF proteins, ZnT5, ZnT6, and ZnT7, seem to work on the activation of TNAP. We therefore investigated the contribution and relationship among these transporters to the expression of TNAP activity by means of the multiple gene disruptions of these transporters. First, we measured the TNAP activity using the total cellular protein prepared from single gene disruptant cell lines, namely ZnT5−/− cells, ZnT6−/− cells, and ZnT7−/− cells. The TNAP activity in ZnT7−/− cells was reduced only by 30%, whereas the activity in ZnT5−/− cells was reduced by 70%. The TNAP activity in ZnT6−/− cells was reduced by 80%, which was somewhat similar to the case of ZnT5−/− cells (Fig. 2A, upper section). Then, we assayed double gene disruptant cell lines, specifically ZnT5−/−ZnT6−/− cells, ZnT5−/−ZnT7−/− cells, and ZnT6−/−ZnT7−/− cells. The TNAP activity in ZnT6−/−ZnT7−/− cells was severely diminished to <1% of that in wild-type cells, as was the case in ZnT5−/−ZnT7−/− cells (Fig. 2A, upper section) (also see Ref. 23), whereas the activity in ZnT5−/−ZnT6−/− cells maintained ~30% of the TNAP activity in wild-type cells (Fig. 2A, upper section), which is not lower than that in the single gene disruptants, ZnT5−/− cells and ZnT6−/− cells, suggesting that ZnT5 and ZnT6 work in the same pathway supplying zinc to TNAP. In addition, the TNAP activity in ZnT5−/−ZnT6−/−ZnT7−/− cells was severely reduced (<1% of that in wild-type cells; Fig. 2A, upper section), implying that ZnT7 works in different pathways from those of ZnT5 and ZnT6 to supply zinc to TNAP. In all cell lines, the expression levels of TNAP mRNA were unaffected (Fig. 2A, lower section).

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Fig. 3. ZnT5 and ZnT6 work in the same pathway, but ZnT7 works alone. A, co-expression of ZnT5 and ZnT6 is required for the restoration of the reduced TNAP activity in ZnT5−/−ZnT6−/−ZnT7−/− cells. TNAP activity was measured using total cellular protein prepared from wild-type (WT) cells, ZnT5−/−ZnT6−/−ZnT7−/− cells, and ZnT5−/−ZnT6−/−ZnT7−/− cells expressing FLAG-hZnT5 and HA-hZnT6 alone or together (upper section). The expression of FLAG-hZnT5 and HA-hZnT6 in these cells was confirmed by immunoblot analysis using membrane protein prepared from the indicated cells. Calnexin (CNX) is shown as a control (lower section). B, the expression of ZnT7 alone is functional for the activation of TNAP in ZnT5−/−ZnT6−/−ZnT7−/− cells. TNAP activity was measured using total cellular protein prepared from wild-type (WT) cells, ZnT5−/−ZnT6−/−ZnT7−/− cells, and ZnT5−/−ZnT6−/−ZnT7−/− cells expressing hZnT7-FLAG (upper section). Expression of hZnT7-FLAG in these cells was confirmed by immunoblot analysis using membrane protein prepared from the indicated cells. Calnexin (CNX) is shown as a control (lower sections). Each value in the upper sections of panels A and B is the mean ± S.D. of triplicate experiments, and the immunoblots in the lower sections of panels A and B show representative results.
ZnT5 and ZnT6 are co-localized in ZnT5 ZnT6−/− cells. Exogenously expressed FLAG-hZnT5 and HA-hZnT6 were double-stained as described under "Materials and Methods." The subcellular localizations of FLAG-hZnT5 (upper section) and HA-hZnT6 (middle section) are shown. The merged image is shown in the lower section. B, ZnT5 and ZnT6 form hetero-oligomers in DT40 cells. Whole cell lysates were prepared from the indicated cells. FLAG-hZnT5 and HA-hZnT6 were immunoprecipitated with antibodies against the FLAG and HA epitopes, respectively. The immunoprecipitates (IP) were subjected to immunoblot analysis using antibodies against the FLAG or HA epitopes. For an estimation of the amount of FLAG-hZnT5 and HA-hZnT6 in whole cell lysates, 50% of the aliquot of lysates was subjected to immunoblot analysis in parallel after acetone precipitation (Input, lower section).

ZnT5 and ZnT6 form hetero-oligomeric complexes (18, 27). As shown in Fig. 5, the co-immunoprecipitation experiments revealed that hZnT7-FLAG and hZnT7 C-terminally tagged with the HA epitope (hZnT7-HA) physically interacted with each other when we expressed hZnT7-FLAG and hZnT7-HA in ZnT5 ZnT6−/− ZnT7−/− cells. This result indicates that ZnT7 forms homo-oligomeric complexes. Unlike ZnT7, neither ZnT5 nor ZnT6 formed homo-oligomeric complexes in similar experimental settings (data not shown).

The Ser-rich Loop in ZnT6 Is Not Essential for ZnT5/ZnT6 Hetero-oligomeric Complexes to Activate TNAP—As mentioned above, most of the ZnT transporters characteristically possess the cytoplasmic His-rich loop (2, 3, 6), which is proposed to be a potential zinc-binding site (16, 28), although its precise role has not yet been elucidated. ZnT6 lacks most of the histidine residues within this loop. Thus, we examined whether the loops in ZnT5 and ZnT6 were essential for their functions based on our findings described above (the hetero-oligomer formation of ZnT5 and ZnT6). As mentioned above, most of the ZnT transporters characteristically possess the cytoplasmic His-rich loop (2, 3, 6), which is proposed to be a potential zinc-binding site (16, 28), although its precise role has not yet been elucidated. ZnT6 lacks most of the histidine residues within this loop. Thus, we examined whether the loops in ZnT5 and ZnT6 were essential for their functions based on our findings described above (the hetero-oligomer formation of ZnT5 and ZnT6). For this purpose, we made mutant proteins lacking these loops that we designated as hZnT5 His and hZnT6ΔSer mutant proteins; the amino acids from 542 to 578 in the His-rich loop in hZnT5 were deleted in hZnT5 His, those in 542 to 578 in the His-rich loop in hZnT6 were deleted in hZnT6ΔSer. From 184 to 189 in the Ser-rich loop in hZnT5 were deleted in hZnT5ΔHis, those from 164 to 189 in the Ser-rich loop in hZnT6 were deleted in hZnT6ΔSer (Fig. 6 A), and the TNAP activity in the cells expressing these mutants was assayed. The TNAP activity was not restored by the co-expression of hZnT5ΔHis with HA-hZnT6 in ZnT5−/− ZnT6−/− ZnT7−/− cells (Fig. 6 B left). The data not shown.)
plex formation was not impaired because hZnT5/H9004His was co-immunoprecipitated with HA-hZnT6 (Fig. 6B right), suggesting that the mutation introduced in the ZnT5 gene did not affect the conformation of ZnT5; if any, the mutation affected the zinc-supplying function of ZnT5. In contrast, the co-expression of HA-hZnT6ΔSer and FLAG-hZnT5 partly restored TNAP.
activity in ZnT5−/ZnT6−/−ZnT7−/− cells (up to 40% of that in HA-hZnT6-expressing cells) (Fig. 6C left). Again, the mutation introduced in the ZnT6 gene had little effect on the conformational changes of ZnT6, as HA-hZnT6ΔSer was co-immunoprecipitated with FLAG-hZnT5 (Fig. 6C right). These results indicate that the His-rich loop in ZnT5 is essential for activating TNP, whereas the Ser-rich loop in ZnT6 seems to have more moderate roles so that ZnT6 seems to lose zinc-transductional changes of ZnT6, as HA-hZnT6ΔHis protein showed dominant negative effects in the activation of TNP when expressed in either wild-type cells, ZnT5−/−ZnT6−/− or ZnT5−/−ZnT7−/− cells (Fig. 6D), which implied that the introduced mutant proteins may interfere with the endogenous hetero-oligomeric formation of ZnT5 and ZnT6 in DT40 cells.

DISCUSSION

In vertebrate cells, three zinc transporters, ZnT5, ZnT6, and ZnT7, have been shown to be localized to the Golgi apparatus and the vesicular compartments and thought to be implicated in the entry of zinc into their lumens, thereby supplying zinc to numerous enzymes biosynthesized and matured in the secretory pathway (12–14). Of these three, we recently reported that ZnT5 and ZnT7 are required for the activation of TNP (29). In the case of ZnT6, however, there was no direct evidence showing that ZnT6 supplies zinc to zinc-requiring enzymes in the secretory pathway. Here, we showed that ZnT6 is involved in the expression of TNP activity by the use of gene disruption studies in DT40 cells. Why then are the three transporters localized to the same compartments? The three might have different specificities to target proteins. However, the actual number of functional zinc transporters in the secretory pathway known to date is two and not three, because we have shown direct evidence that ZnT5 and ZnT6 form hetero-oligomeric complexes, which was also suggested in a most recent report by Ellis et al. (29).

The formation of ZnT5/ZnT6 hetero-oligomeric complexes has been considered to be essential for their functions, because both genes need to be expressed to activate TNP. There are many transporters or channels that form hetero-oligomers to manifest their own biological activity, and some of them form hetero-oligomers not only for their activity but also for their subcellular translocation. For example, the ATP-binding cassette transporters ABCG5 and ABCG8 are not trafficked to plasma membrane for excretion of their substrate out of the cells until the heterodimers are formed; each of them is retained in the endoplasmic reticulum if expressed alone (30). This is not the case for ZnT5 and ZnT6. The subcellular localization of ZnT5 and ZnT6 was the same when they were expressed alone or together (data not shown and Fig. 4A). Rather, they require each other for the zinc-supplying function.

They require each other for the zinc-supplying function.

REFERENCES

1. Nies, D. H., and Silver, S. (1995) J. Ind. Microbiol. 14, 186–199
2. Paulsen, I. T., and Saier, M. H., Jr. (1997) J. Membr. Biol. 156, 99–103
3. Gaither, L. A., and Eide, D. J. (2001) Biomolecules 14, 251–270
4. Chipman, P., Aoufoun, M., Favier, A., and Seve, M. (2003) Curr. Drug Targets 4, 323–338
5. Luzzi, J. P., and Cousins, R. J. (2004) Annu. Rev. Nutr. 24, 151–172
6. Rame, T., Yamaguchi-Iwai, Y., Sasaki, R., and Nagao, M. (2004) Cell. Mol. Life Sci. 61, 49–68
7. Palmiter, R. D., and Huang, L. (2004) Pfluegers Arch. Eur. J. Physiol. 447, 744–751
8. Palmiter, R. D., and Fendley, S. D. (1995) EMBO J. 14, 639–649
9. Palmiter, R. D., Cole, T. B., and Fendley, S. D. (1996) EMBO J. 15, 1784–1791
10. Palmiter, R. D., Cole, T. B., Quaiife, C. J., and Fendley, S. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14834–14839
11. Huang, L., and Gitschier, J. (1997) Nat. Genet. 17, 292–297
12. Rame, T., Nara, H., Yamaguchi-Iwai, Y., Hirose, J., Amano, T., Sugita, N., Sasaki, R., Mori, K., Iwanaga, T., and Nagao, M. (2004) J. Biol. Chem. 277, 19049–19055
13. Huang, L., Kirschke, C. P., and Gitschier, J. (2002) J. Biol. Chem. 277, 26388–26395
14. Kirschke, C. P., and Huang, L. (2003) J. Biol. Chem. 278, 4096–4102
15. Chimienti, F., Devergnas, S., Favier, A., and Seve, M. (2004) Diabetes 53, 2330–2337
16. Murgia, C., Vestpignani, I., Cerese, J., Nobili, F., and Perozzi, G. (1999) Am. J. Physiol. 277, G1231–G1239
17. Li, L., and Kaplan, J. (1998) J. Biol. Chem. 273, 22181–22187
18. Blau-dez, D., Kohler, A., Martin, F., Sanders, D., and Chalot, M. (2003) Plant Cell 15, 2911–2928
19. Ellis, C. D., Wang, F., MacDiarmid, C. W., Clark, S., Lyons, T., and Eide, D. J. (2005) Plant Cell 17, 3230–3237
20. Ellis, C. D., and Lopez-Otin, C. (2002) J. Biol. Chem. 277, 13235–13239
21. Zhang, Y., Wienands, J., Zurn, C., and Reth, M. (1998) EMBO J. 17, 48275–48282
22. Haas, R., Schwager, S. L., Sturrock, E. D., and Acharya, K. R. (2003) J. Ind. Microbiol. 27, 551–554
23. Suzuki, T., Ishihara, K., Matsuura, W., Kohda, A., Okumura, K., Nagao, M., Yamaguchi-Iwai, Y., and Kambe, T. (2005) J. Biol. Chem. 280, 637–643
24. Arakawa, H., Lozygin, D., and Buerstedde, J. M. (2001) BMC Biotechnol. http://www.biomedcentral.com/1472-6740/1/17
25. Zhang, Y., Wienands, J., Zurn, C., and Reth, M. (1998) EMBO J. 17, 7304–7310
26. Kobayashi, T., Beuchat, M. H., Lindsay, M., Frias, S., Palmiter, R. D., Sakuraba, H., Parton, R. G., and Gruenberg, J. (1999) Nat. Cell Biol. 1, 113–118
27. Wei, Y., Li, H., and Fu, D. (2004) J. Biol. Chem. 279, 39251–39259
28. Blos, T., Clemens, S., and Nies, D. H. (2002) Planta 214, 783–791
29. Ellis, C. D., MacDiarmid, C. W., and Eide, D. J. (2005) J. Biol. Chem. 280, 28831–28838
30. Graf, G. A., Yu, L., Li, W. P., Gerard, R., Tuma, P. L., Cohen, J. C., and Hobbs, H. H. (2003) J. Biol. Chem. 278, 48275–48282
31. Yamashita, S., Miyagi, C., Fukada, T., Kagawa, N., Che, Y. S., and Hirano, T. (2004) Nature 429, 296–302
Two Different Zinc Transport Complexes of Cation Diffusion Facilitator Proteins Localized in the Secretory Pathway Operate to Activate Alkaline Phosphatases in Vertebrate Cells

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