Zinc Transporters, ZnT5 and ZnT7, Are Required for the Activation of Alkaline Phosphatases, Zinc-requiring Enzymes That Are Glycosylphosphatidylinositol-anchored to the Cytoplasmic Membranestr

Numerous proteins are properly folded by binding with zinc during their itinerary in the biosynthetic-secretory pathway. Several transporters have been implicated in the zinc entry into secretory compartments from cytosol, but their precise roles are poorly understood. We report here that two zinc transporters (ZnT5 and ZnT7) localized in the secretory apparatus are responsible for loading zinc to alkaline phosphatases (ALPs) that are glycosylphosphatidylinositol-anchored membrane proteins exposed to the extracellular site. Disruption of the ZnT5 gene in DT40 cells decreased the ALP activity to 45% of that in the wild-type cells. Disruption of the ZnT7 gene lowered the ALP activity only by 20%. Disruption of both genes markedly decreased the ALP activity to <5%. Overexpression of human ZnT5 or ZnT7 in DT40 cells deficient in both ZnT5 and ZnT7 genes recovered the ALP activity to the level comparable to that in the wild-type cells. The inactive ALP protein in DT40 cells deficient in both ZnT5 and ZnT7 genes was transported to cytoplasmic membrane like the active ALP protein in the wild-type cells. Thus both ZnT5 and ZnT7 contribute to the conversion of apo-ALP to holo-ALP.

Zinc is an essential trace element required as a structural component of a large number of proteins such as metalloenzymes and transcription factors (1, 2). Because zinc can not freely pass the membrane of cells and intracellular organelles, zinc transporter proteins have been thought to play a critical role in regulating the zinc concentration in the cytosol and lumen of organelles. Recently, a number of zinc transporters (ZIP family, CDF members, ZnT1–7) have been characterized in mammals (3–8). ZIP family transporters function in zinc influx into cytosol from extracellular sites and the lumen of intracellular organelles. CDF family transporters facilitate zinc efflux from cytosol and mobilize the cytosolic zinc into intracellular organelles. This mobilization of zinc into organelles may supply zinc to zinc-dependent proteins in situ and may also contribute to zinc storage and/or detoxification. To date, eight CDF members, named ZnT1–8 (zinc transporter 1–8), have been characterized in mammals (4–6, 8, 9). Of these mammalian CDF members, ZnT5–7 are located in the Golgi apparatus and implicated in the entry of zinc into the Golgi lumen (10–12). ZnT5–7, if not all, may be responsible for loading zinc to secretory, membrane-bound, or organelle-resident proteins that require zinc for manifestation of their biological activities, but evidence supporting this assumption has been lacking.

There are a number of secretory or membrane-bound enzymes that require zinc as an essential component; e.g. matrix metalloproteinases (13), angiotensin-converting enzyme (14), carboxypeptidases (15), and alkaline phosphatases (ALPs) (16). ALPs are a group of glycosylphosphatidylinositol (GPI)-anchored membrane proteins exposed to the extracellular site (17). In mammals, four types of ALPs, tissue-nonspecific ALP (TNAP), placental ALP (PLAP), intestinal ALP (IAP) and germ cell ALP, have been identified. They contain two tightly bound zinc ions per polypeptide in their active site (17), and the binding of zinc likely occurs in the biosynthetic-secretory pathway before reaching their final destination. Dimerization yields the active enzyme (18).

DT40 is a chicken B lymphocyte-derived cell line with a unique property that the homologous recombination activity is high, and thereby genes of interest can be disrupted at a high frequency (19, 20). Furthermore, as described later, it contains counterparts of mammalian ZnT1–7 genes, and most of their mRNAs are expressed. DT40 cells also express TNAP at the extracellular site of their cytoplasmic membrane. Using DT40 cells and ALPs, here we describe the first evidence that both ZnT5 and ZnT7 are required for loading zinc to ALPs in the biosynthetic-secretory pathway.

* This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T. K.). The costs of publication of this article therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 81-75-753-6273; Fax: 81-75-753-6274; E-mail: kambe1@kais.kyoto-u.ac.jp.

Received for publication, October 1, 2004, and in revised form, November 2, 2004
Published, JBC Papers in Press, November 2, 2004, DOI 10.1074/jbc.M411247200

Published, JBC Papers in Press, November 2, 2004, DOI 10.1074/jbc.M411247200

From the Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502 and the Department of Life Science, Faculty of Bioreources, Mie University, Mie 514-8507, Japan

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AY703476 and AY703477.

§ Supported by the 21st Century Center of Excellence Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y. Y.-I. and T. K.) and by the Novartis Foundation (Japan) for the Promotion of Science (to T. K.). 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.

1 The abbreviations used are: ZIP, ZRT/IRT-related protein; ZnT, zinc transporter; CDF, cation diffusion facilitator; ALP, alkaline phosphatase; TNAP, tissue nonspecific ALP; PLAP, placenta ALP; IAP, intestinal ALP; GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum; FCS, fetal calf serum; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI-PET, GPI-phosphoethanolamine transferase.

2 This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y. Y.-I. and T. K.) and by the Novartis Foundation (Japan) for the Promotion of Science (to T. K.). 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.

3 Supported by the 21st Century Center of Excellence Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y. Y.-I. and T. K.) and by the Novartis Foundation (Japan) for the Promotion of Science (to T. K.). 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.
**ZnT5 and ZnT7 Are Essential for ALP Activity**

**Cell Culture and Transfection**—Fetal calf serum (FCS, Trace Scientific Ltd.) was heat-inactivated. HeLa and HEp2 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FCS at 37 °C. DT40 cells (gift from Dr. Shunichi Takeda) were maintained in RPMI 1640 (Sigma) supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco BRL). The membrane was hybridized to a non-radioactively labeled cDNA probe. Radioimages were obtained as described above.

**RNA Preparation and Northern Blotting**—Total RNA was extracted from DT40 cells using Sepasol R I (Nacalai). The RNA (20 µg) was electrophoresed on agarose gel and transferred to nitrocellulose membrane filter. An FCS buffer was used, because the membrane was not hybridized to an appropriately radiolabeled cDNA probe. Radioimages were obtained as described above.

**Preparation of Membrane Fraction—**DT40 cells (2 × 10^7) were collected and washed with PBS. These cells were resuspended in 2 ml of cold homogenizing buffer (0.25 M sucrose, 20 mM HEPES, and 1 mM EDTA) and homogenized with 20 strokes of a 1 ml Dounce homogenizer. ALPs firmly bind with zinc, and therefore the presence of 1 mM EDTA in the homogenizing buffer does not affect the enzyme activity. To remove the nucleoside, the homogenate was centrifuged at 2,200 rpm for 10 min at 4 °C. The post-nuclear supernatant was centrifuged at 12,000 rpm for 60 min at 4 °C. The pellet, which contained cytoplasmic and organelle membranes, was lysed in ALP lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, and 0.1% Triton X-100), and stored at −70 °C until use. For fractionation of the organelles, the sample was centrifuged on a 0.7–1.7 M sucrose gradient as described previously (10) and separated into 10 fractions. The concentration of protein was determined with a protein assay kit (Bio-Rad) using bovine serum albumin as a standard.

**Immunoblot Analysis**—Membrane proteins were mixed with 6× SDS sample buffer and then incubated at 70 °C for 10 min, or total cellular proteins were lysed in the same buffer and then boiled at 100 °C for 5 min before electrophoresis. Proteins were separated with electrophoresis through 8% SDS-polyacrylamide gels. After transfer of proteins to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences), the blot was blocked with blocking solution (5% skim milk and 0.1% Tween 20 in PBS) and then incubated with anti-ZnT5 (1:2000 dilution), anti-ZnT7 (1:5000 dilution), anti-calreticulin (Affinity Bioreagents; 1:1000 dilution), anti-calnexin (Stressgen; 1:1000 dilution) antibody in blocking solution. Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) were used at a dilution of 1:2000. The stained cells were observed under a confocal laser-scanning microscope (Fluoview, Olympus).

**Immunofluorescence Staining—**Immunofluorescence for ZnT5 was performed as described previously (10). Briefly, HeLa or HEp2 cells were fixed with 4% paraformaldehyde or methanol at −20 °C. The cells were incubated with anti-ZnT5 antibody followed by goat anti-mouse IgG (Jackson ImmunoResearch; 1:100 dilution) or anti-rabbit IgG (Molecular Probes; 1:40 dilution) conjugated with fluorescein isothiocyanate. The stained cells were observed by confocal microscopy (Fluoview, Olympus).

**Measurement of ALP Activity—**The membrane protein (1 µg) or total cellular protein (10 µg) lysed in ALP lysis buffer was preincubated for 10 min at room temperature. A 100-µl substrate solution (2 mg/ml p-nitrophenyl phosphate in 1 M diethanolamine buffer, pH 9.8, containing 0.5 mM MgCl₂) was added. After incubation for 10 min at room temperature, p-nitrophenol released by TNP was measured by the absorbance at 405 nm. For measurement of ALP activity, samples were kept at 65 °C for 30 min to inactivate TNP. Shrimp ALP (Roche Applied Science) was used as a standard.

**PI-PLC Digestion and Immunoprecipitation—**DT40 cells (2 × 10⁶) expressing PLAP were incubated in culture medium for 3 h and collected. These cells were washed with PBS and incubated with or without 0.2 unit of phosphatidylinositol-specific phospholipase C (PI-PLC) (Molecular Probes) in 0.5 ml of PBS for 1 h at 4 °C. The cell suspension was centrifuged for 10 min at 4 °C, and the resultant supernatant was subjected to immunoprecipitation with anti-PLAP antibody conjugated to agarose (Sigma) to concentrate PLAP detached from cell surface by PI-PLC digestion. The PLAP-complex beads were treated with 6× SDS sample buffer at 100 °C for 5 min, electrophoresed as described above, and immunoblotted.

**RESULTS**

**Subcellular Localization of ZnT5 in HeLa and HEp2—**We previously identified ZnT5 as a zinc transporter that was abundantly expressed in pancreatic β cells and was associated with...
insulin granules in the cells, which suggested that ZnT5 transports zinc into insulin granules to form an insulin hexamer containing two zinc ions (10). Messenger RNA of ZnT5, however, was detected ubiquitously (10, 24, 25), indicating that ZnT5 possesses other functions. When ZnT5 was overexpressed, its localization overlapped with that of the Golgi marker protein, GM130 (10), or Golgi 58K protein (24). Because localization of the endogenous ZnT5 remains to be determined, we immunostained the cells using antibodies against hZnT5 and various marker proteins of organelles. As shown in Fig. 1A, ZnT5 was found in the cytoplasmic vesicles and perinuclear portion in HeLa and HEp2 cells. The immunostaining against marker proteins, however, failed to reveal the specific marker that was completely superimposed with ZnT5 (data not shown). Then, we fractionated HeLa-derived organelles by sucrose gradient centrifugation, and the distribution of ZnT5 was compared with that of various organelle markers. Fig. 1B shows the immunoblot of proteins in the fractionated organelles. ZnT5 was mainly detected in fractions 5 and 6, which was similar to Sec23, a marker protein of COPII vesicles that bud from endoplasmic reticulum (ER) and carry cargo to the trans side of the Golgi apparatus, which faces cytoplasmic membrane where secretion occurs. The antibody against γ-adaptin used here recognizes two types of γ-adaptin with different sizes. Because ER produces vesicles with various densities during preparation, its marker protein (calreticulin) was detected in most fractions. The same results were obtained when another marker, calnexin, was used (data not shown). These results indicate that the endogenous ZnT5 is concentrated in early compartments of the secretory pathway such as COPII-coated vesicles and Golgi apparatus.

Expression of ZnT Members in DT40 Cells—To find whether the chicken-derived DT40 cell line expresses the orthologues of mammalian CDF family members, we examined their mRNA expression in DT40 cells. Comparison of the chicken expressed sequence tag sequences with mammalian chicken expressed sequence tag sequences with mammalian

### Table 1

| Gene     | Chicken ESTs | Expression in DT40 cells |
|----------|--------------|--------------------------|
| cZnT1    | +            | D                        |
| cZnT2    | +            | D                        |
| cZnT3    | +            | UD                       |
| cZnT4    | +            | D                        |
| cZnT5    | +            | D                        |
| cZnT6    | +            | D                        |
| cZnT7    | +            | D                        |
| cZnT8    | +            | NE                       |

*+, -, D, UD, and NE indicate presence, absence, detectable, undetectable, and not examined, respectively.*

#### Fig. 1. Subcellular localization of ZnT5. A, immunostaining of ZnT5 in HeLa cells (upper) and HEp2 cells (lower). HeLa cells were fixed with 4% paraformaldehyde and stained with anti-ZnT5 antibody, followed by second and third antibodies conjugated with Alexa 594. HEp2 cells were fixed with methanol at −20 °C and stained with anti-ZnT5 antibody, followed by second and third antibodies conjugated with Alexa 488. B, ZnT5 protein is concentrated into the fractions of secretory apparatus. The post-nuclear supernatant derived from HeLa cells was separated into 10 fractions by centrifugation on a 0.7–1.7 m sucrose gradient. Membrane protein from each fraction was analyzed by immunoblotting, and the distribution of ZnT5 in the gradient was compared with that of indicated proteins. Indicated proteins are markers of the following organelles: GM130 for Golgi apparatus, SEC23 for COPII vesicles, EEA1 for endosomes, γ1-adaptin for AP-1 vesicles, and Calreticulin for ER. The asterisk in the panel of GM130 indicates a nonspecific band.
panels are as described in Hisr disrupted, the...}

...that of wild-type cells. TNAP activity in ZnT5 -cells was reduced to about 45% of that in wild-type cells (Fig. 3B, upper panel) despite no significant difference in expression of TNAP mRNA (Fig. 3B, lower panel). This reduction was restored by expressing the exogenous ZnT5 cDNA from either chicken or human (about 80% or 90% activity compared with that of wild-type cells) (Fig. 3B, upper panel). Overexpression of hZnT5 in the wild-type DT40 did not increase TNAP activity (Fig. 3B, upper panel), indicating that zinc influx to secretory compartments by endogenous transporters is sufficient for the full expression of TNAP activity.

The overexpressed hZnT5 in DT40 cells was localized mainly in secretory compartments such as Golgi apparatus with immunofluorescence staining of the cells or immunoblotting after fractionation of organelles by sucrose gradient centrifugation (data not shown). Similar localization in DT40 cells was found for hZnT7 tagged with FLAG epitope (data not shown).

ZnT5 and ZnT7 Are Essential for the Full Activation of TNAP—Disruption of ZnT5 gene reduced TNAP activity, but 45% of the activity remained, suggesting that other transporters may also be involved in expression of TNAP activity. Kirschke and Huang (12) previously reported that ZnT7 residing in Golgi apparatus may transport zinc into the Golgi lumen, because overexpression of ZnT7 in CHO cells accumulates zinc in Golgi apparatus in a high zinc condition. To examine contribution of ZnT7 to TNAP activity in DT40 cells, we generated ZnT7-deficient DT40 cells (ZnT7 -/- cells) (Fig. 2B) and compared their phenotypes with those of ZnT5 -cells and wild-type cells. ZnT7 -/- cells showed lower TNAP activity than wild-type cells (80% of the wild-type), but this reduction was smaller than that found in ZnT5 -cells (Fig. 4A, upper panel). Thus contribution of ZnT7 to TNAP activity appears to be minor. TNAP activity was severely decreased in DT40 cells deficient in both ZnT5 and ZnT7 (ZnT5 -/- ZnT7 -/- cells) (Fig. 4A, upper panel). The TNAP activity in ZnT5 -ZnT7 -/- cells was restored to 75 and 85% of that in the wild-type cells by expression of human ZnT5 and ZnT7, respectively. In all cases, the level of TNAP mRNA was unchanged (Fig. 4A, lower panel). ZnT5 -ZnT7 -/- cells showed slightly slow growth; doubling time for ZnT5 -ZnT7 -/- cells was 9.5 h, while that for wild-type cells was 7.3 h. Microscopic morphology of ZnT5 -ZnT7 -/- cells was normal and the sensitivity to a high zinc concentration was similar to that of the wild-type cells. Like the wild-type DT40 cells (see Fig. 3A, upper panel), the TNAP activity in ZnT5 -ZnT7 -/- cells restored by expressing hZnT5 or hZnT7 disappeared when cultured in the zinc-free medium, but the
addition of zinc to the medium maintained the activity (Fig. 4B, upper panel). Levels of mRNAs for hZnT5 and hZnT7 expressed in ZnT5 ZnT7−/− cells were unchanged under the culture conditions tested (Fig. 4B, lower panel). The addition of cobalt or manganese to the zinc-free medium had no effects (data not shown). We conclude from these results that both ZnT5 and ZnT7 are required for expression of the full TNAP activity.

ZnT5 and ZnT7 Contribute to the Conversion of Apo-ALP to Holo-ALP—Marked reduction of TNAP activity in ZnT5 ZnT7−/− cells (Fig. 4A, upper panel) raises two possibilities; TNAP protein may not be present in this mutant cell although its mRNA is present at a normal level (Fig. 4A, lower panel) or TNAP protein may be present as an inactive enzyme. To examine which is the case, we used PLAP as a reporter protein, because the antibody against PLAP was available but the antibody against PLAP protein may have a slow turnover rate so that the active enzyme bound with zinc before cell culture in the zinc-free medium remains to exhibit PLAP activity. It is also possible that PLAP protein has a high affinity to zinc as compared with that of TNAP and therefore some active PLAP molecules can be formed even in zinc-free medium by binding with zinc that would exist at a very low concentration. The PLAP activity was fully restored in the cells cultured in the medium supplemented with zinc (Fig. 5B).

When PLAP was expressed in ZnT5 ZnT7−/− cells, its activity was very low (Fig. 5D). It is noted that immunoblot analysis indicates that PLAP protein was present in ZnT5 ZnT7−/− cells at a level similar to that in wild-type cells (Fig. 5E). Expression of hZnT5 or hZnT7 in ZnT5 ZnT7−/− cells restored PLAP activity to the level comparable to that in wild-type cells (Fig. 5D) without affecting PLAP protein level (Fig. 5E). These results suggest that PLAP in ZnT5 ZnT7−/− cells fails in formation of the active conformation due to deficiency of zinc in the biosynthetic-secretory pathway. The same would be true for the endogenous TNAP.

Subsequently, we examined the localization of PLAP in the wild-type and ZnT5 ZnT5−/− cells. Immunofluorescence analysis showed that PLAP protein was mainly localized to cytoplasmic membrane in both cells, but some vesicles were also positive (Fig. 6A). This localization of PLAP was ensured by treating the cells with PI-PLC that releases GPI-anchored protein from the cell surface. PLAP released from the cell surface by PI-PLC digestion was detected by immunoblot analysis after immunoprecipitation with anti-PLAP antibody. As shown in Fig. 6B, the amount of PLAP released from ZnT5 ZnT7−/− cells was similar to that released from the wild-type cells. PLAP released from the wild-type cells showed enzyme activity, but that from ZnT5 ZnT7−/− cells did not (Fig. 6C), indicating that PLAP was transported to plasma membrane despite having little activity.

Lastly, we investigated whether the addition of zinc into the culture medium restores PLAP activity in ZnT5 ZnT7−/− cells, because the culture medium contains low micromolar level of zinc derived from FCS (27), and this level may be insufficient for formation of the active enzyme. When the wild-type cells and ZnT5−/− ZnT7−/− cells were cultured for 18 h in the medium containing 50 μM ZnSO₄, a slight increase of PLAP activity was seen in both cells but the enzyme activity of ZnT5 ZnT7−/− cells was much less than that of the wild-type cells (Fig. 6D). Taken together, both ZnT5 and ZnT7 are essential for zinc incorporation into ALPs in secretory pathway to convert apo-ALPs to holo-ALPs.

DISCUSSION

CDF family members transport cytosolic zinc to the extracellular domain or into the lumens of intracellular organelles (3–6, 8). Of these members, ZnT5–7 have been shown to reside in the Golgi apparatus (10–12), suggesting that they are responsible for the zinc entry into the lumens thereby loading zinc to proteins in the biosynthetic-secretory pathway, but this hypothesis has not been verified. In this study, we focused on ZnT5 and ZnT7, because they are highly homologous in their cation efflux domains (pfam01545) that are essential for zinc.
transport, although ZnT5 has a unique and very long amino-terminal domain whose function is unknown (5, 8, 25). DT40 cells were examined for their usefulness to investigate the role of ZnT5 and ZnT7 in the secretory pathway. Fortunately, this cell line expressed both transporters and TNAP, a member of the ALP family, which is anchored to the extracellular domain of cytoplasmic membrane via GPI (17). ALPs contain tightly bound zinc essential for the enzyme activity, and zinc is thought to be loaded in the biosynthetic-secretory pathway. The activity of ALPs, therefore, can be a reporter of zinc metabolism in this pathway. Clear dependence of the TNAP activity in DT40 on zinc in the culture medium indicated that this cell line would be competent for our study.

A partial decrease of the TNAP activity in DT40 deficient in ZnT5 or ZnT7 and marked loss of the activity in the mutant lacking both genes clearly indicate that both transporters are required for the full expression of TNAP activity. Disruption of the ZnT5 gene caused a partial decrease of TNAP activity, and the residual activity was 45% of that in the wild-type DT40 cells. Disruption of ZnT7 gene resulted in a partial but small decrease; 80% of the TNAP activity remained. These results suggest that the total zinc-mobilizing activity by ZnT5 and ZnT7 is sufficient for the full activation of TNAP molecules produced in DT40 cells. This notion is supported by the fact that overexpression of ZnT5 in the wild-type cells does not increase TNAP activity. Partial responsibility of ZnT5 in the activation of TNAP appears to hold true in mammals. TNAP is crucial for postnatal bone mineralization (28). The ZnT5−/− mouse shows osteopenia due to impairment of osteoblast maturation, and partial reduction of TNAP activity is seen in osteoblasts differentiated in vitro from precursor cells prepared from this mouse (24).

DT40 cells express ZnT5 and ZnT7 that both function to activate TNAP. Such redundant expression is not necessarily seen in all types of mammalian cells; ZnT5 is ubiquitously expressed, but ZnT7 is less widely expressed (10, 12, 25). Although the physiological significance of the redundant expression of zinc transporters is not known, another transporter(s) may substitute for ZnT7 in ZnT5-positive but ZnT7-negative cells. The best candidate is ZnT6, because it resides around the trans-Golgi network (11). However, ZnT6 lacks the His-rich region in the cytoplasmic loop, a characteristic of ZnT members, and two His residues in transmembrane domains, which
are highly conserved among all ZnT members and thought to be important for transporter function, have been altered to Leu and Phe (5). These structural features suggest that ZnT6 may not be as efficient as ZnT5 and ZnT7. This may be supported by our observation that TNP activity is largely lost in ZnT5−/−ZnT7−/− cells, although they express ZnT6.

In this study, we examined two ALPs as reporter proteins of zinc metabolism in the secretory pathway. There are many zinc proteins that travel in the biosynthetic-secretory pathway. To know whether ZnT5 and ZnT7 also participate in zinc loading to these proteins, further studies using these proteins as reporters are needed. In this respect, GPI-phosphoethanolamine transferase (GPI-PET), a member of the ALP superfamily enzymes (29), which adds phosphoethanolamine groups to GPI anchors in ER and requires zinc (30), is of interest. The activity of PLAP expressed in ZnT5−ZnT7−/− cells was significantly reduced, but the PLAP protein was exposed to the activity of GPI-PET in ZnT5.ZnT7−/− cells, one is that there is one or more unidentified zinc transporters in the ER. It is also possible that zinc in the ER is supplied from Golgi apparatus through the retrieval pathway. If this is the case, zinc in the ER of ZnT5.ZnT7−/− cells may originate from that transported into Golgi by ZnT6. Affinity of GPI-PET to zinc may be very high and therefore GPI-PET can bind with zinc that may be present at a low concentration in ER in ZnT5−ZnT7−/− cells, resulting in the formation of active conformation of the enzyme.

Instead of TNAP, PLAP expressed in DT40 cells successfully acted as a reporter that conveyed zinc metabolism in the biosynthetic-secretory pathway. Surprisingly, like the active PLAP in the wild-type DT40, the inactive PLAP protein in ZnT5.ZnT7−/− cells was transported to cytoplasmic membrane where it was anchored via GPI. It is believed in eukaryotes that misfolded or incompletely assembled proteins are eventually degraded by the ER-associated degradation pathway after transported back into the cytoplasm, or lysosomal pathway (31). The inactive PLAP in ZnT5.ZnT7−/− cells may be misfolded or incompletely assembled through unsuccessful binding with zinc and would be a target of cellular degradation pathways. At the present time we have no evidence for misfolding of PLAP, but if we assume this is the case, the result that PLAP in ZnT5.ZnT7−/− cells is transported to the cytoplasmic membrane may indicate that ZnT5 and/or ZnT7 are responsible not only for activation of secretory zinc proteins but also for zinc transport to keep the degradation pathways fully active. Very recently, Ellis et al. (32) reported that Saccharomyces cerevisiae msc2, which is highly homologous to the C-terminal portion of ZnT5 and ZnT7, is one route of zinc entry into ER in yeast. The msc2 mutants are defective in ER-associated degradation pathway under zinc-limited conditions.

Redundant expression of zinc transporters has made it difficult to verify the precise role of individual transporters. Because DT40 has high homologous recombination activity and expresses most of the ZnT members, this cell line is expected to be useful for elucidating the cellular functions of zinc transporters, including the molecular mechanism of zinc transport and interplay of transporters.

Acknowledgments—We thank Dr. Shunichi Takeda (Kyoto University) for providing us the DT40 cells and drug selection marker cas- settes used in this work and Dr. Ryuzo Sasaki (The University of Shiga Prefecture) for critical reading of the manuscript.

REFERENCES

1. Vallee, B. L., and Falchuk, K. H. (1993) Physiol. Rev. 73, 79–118
2. Berg, J. M., and Shi, Y. (1996) Science 271, 1081–1085
3. Winding, P., and Berchtold, M. W. (2001) J. Biol. Chem. 276, 2330–2337
4. Kambe, T., Yamaguchi-Iwai, Y., Sasaki, R., and Nagao, M. (2004) Cell Mol. Life Sci. 61, 49–68
5. Liu, J. P., and Cousins, R. J. (2004) Annu. Rev. Nutr. 24, 151–172
6. Eide, D. J. (2004) Pflugers Arch. 447, 786–800
7. Le Du, M. H., and Millan, J. L. (2002) J. Biol. Chem. 277, 26389–26396
8. Kilshtain-Vardi, A., Glick, M., Greenblatt, H. M., Goldblum, A., and Shoham, G. (2003) Acta Crystallogr. Sect. D Biol. Crystallogr. 59, 323–333
9. Le Du, M. H., Stibbrand, T., Taussig, M. J., Menez, A., and Stura, E. A. (2001) J. Biol. Chem. 276, 9158–9165
10. Millan, J. L., and Fishman, W. H. (1995) Crit. Rev. Clin. Lab. Sci. 32, 1–39
11. Le Du, M. H., and Millan, J. L. (2002) J. Biol. Chem. 277, 49808–49814
12. Buerstedde, J. M., and Takeda, S. (1991) Cell 67, 179–188
13. Overall, C. M., and Lopez-Otin, C. (2002) Nat. Rev. Cancer 2, 657–672
14. Natesh, R., Schwager, S. L., Sturrock, E. D., and Acharya, K. R. (2004) Nature 431, 551–554
15. Kilshtain-Vardi, A., Gluck, M., Greenblatt, H. M., Goldblum, A., and Shoham, G. (2003) Acta Crystallogr. Sect. D Biol. Crystallogr. 59, 323–333
16. Le Du, M. H., Stibbrand, T., Taussig, M. J., Menez, A., and Stura, E. A. (2001) J. Biol. Chem. 276, 9158–9165
17. Millan, J. L., and Fishman, W. H. (1995) Crit. Rev. Clin. Lab. Sci. 32, 1–39
18. Le Du, M. H., and Millan, J. L. (2002) J. Biol. Chem. 277, 49808–49814
19. Buerstedde, J. M., and Takeda, S. (1991) Cell 67, 179–188
20. Overall, C. M., and Lopez-Otin, C. (2002) Nat. Rev. Cancer 2, 657–672
21. Natesh, R., Schwager, S. L., Sturrock, E. D., and Acharya, K. R. (2004) Nature 431, 551–554
22. Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., and Kuroski, H. (1994) EMBO J. 13, 1341–1349
23. Okumura, K., Nogami, M., Matsushima, Y., Matsumura, K., Nakamura, K., Taguchi, H., and Kitagawa, Y. (1998) Biosci. Biotech. Biochem. 62, 1640–1642
24. Inoue, K., Matsuda, K., Itoh, M., Kagawauchi, H., Tonomike, H., Azayagi, T., Nagai, R., Horii, M., Nakamura, Y., and Tanaka, T. (2002) Hum. Mol. Genet. 11, 1775–1784
25. Seve, M., Chimenti, F., Devergnans, S., and Pavier, A. (2004) BMC Genomics 5, 32
26. Crawford, K., Weissig, H., Binette, F., Millan, J. L., and Goetinck, P. F. (1995) Dev. Dyn. 204, 48–56
27. Palmeter, R. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1219–1223
28. Pedde, K. N., Blair, L. R., Silverstein, J., Coburn, S. P., Ryan, L. M., Weinstein, R. S., Veyhman, K., Nantzas, S., Onken, J. L., MacGregor, G. R., and Whyte, M. P. (1999) J. Bone Miner. Res. 14, 2015–2026
29. Galperin, M. Y., and Jedrzejas, M. J. (2001) J. Biol. Chem. 276, 551–554
30. Mann, K. J., and Selever, D. (2001) Biochemistry 40, 1205–1213
31. Trembetta, E. S., and Parodi, A. J. (2003) Annu. Rev. Cell Dev. Biol. 19, 649–670
32. Ellis, C. D., Wang, F., MacDiarmid, C. W., Clark, S., Lyon, T., and Eide, D. J. (2004) J. Cell Biol. 166, 325–335
33. Boardman, P. E., Sanz-Ezquerro, J., Overton, I. M., Burt, D. W., Bosch, E., Fong, W. T., Tickle, C., Brown, W. R., Wilson, S. A., and Hubbard, S. J. (2002) Curr. Biol. 12, 1965–1969

Downloaded from http://www.jbc.org/ by guest on July 23, 2018
Zinc Transporters, ZnT5 and ZnT7, Are Required for the Activation of Alkaline Phosphatases, Zinc-requiring Enzymes That Are Glycosylphosphatidylinositol-anchored to the Cytoplasmic Membrane

Tomoyuki Suzuki, Kaori Ishihara, Hitoshi Migaki, Wataru Matsuura, Atsushi Kohda, Katsuzumi Okumura, Masaya Nagao, Yuko Yamaguchi-Iwai and Taiho Kambe

J. Biol. Chem. 2005, 280:637-643.
doi: 10.1074/jbc.M411247200 originally published online November 2, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411247200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 10 of which can be accessed free at http://www.jbc.org/content/280/1/637.full.html#ref-list-1