The majority of CDF/ZnT zinc transporters form homo-oligomers. However, ZnT5, ZnT6, and their orthologues form hetero-oligomers in the early secretory pathway where they load zinc onto zinc-requiring enzymes and maintain secretory pathway functions. The details of this hetero-oligomerization remain to be elucidated, and much more is known about homo-oligomerization that occurs in other CDF/ZnT family proteins. Here, we addressed this issue using co-immunoprecipitation experiments, mutagenesis, and chimera studies of hZnT5 and hZnT6 in chicken DT40 cells deficient in ZnT5, ZnT6, and ZnT7 proteins. We found that hZnT5 and hZnT6 combine to form heterodimers but do not form complexes larger than heterodimers. Mutagenesis of hZnT6 indicated that the sites present in transmembrane domains II and V in which many CDF/ZnT proteins have conserved hydrophilic amino acid residues are not involved in zinc binding of hZnT6, although they are required for zinc transport in other CDF/ZnT family homo-oligomers. We also found that the long N-terminal half of hZnT5 is not necessary for its functional interaction with hZnT6, whereas the cytosolic C-terminal tail of hZnT5 is important in determining hZnT6 as a partner molecule for heterodimer formation. In DT40 cells, cZnT5 variant lacking the N-terminal half was endogenously induced during periods of endoplasmic reticulum stress and so seemed to function to supply zinc to zinc-requiring enzymes under these conditions. The results outlined here provide new information about the mechanism of action through heterodimerization of CDF/ZnT proteins that function in the early secretory pathway.

Zinc transporter (SLC30A) proteins, also known as CDF/ZnTs, are responsible for the efflux of cytosolic zinc from cells and the mobilization of zinc into intracellular organelles to remove the excess zinc and to ensure a supply of zinc to zinc-binding proteins (1–8). Recently, more data about the biochemical properties of these transporter molecules have been published, including their substrate specificity, structural conformation, metal transport mechanisms, and cellular or physiological functions (1, 5, 8–13). CDF/ZnT proteins perform many cellular functions, but the supply of zinc to the early secretory pathway is one of their main tasks. This is because numerous zinc-binding proteins that are secreted out of the cells or are resident within the intracellular organelles capture zinc as they are transported in the early secretory pathway, although a recent study suggests the existence of an alternative mechanism (14). ZnT5, ZnT6, ZnT7, and their orthologues have been shown to play key roles in the performance of this task (15–19).

Most CDF/ZnT proteins form homo-oligomers (dimers) to perform their functions (12, 13, 16, 20–22). The x-ray structure of the YiiP, Escherichia coli CDF transporter, has revealed important characteristics of these CDF/ZnT homodimers (12). The YiiP orthologous has three tetrahedral zinc-binding sites formed by the cytosolic C-terminal tail and one tetrahedral zinc-binding site formed by four conserved hydrophilic residues (Asp\(^{159}\), Asp\(^{49}\), His\(^{153}\), and Asp\(^{157}\)) in transmembranes (TMs) II and V (12). Information obtained from a number of mutagenesis studies has shown that the zinc-binding site embedded in the TM domains (TMDs) is essential for zinc transport across the cellular membrane by the CDF/ZnT proteins, suggesting that this may be true for all of the CDF/ZnT homo-oligomers (reviewed in Ref. 23).

In contrast, the characteristics of the hetero-oligomerization between ZnT5 and ZnT6 and their functional orthologues are poorly understood (19, 24, 25). This is because ZnT5 and ZnT6 differ from other CDF/ZnT proteins in two important respects. First, hZnT5 and its orthologues have an unusually long N-terminal sequence fused to the C-terminal half of six TMDs termed cation efflux domains (19, 26, 27). Second, vertebrate orthologues of ZnT6 lack two of the four conserved hydrophilic residues in TMs II and V (28) (Fig. 1). Moreover, the functional yeast orthologues Zrg17p (Saccharomyces cerevisiae) and Zrg17 (S. pombe) show little homology to those of vertebrates. These discrepancies make it dif-
Characterization of ZnT5/ZnT6 Heterodimerization

| Name     | TM II | TMV |
|----------|-------|-----|
| YiP      | 45 49 | 153 157 |
| Mtp1     | 85    | 253 266 |
| ZnT1     | 39    | 244 257 |
| ZnT8     | 102   | 213 226 |
| ZnT5     | 447   | 588 601 |
| ZnT7     | 66    | 233 246 |
| Msc2p    | 416   | 524 537 |
| Cis4     | 378   | 582 595 |
| ZnT6     | 62    | 194 207 |

![Figure 1. Comparison of the conserved hydrophilic residues in TMs II and V among CDF/ZnT proteins known to form oligomeric complexes. BLASTp analyses of the following sequences were performed using Yip amino acid sequence. Asp40, Asp32, His153, and Asp157 contribute to the zinc-binding site in Yip (12, 33). The sequences used are as follows: NP418350 for Yip (E. coli), AAR23528 for Mtp1 (poplar), NP067017 for Znt1 (human), NP776230 for Znt8 (human), AAP21969 for Znt7 (human), NP075053 for Znt5 (human), NP010491 for Msc2p (S. cerevisiae), NP94694 for Cis4 (S. pombe), NP060434 for Znt6 (human). Znt6 has two hydrophilic residues in these four conserved positions. Zrg17p, a functional orthologue of Znt5 in S. cerevisiae (NP14437), and Zrg17, a functional orthologue of hZnt6 in S. pombe (NP59576), are not applicable to this alignment. The residues His90 and His129 of Znt5 in TMs XI and XIV (corresponding to the TMs II and V of others) have recently been shown to be involved in the zinc-binding site of Znt5 (31). Conserved hydrophilic residues are shaded in black or in gray. Nonconserved hydrophilic residues of Znt6 are indicated in open boxes.](image)

We have previously reported that DT40 cells are a useful system in which to examine oligomer formation between hZnt5, hZnt6, and hZnt7 using a combination of gene disruption/re-expression and co-immunoprecipitation experiments (24). Therefore, we used this system for the mutagenesis and chimera studies of hZnt5 and hZnt6. We found that hZnt5 and hZnt6 form heterodimers to transport zinc into the early secretory pathway. We also found that the positions of the conserved His or Asp residues within TMs II and V of the CDF/ZnT proteins do not form part of the zinc-binding site in hZnt6. This suggests that hZnt6 is unlikely to function as part of the zinc-binding sites embedded in the TMDs of the hZnt5/hZnt6 heterodimers. The unique long N-terminal half of hZnt5 appears to be dispensable for the formation of hZnt5/hZnt6 heterodimers, but the cytosolic C-terminal tail is important as a dominant factor in determining the binding of hZnt6 to form a heterodimer. Consistent with this finding is the fact that the chicken-specific cZnt5 variant lacking the N-terminal half was endogenously induced during periods of endoplasmic reticulum stress and seemed to function to supply zinc-requiring enzymes with zinc. These results aid in the understanding of the unique properties of CDF/ZnT proteins that lead to the formation of heterodimers.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Chicken B lymphocyte-derived DT40 cells were maintained in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum (Biowest), 1% chicken serum (Invitrogen), and 50 μM 2-mercaptoethanol (Sigma) at 39.5 °C. DNA transfection into DT40 cells was carried out as previously described (16, 24). More than three independent clones were established per disruptant.

Plasmid Construction—Plasmids used for the expression of FLAG-tagged human Znt5 (FLAG-hZnt5), HA-tagged human Znt6 (HA-hZnt6), and FLAG- or HA-tagged human Znt7 (hZnt7-FLAG or hZnt7-HA) have been described previously (24). Plasmids used for the expression of HA-hZnt5, Myc-hZnt6, and FLAG-hZnt6 were constructed by inserting each cDNA into pA-Puro, pA-Ecopt, or pA-Zeo vectors. The plasmid used to express ANZnt5 was constructed by deleting the N-terminal half of hZnt5 (amino acids 1–387) in which the start Met corresponds to Met388 in the full-length hZnt5. The plasmid used for the expression of the hZnt5/hZnT7 chimera protein was constructed by fusing the DNA fragment encoding amino acids 1–398 of hZnt5 to the fragment encoding amino acids 18–376 of hZnt7 using a two-step PCR method as previously described (24, 29). Other plasmids used for the expression of the hZnt5/hZnt7 chimera (hZnt7(TM1–6)pA-Puro) or the hZnt6 mutants (HA-hZnt6L66H-F201H and HA-hZnt6D70A-D205A) were also constructed using this two-step PCR method. The mutated cDNAs were inserted into pA-Puro, pA-Zeo, or pA-Ecopt vectors (24). All of the plasmids were linearized with appropriate restriction enzymes prior to electro precipitation.

Measurement of TNAP Activity—Total cellular protein extracts were prepared from cells lysed in alkaline phosphatase lysis buffer and 2 or 3 μg of protein was used for measuring TNAP activity as previously described (24). Shrimp alkaline phosphatase (Roche Applied Science) was used as the standard.

Immunoblotting and Immunoprecipitation Experiments—Immunoblotting and immunoprecipitation were performed as previously described (24). Briefly, the blotted polyvinylidene difluoride membrane (Pall) was blocked with a solution of 5% skimmed milk and 0.1% Tween 20 in phosphate-buff ered saline prior to incubation with anti-hZnt5 (26) (1:1000 dilution), anti-FLAG M2 (Sigma; 1:2000 dilution), anti-HA 12CA5 (Roche Applied Science; 1:200 dilution), anti-HA 3F10 (Roche Applied Science; 1:20000 dilution), anti-HA HA-11 (Covance; 1:2000 dilution), or anti-Myc 9E10 (Santa Cruz; 1:1000) antibodies in blocking solution. For immunoprecipitation, the DT40 cell lysates were rotated with anti-FLAG M2 (1:200 dilution), anti-HA 3F10 (1:200 dilution), or anti-HA HA-11 (1:200 dilution) antibodies for 1 h prior to the addition of 10 μl of protein G-Sepharose beads (Amer sham Biosciences). After incubating for 2 more hours, immunoprecipitates were subjected to immunoblotting.
Characterization of ZnT5/ZnT6 Heterodimerization

ZnT5 and ZnT6 Form Heterodimers—To address the question of whether ZnT5 and ZnT6 form heterodimers or larger complexes, we established a series of ZnT5−/−ZnT6−/−DT40 cell lines expressing various combinations of hZnT5 and/or hZnT6 all carrying different tags (or no tag) on the N terminus (Figs. 2 and 3). We then performed co-immunoprecipitation experiments. First, we confirmed that no functional interaction occurs between FLAG-hZnT6 and HA-hZnT6 (Fig. 2A) or FLAG-hZnT5 and HA-hZnT5 (Fig. 2B) when we co-expressed their trans-genes in ZnT5−/−ZnT6−/−ZnT7−/− cells. However, a functional interaction was seen between FLAG-hZnT5 and HA-hZnT6 when both were co-expressed in the ZnT5−/−ZnT6−/−ZnT7−/− cells (Fig. 2, A and B). To determine which particular complexes were formed (heterodimers or larger complexes), we next examined whether FLAG-hZnT6 and HA-hZnT6 co-immunoprecipitated with the anti-FLAG or HA antibody when simultaneous expressed alongside hZnT5. Co-expression of hZnT5 in the ZnT5−/−ZnT6−/−ZnT7−/− cells already expressing both FLAG-hZnT6 and HA-hZnT6 restored TNAP activity (a marker for zinc transport into the early secretory pathway) to the same level as that seen in wild-type DT40 cells. These results suggest the formation of ZnT5/ZnT6 hetero-oligomers in the cells (Fig. 3A). Co-immunoprecipitation experiments with anti-FLAG or anti-HA antibody did not show any functional interaction between FLAG-hZnT6 and HA-hZnT6 but did show an interaction between FLAG-hZnT6 and hZnT5 or HA-hZnT6 and hZnT5 (Fig. 3B, middle and right panels). These results indicate that hZnT5/hZnT6 hetero-oligomers containing one hZnT6 monomer were formed in the ZnT5−/−ZnT6−/−ZnT7−/− cells co-expressing FLAG-hZnT6, HA-hZnT6, and hZnT5 (Fig. 3C). To examine the possibility that the hZnT5/hZnT6 hetero-oligomers include hZnT5 over two protomers, we repeated the experiment using FLAG-hZnT5, HA-hZnT5, and Myc-hZnT6. Co-immunoprecipitation experiments with anti-FLAG or anti-HA antibodies did not show any functional interaction between FLAG-hZnT5 and HA-hZnT5 even with the co-expression of Myc-hZnT6, suggesting that hZnT5/hZnT6 hetero-oligomers do not include hZnT5 over two protomers (Fig. 3, D, middle and right panels, and E). Taken together, these results show that ZnT5/ZnT6 hetero-oligomers are composed of one ZnT5 monomer and one ZnT6 monomer. Thus, ZnT5 and ZnT6 form a heterodimer that is then able to supply zinc into the early secretory pathway.

The Two Conserved Hydrophilic Amino Acid Residues in TMs II and V of hZnT6 Are Not Involved in the Zinc-binding Site—Many CDF/ZnT proteins form homo-oligomers with the zinc-binding site being formed by four conserved hydrophilic residues (Asp and 2×His in vertebrates) present in TMs II and V (12, 23) (Fig. 1). From our sequence comparison (30), we found that almost all vertebrate ZnT6 proteins substitute two of these hydrophilic residues with two hydrophobic residues (Leu66 and Phe201 in hZnT6) (Fig. 1), whereas these four hydrophilic residues are conserved in vertebrate ZnT5 proteins. This leads to the question

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**FIGURE 3. Human ZnT5 and hZnT6 form heterodimers.** A, TNAP activity was measured using total cellular protein prepared from the indicated cell lines. Each value is the mean ± S.D. of triplicate experiments. WT, wild-type DT40 cells.

B, FLAG-hZnT6 and HA-hZnT6 do not form complexes even in the presence of hZnT5. Whole cell lysates were subjected to immunoprecipitation (IP) as described in the legend for Fig. 2 followed by immunoblot analysis using anti-FLAG, anti-HA, or anti-hZnT5 antibodies. To estimate the amount of FLAG-hZnT5 and HA-hZnT6 in the whole cell lysates, 10% of each aliquot was subjected to immunoblot analysis (Input panels). C, schematic summarizing the IP experiments in B. Human ZnT6 exists as a monomer within the hZnT5/hZnT6 hetero-oligomers. *, complexes not detected in D. D, FLAG-hZnT5 and HA-hZnT6 do not form complexes even in the presence of Myc-hZnT6. Whole cell lysates prepared from the indicated cell lines were subjected to immunoprecipitation as described for B. E, schematic summarizing the IP experiments in D. The two protomers of hZnT5 do not exist in the hZnT5/hZnT6 hetero-oligomers. C and E suggest that hZnT5/hZnT6 hetero-oligomers consist of only one monomer of each protein. F, H, and M, mean FLAG, HA, and Myc tags, respectively.
Characterization of ZnT5/ZnT6 Heterodimerization

Because the zinc-binding amino acid residues of hZnT5 (His<sup>451</sup> and Asp<sup>599</sup>) are essential for its zinc transporting activity (31), hZnT5/hZnT6 heterodimers may form a zinc-binding site embedded in TMDs using only the four hydrophilic residues present in TMs II and V of the hZnT5 component.

The N-terminal Portion of ZnT5 Is Not Required for Heterodimer Formation with ZnT6—The C-terminal halves of the ZnT5 orthologues share homology between vertebrates and yeast, but the N-terminal halves of them are less similar (19, 26, 27). ZnT6 and its functional orthologues show much less homology between vertebrates and yeast (19, 25, 28). Ellis et al. (25) found that hZnT5 failed to complement msc2 mutant yeast, even in the presence of Zrg17, suggesting that the N-terminal half of the ZnT5 orthologues may determine the partner ZnT6 orthologue and thus be required for heterodimer formation. To investigate the importance of the N-terminal half of hZnT5 in heterodimer formation with hZnT6, we expressed a FLAG-tagged N-terminal deletion mutant of hZnT5 that still contained the C-terminal half of hZnT5 (FLAG-ΔNhZnT5). The FLAG-ΔNhZnT5 was co-localized with HA-hZnT6 to the vesicular compartments when expressed in ZnT5<sup>−/−</sup>ZnT6<sup>−/−</sup>ZnT7<sup>−/−</sup> cells (Fig. 5A), indicating that the N-terminal half of hZnT5 is not required for its subcellular localization. Co-immunoprecipitation experiments showed that FLAG-ΔNhZnT5 formed a heterodimer with HA-hZnT6 in the same manner as wild-type FLAG-hZnT5 (Fig. 5B). TNAP activity in the ZnT5<sup>−/−</sup>ZnT6<sup>−/−</sup>ZnT7<sup>−/−</sup> cells expressing both the FLAG-ΔNhZnT5 mutant and HA-hZnT6 was also restored, although the activity was lower compared with that of ZnT5<sup>−/−</sup>ZnT6<sup>−/−</sup>ZnT7<sup>−/−</sup> cells expressing both wild-type FLAG-hZnT5 and HA-hZnT6 (Fig. 5C). These results suggest that the N-terminal half of hZnT5 is not required for heterodimerization with hZnT6 and the ability to supply zinc to TNAP. We found that ΔNcZnT5 mRNA was induced in DT40 cells during periods of endoplasmic reticulum stress along with small increases in full-length cZnT5 mRNA (supplemental Fig. S1) (17). This was not seen in human and mouse cell lines (data not shown). The ΔNcZnT5 induced in DT40 cells probably functions to supply zinc into the early secretory pathway (supplemental Fig. S1).

We also examined the possibility that the N-terminal half of hZnT5 may interfere with its homo-oligomerization by using a chimera protein of hZnT7 fused to the N-terminal half of hZnT5 (hZnT5N-hZnT7). TNAP activity in ZnT5<sup>−/−</sup>ZnT6<sup>−/−</sup>ZnT7<sup>−/−</sup> cells expressing hZnT5N-hZnT7 was restored to the same extent as that in ZnT5<sup>−/−</sup>ZnT6<sup>−/−</sup>ZnT7<sup>−/−</sup> cells expressing hZnT7 alone (data not shown). This suggests that the N-terminal half of hZnT5 is not responsible for the inability of hZnT5 to form homo-oligomers.

The Cytosolic C-terminal Tail of hZnT5 Is Important for Interaction with hZnT6—The C-terminal half of ZnT5 (ΔNcZnT5) is highly homologous to ZnT7, particularly in the cation efflux domains (pfam01545; 48% sequence identity in humans) (Fig. 6A) (26, 32), but it does not form heterodimer with ZnT7 (Fig. 6B). This suggests that ZnT5 may have specific domain(s) responsible for interaction with ZnT6. Reduced homology (~20%) within the short cytosolic N terminus and

**Figure 4.** The conserved Asp residues in TMs II and V of hZnT6 are not involved in the zinc-binding site of hZnT5/hZnT6 heterodimers. A, whole cell lysates prepared from the indicated cell lines were subjected to immunoprecipitation (IP). B, TNAP activity was measured using total cellular protein prepared from the cells. TNAP activity is expressed as milliunits (mU)/mg of cellular protein. Each value is the mean ± S.D. of triplicate experiments.
Characterization of ZnT5/ZnT6 Heterodimerization

A. FLAG-ΔNhZnT5 and HA-hZnT6 were co-localized in ZnT5/ZnT6−/− cells. Stably expressed FLAG-ΔNhZnT5 and HA-hZnT6 were double stained as described under "Experimental Procedures." The subcellular localization of FLAG-ΔNhZnT5 (left-hand panel) and HA-hZnT6 (middle panel) is shown. The merged image is shown in the right-hand panel. B, FLAG-ΔNhZnT5 forms heterodimers with HA-hZnT6. Whole cell lysates prepared from the indicated cell lines were subjected to immunoprecipitation (IP) as described. C, the C-terminal half of hZnT5 is essential for TNAP activation. TNAP activity was measured using total cellular protein prepared from wild-type cells, ZnT5−/−ZnT7−/− cells expressing FLAG-hZnT5 and HA-hZnT6, or FLAG-ΔNhZnT5 and HA-hZnT6. TNAP activity is expressed as milliunits (mU)/mg of cellular protein. Each value is the mean ± S.D. of triplicate experiments.

The N-terminal half of hZnT5 is not required for either heterodimer formation or TNAP activation. A, ΔNhZnT5 and hZnT6 are co-localized in ZnT5/ZnT6−/− cells. Stably expressed FLAG-ΔNhZnT5 and HA-hZnT6 were double stained as described under "Experimental Procedures." The subcellular localization of FLAG-ΔNhZnT5 (left-hand panel) and HA-hZnT6 (middle panel) is shown. The merged image is shown in the right-hand panel. B, FLAG-ΔNhZnT5 forms heterodimers with HA-hZnT6. Whole cell lysates prepared from the indicated cell lines were subjected to immunoprecipitation (IP) as described. C, the C-terminal half of hZnT5 is essential for TNAP activation. TNAP activity was measured using total cellular protein prepared from wild-type cells, ZnT5−/−ZnT7−/− cells expressing FLAG-hZnT5 and HA-hZnT6, or FLAG-ΔNhZnT5 and HA-hZnT6. TNAP activity is expressed as milliunits (mU)/mg of cellular protein. Each value is the mean ± S.D. of triplicate experiments.

DISCUSSION

Oligomerization is critical for the functioning of CDF/ZnT proteins (12, 13, 19–22, 24, 25, 33). However, ZnT5 and ZnT6, along with their orthologues, are different from other CDF/ZnT family members in that they form hetero-oligomers rather than homo-oligomers (dimers).

In this study, we hypothesized that ZnT5 and ZnT6 would form hetero-tetramers or bigger oligomers because ZnT6 lacks two of the four conserved hydrophilic residues in TMs II and V known to be essential both for the formation of a zinc-binding site embedded within the TMDs and for transportation of zinc across the cell membrane (12, 23). However, we found that hZnT5 and hZnT6 form heterodimers and that they are unlikely to use hZnT6 to form the zinc-binding site embedded in the TMDs. Thus, although hZnT5/hZnT6 heterodimers work as Zn2+/H+ exchangers in the same manner as other CDF/ZnT proteins (31, 34–36), they would seem to use a different mechanism for transporting zinc across the cell membrane.

Recently, Fu and co-workers (12, 33) reported important information about the zinc-binding site and mechanism of zinc expression of hZnT7(TM1–6)5(tail) restored TNAP activity up to almost 20% of that in wild-type cells, and this restoration was similar with or without co-expression of HA-hZnT6 (Fig. 6D). This suggests that hZnT7(TM1–6)5(tail) functions as a homo-oligomer in a similar manner to hZnT7 and that hZnT7(TM1–6)5(tail)/hZnT6 heterodimer complexes are almost inactive.

We also expressed a chimera of ΔNhZnT5 in which the short cytosolic N terminus was replaced by the corresponding portion of hZnT7 (Fig. 6A) and expressed it in ZnT5−/−ZnT7−/− (expressing cZnT6) and ZnT5−/−ZnT6−/−ZnT7−/− (lacking cZnT6) cell lines. TNAP activity was restored in ZnT5−/−ZnT7−/− cells to a level comparable with that seen in the nonchimeric ΔNhZnT5, but this was not the case in the ZnT5−/−ZnT6−/−ZnT7−/− cells (data not shown). These results indicate that the short cytosolic N terminus of ΔNhZnT5 is not so important for its interaction with hZnT6. Taken together, these results show that the cytosolic C-terminal tail of hZnT5, but not the short cytosolic N terminus of ΔNhZnT5 corresponding to the cytosolic loop between TMDs IX and X of full-length hZnT5, is important for its interaction with hZnT6.
Characterization of ZnT5/ZnT6 Heterodimerization

A

hZnT5 388
hZnT7 1
hZnT5 420
hZnT7 39
hZnT5 479
hZnT7 98
hZnT5 539
hZnT7 99
hZnT5 581
hZnT7 158
hZnT5 581
hZnT7 581
hZnT5 218
hZnT7 592
hZnT5 633
hZnT7 218
hZnT5 278
hZnT7 693
hZnT5 338
hZnT7 733
hZnT5 376
hZnT7 376

B

Input IP: αFLAG IP: αHA
αhZnT5
αHA
αFLAG
hZnT5: + - + - + - HA-hZnT6:
HA-hZnT6: + - - + - + hZnT5 chimera: Tail ΔNhZnT5
hZnT7-HA: - + - + - + ZnT5−ZnT6−/−ZnT7−/−
hZnT7-FLAG: + + - + + +

C

Input
αHA
αZnT5

HA-hZnT6:

hZnT5 chimera:

ZnT5−ZnT6−/−ZnT7−/−

D

HA-hZnT6: + - + - + +
hZnT5 chimera: Tail ΔNhZnT5

WT

ZnT5−ZnT6−/−ZnT7−/−

TNAP activity [μM/mg protein]

0 5 10 15 20 25 30

NS
transport by YiiP homodimers. From their report, we could speculate that two mechanisms are available for zinc transport across the cell membrane by YiiP homodimers: one mechanism is that two tetrahedral zinc-binding sites formed by the four conserved amino acid residues (Asp\(^49\), Asp\(^59\), His\(^139\), and Asp\(^157\)) within TMs II and V of each protomer work separately (12). The other is that the eight zinc-binding amino acid residues (four conserved residues within each protomer) cooperatively form zinc-binding sites at the dimeric interface (termed a bimetal-binding center) and then transport zinc (33). Our results strongly suggest that hZnT5/hZnT6 heterodimers use a mechanism similar to the former for zinc transport because the sites present in TMs II and V corresponding to the conserved Asp and His residues in many CDF/ZnT proteins are unlikely to contribute to the zinc-binding site of hZnT6. Thus, the hZnT6 component is not involved in zinc transport across the cell membrane. This is supported by the recent studies of Ohana et al. (31), which show hZnT6 to be catalytically nonfunctional. So what is the physiological relevance of ZnT6 to the ZnT5/ZnT6 heterodimers? We have previously reported that hZnT6\(\Delta\)Ser, in which the Ser-rich loop between TMs IV and V was deleted, had only 40% of the TNAP activity seen in the wild-type hZnT6 when co-expressed with hZnT5 (24). These results suggest the Ser-rich loop is not essential for zinc transport by hZnT5/hZnT6 heterodimers but is required if optimum activity is to be achieved. Interestingly, several human cell types and cell lines express a splice variant of hZnT6 mRNA lacking exon 9, which encodes most of this Ser-rich loop. It is plausible that hZnT6 and its vertebrate orthologues function as modulators of zinc transport activity by ZnT5/ZnT6 heterodimers via this Ser-rich loop. Further work is needed to investigate this hypothesis. The results of our mutational analysis using hZnT5 show that the cytosolic C-terminal tail of hZnT5 is important for its interaction with hZnT6 within the heterodimer. Because the hZnT7\(^{5a}(T M 1–6}\delta(t a i l)) chimera used in our study retains all the TMDs of hZnT7, we assumed that it would only form homooligomers in a manner analogous to hZnT7. However, our co-immunoprecipitation experiments clearly show that hZnT7\(^{5a}(T M 1–6}\delta(t a i l)) forms heterodimers with hZnT6, suggesting that the cytosolic C-terminal tail of hZnT5 is the dominant factor determining its interaction with hZnT6. Comparison of the amino acid sequences in the cytosolic C-terminal tails of ZnT5 orthologues between human, chicken, and yeast (S. cerevisiae) using BLASTp showed 84% identity (92% similarity) between human and chicken but only 10% identity (15% similarity) between human and yeast. This suggests that an interaction between hZnT5 and cZnT6 is possible, but an interaction between hZnT5 and Zrg17p is not. This may be one reason why the expression of hZnT5 complemented the defect in cZnT5-deficient DT40 cells but not in msc2 mutant yeast. A recent study reporting the crystallization of CzrB, *Thermus thermophilus* CDF transporter, showed that the cytosolic fragment of CzrB is sufficient for the formation of homodimers both in the apo or holo form without the need for TMDs (13). Taken together with our results, this suggests that the cytosolic C-terminal tail of CDF/ZnT proteins is important in determining their oligomeric partner molecules. The cytosolic C-terminal tail of CDF/ZnT proteins has been shown to be important for binding of both zinc and a signal transduction molecule (12, 13, 37). Further studies may reveal additional functions for the C-terminal region.

In contrast, the long N-terminal half of hZnT5 is not essential for the function of ZnT5/ZnT6 heterodimers. This finding was unexpected because the long N-terminal portion is the main feature of hZnT5 and its orthologues (19, 26, 27). However, we found that the endogenously expressed, N-terminally deleted form of ZnT5 (\(\Delta\)Nznt5) plays a role in zinc transport in DT40 cells during endoplasmic reticulum stress conditions (supplemental Fig. S1). This suggests that the N-terminal portion of ZnT5 orthologues is not required for its interaction with ZnT6 orthologues in all cells co-expressing both proteins. So what is the function of the long N-terminal half of hZnT5? We have previously reported that the growth of yeast expressing full-length hZnT5 was inhibited by zinc at a concentration of 10 \(\mu\)M, but yeast expressing \(\Delta\)NhZnT5 were unaffected (26). This result may suggest that the N-terminal half of ZnT5 has some function. Several studies using human cell lines have indicated that, when overexpressed, hZnT5 is able to transport zinc without overexpressing hZnT6 (26, 31). In such cases, the N-terminal half of hZnT5 may be important in allowing hZnT5 to form complexes other than hZnT5/hZnT6 heterodimers. Further investigation is required to clarify the physiological and cellular functions of the N-terminal half of hZnT5.

Human ZnT5 has one splice variant, ZnT5B (hZTL1), that has quite different characteristics from hZnT5 (38, 39). ZnT5B (hZTL1) is localized to the plasma membrane and functions to enable zinc uptake from the extracellular environment - the opposite direction of zinc transport by hZnT5/hZnT6 heterodimers. To date there has been no explanation of these differences, but our present results may shed some light on them. The sequence of ZnT5B (hZTL1) is unique in several respects. First, it lacks both the N-terminal 171 amino acids and the cytosolic C-terminal tail of canonical hZnT5 and instead has a nonconserved extra putative TMD at the C terminus that

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3 M. L. Ackland, personal communication.
4 A. Fukunaka, Y. Kurokawa, and T. Kambe, unpublished data.

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**Characterization of ZnT5/ZnT6 Heterodimerization**

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**FIGURE 6.** The cytosolic C-terminal tail of hZnT5 is important for interaction with hZnT6. A. An optimal alignment of amino acid sequences between \(\Delta\)NhZnT5 (amino acids 388–765 of hZnT5) and hZnT7. The shaded amino acids are identical. The six putative TMDs are indicated by the solid line below the amino acid sequences. Gray arrowheads indicate domain-swapping positions between \(\Delta\)NhZnT5 and hZnT7. B, human ZnT7 does not form hetero-oligomers with either hZnT5 or hZnT6. The indicated FLAG- or HA-tagged hZnT6, hZnT7, and untagged hZnT5 were expressed in ZnT5\(^{5a}(\Delta Nznt5)\) \(\times\) ZnT7\(^{-}\) cells and whole cell lysates were subjected to immunoprecipitation (IP) as described. C, the cytosolic C-terminal tail of hZnT5 is important for the interaction with hZnT6. Whole cell lysates prepared from the indicated cell lines were subjected to immunoprecipitation (IP) as described. The hZnT7\(^{5a}(\Delta Nznt5)\) chimera activates with HA-hZnT5. A nonspecific band (NS) is partially overlaps the immunoprecipitated ZnT5 mutants. D, the hZnT7\(^{5a}(\Delta Nznt5)\) chimera activates TNAP in ZnT5\(^{5a}(\Delta Nznt5)\) \(\times\) ZnT7\(^{-}\) cells with or without co-expression of hZnT6. TNAP activity was measured using total cellular protein prepared from the indicated cell lines. TNAP activity is expressed as milliunits (mU)/mg of cellular protein. Each value is the mean \(\pm\) S.D. of triplicate experiments. WT, wild-type DT40 cells; Tail, the hZnT7\(^{5a}(\Delta Nznt5)\) chimera, respectively.
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changes the topology of its cation efflux domains. Considering our present results showing that the cytosolic C-terminal tail is important for the functional interaction with hZnT6 and that the N-terminal half is not required for localization to the secretory compartments, ZnT5B (hZTL1) would fail to form heterodimers with hZnT6 and so the extra putative TMD in the C terminus would probably function as a localization signal to the plasma membrane. We have attempted to confirm this theory but have not been able to obtain ZnT5B (hZTL1) cDNA by reverse transcription-PCR from total RNA prepared from human cell lines, including the CaCo-2 cell line used to analyze reverse transcription-PCR from total RNA prepared from properties of ZnT5 and ZnT6 and their orthologues is of significance. The understanding of the unique pathway, 2) hZnT6 is unlikely to be involved in zinc transport as form heterodimers that transport zinc into the early secretory expression and cellular functions. A detailed examination would be required to corroborate their mRNA variants can be found in sequence data bases, a detailed examination would be required to corroborate their expression and cellular functions.

In conclusion, our studies reveal that: 1) hZnT5 and hZnT6 form heterodimers that transport zinc into the early secretory pathway, 2) hZnT6 is unlikely to be involved in zinc transport as a donor for zinc-binding sites embedded within the TMDs, and 3) the cytosolic C-terminal tail of hZnT5 is important for heterodimerization with hZnT6. The understanding of the unique properties of ZnT5 and ZnT6 and their orthologues is of significance because their heterodimer formation is important for the homeostatic maintenance of the early secretory pathway.

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Note Added in Proof—While this manuscript was under review, a paper describing the finding of the homo-oligomerization of hZnT3 and hZnT4 was published (Salazar, G., Falcon-Perez, J. M., Harrison, R., and Faundez, V. (2009) PLoS One 4, e5896).

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