Zinc Transport Complexes Contribute to the Homeostatic Maintenance of Secretory Pathway Function in Vertebrate Cells*

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Zinc transporters play important roles in a wide range of biochemical processes. Here we report an important function of ZnT5/ZnT6 hetero-oligomeric complexes in the secretory pathway. The activity of human tissue-nonspecific alkaline phosphatase (ALP) expressed in ZnT5/ZnT7×/−/− cells was significantly reduced compared with that expressed in wild-type cells as well as in the case of endogenous chicken tissue-nonspecific ALP activity. The inactive human tissue-nonspecific ALP in ZnT5/ZnT7×/−/− cells was degraded by proteasome-mediated degradation without being trafficked to the plasma membrane. ZnT5/ZnT7×/−/− cells showed exacerbation of the unfolded protein response as did the wild-type cells cultured under a zinc-deficient condition, revealing that both complexes play a role in homeostatic maintenance of secretory pathway function. Furthermore, we showed that expression of ZnT5 mRNA was up-regulated by the endoplasmic reticulum stress in various cell lines. The up-regulation of the hZnT5 transcript was mediated by transcription factor XBP1 through the TGACGTGG sequence in the hZnT5 promoter, and this sequence was highly conserved in the ZnT5 genes of mouse and chicken. These results suggest that zinc transport into the secretory pathway is strictly regulated for the homeostatic maintenance of secretory pathway function in vertebrate cells.

A large number of secretory and membrane-bound proteins become properly folded and assembled for their full biological activity in the secretory pathway as typified by the endoplasmic reticulum (ER) (1). To ensure the protein quality in the pathway, the cell has evolved an adaptive coordinated response, which limits further accumulation of unfolded nonnative proteins. This signaling pathway is termed the unfolded protein response (UPR) or the ER stress response (2, 3). The UPR is the conserved cellular response among eukaryotic cells and is composed of three major responses; they are translational attenuations to decrease the protein-folding load on the ER, induction of the transcriptions of a large set genes such as ER chaperones to increase the volume and capacity for protein folding, and the degradation of unfolded proteins called ER-associated degradation to remove the non-native proteins from the ER (4). Moreover, the cells that sustained irreparable levels of damage caused by the ER stress are eliminated by programmed cell death (5).

There are numerous proteins that become functional by binding with zinc during their itinerary in the secretory pathway, e.g. ER chaperones such as Scj1 and calreticulin (6, 7), and secretory or membrane-bound enzymes like matrix metalloproteinases and alkaline phosphatases (8, 9); hence, zinc is a potential regulator of the homeostatic maintenance of the secretory pathway. Recently, Ellis et al. (10) showed that genes of the ER chaperone that are involved in the UPR were induced by zinc deficiency in genome-wide CDNA microarray analysis in yeast (10). The UPR induction by zinc deficiency was exacerbated in mutant yeast deficient in Msc2 and/or Zrg17 that function as a major route of zinc entry into the ER through the formation of the hetero-oligomeric complexes (10, 11), indicating that this zinc transport route is indispensable for the homeostatic maintenance of the ER function. Furthermore, they showed that the UPR was increased by zinc deficiency in mammalian cells by performing transient transfection using the sensitive synthetic ER stress-responsive reporter plasmid (10), which indicates that zinc has important physiological functions in the UPR in mammals.

We have recently shown that two oligomeric zinc transport complexes, ZnT5/ZnT6 hetero-oligomeric complexes and ZnT7 homo-oligomeric complexes, are required for zinc incorporation into alkaline phosphatases (12, 13). In this study we demonstrate that ZnT5/ZnT6 hetero-oligomeric complexes contribute to the homeostatic maintenance of secretory pathway function using DT40 cells deficient in both complexes (specifically ZnT5/ZnT7×/−/− cells in this study). Furthermore, we show that ZnT5 mRNA expression was transcriptionally up-regulated by ER stress and that the up-regulation was mediated by XBP1 via the TGACGTGG sequence in the ZnT5 promoter. These findings suggest that a positive feedback regulation may operate to maintain the homeostasis of secretory pathway function via the ZnT5 transcript in vertebrate cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa and DT40 cells were maintained as described previously (12, 14). MIN6 cells (gift from Dr. Susumu Seino, Chiba University) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum (FCS) (BioWest) and 10 μM 2-mercaptoethanol (Sigma) at 37 °C. TT2 cells (a gift from Dr. Yoichi Shimakai, Kyoto University) were cultured on the feeder cells treated with 10 μg/ml mitomycin C in Dulbecco’s modified
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Eagle’s medium supplemented with heat-inactivated 15% embryonic stem cell-qualified FCS (Invitrogen), 100 µM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 10 µM 2-mercaptoethanol, and 10^4 units/ml leukemia inhibitory factor (LIF) (Chemicon) at 37 °C. DT40 cells were transiently transfected by electroporation. Briefly, plasmid DNA was electroporated into the cells (5 × 10^6 cells) suspended in 0.4 ml of cold potassium phosphate-buffered saline (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 5 mM MgCl_2) with a Gene Pulser apparatus (Bio-Rad) at 600 V and 25 microfarads. After electroporation, the cell was transferred into fresh growth medium for 3 h and then incubated with growth medium containing indicated chemicals or not for another 13 h. In a zinc-deficient condition, the cells were precultured for 24 h in zinc-free medium containing Chelex-treated FCS (12) and then cultured for 13 h in the same medium after electroporation. Transient transfection into HeLa cells was performed as described previously (15). Briefly, HeLa cells grown to 70% confluency in 12-well plates were transfected with plasmid DNA using Trans IT-LL1 (PanVera) at 600 V and 25 microfarads. After electroporation, the cells were transferred into fresh growth medium for 3 h and then incubated with growth medium containing indicated chemicals or not, and the plates were incubated for another 18 h. DNA transfection into DT40 cells to establish stable transformants was carried out as described previously (12). For screening, we used 0.25–0.35 mg/ml Zeocin (Invitrogen), 0.5 µg/ml for Puromycin (Sigma). More than three independent clones per transfectant were established.

**Immunoblotting and Lectin Blotting**—Proteins were separated by electrophoresis through 8% SDS-polyacrylamide gels as described previously. After transfer of proteins to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences), the blot was blocked with blocking solution (0.5 M NaCl and 0.05% Tween 20 in 10 mM Tris-HCl, pH 7.4). The avidin-biotin complex was formed with a Vectastain ABC kit (Vector Laboratories). Super Signal chemiluminescent substrate (Pierce) was used for detection. The fluorographic bands were obtained using LAS1000 plus (Fuji).

**Metabolic Labeling**—Cells (3 × 10^6 cells/ml) were precultured in the medium supplemented with dialyzed FCS for 45 min. Then the cells were pulse-labeled with 100 µCi/ml [35S]methionine/cysteine (PerkinElmer Life Sciences) for 10 min and chased with the regular culture medium containing 1 mM cold methionine/cysteine for the indicated period after washing with phosphate-buffered saline. For immunoprecipitation cells were lysed with lysis buffer (1% (w/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, and 0.05% SDS in phosphate-buffered saline) and rotated with anti-hTNAP antibody for 2 h at 4 °C. After rotating, 15 µl of protein A-Sepharose beads (Amersham Biosciences) were added and rotated for 3 h at 4 °C. The Sepharose beads containing immunoprecipitates were collected by centrifugation at 400 × g for 5 min at 4 °C. Washed 3 times with lysis buffer, and lysed with 6× SDS sample buffer at 95 °C followed by electrophoresis through 7.5% SDS-polyacrylamide gels. The radiographic images were obtained using a BAS 2500 Bioimaging analyzer (Fuji) after enhancement by autoradiography using an image analyzer (BAS 2500 Bioimaging analyzer). Quantification of the band intensity was performed on a Macintosh iBook G4 computer using the public domain NIH Image program (developed at the United States National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov).

**RNA Preparation and Northern Blotting**—Total RNA was extracted from DT40, HeLa, MIN6, and TT2 cells cultured in the presence or absence of tunicamycin (Tm) for the indicated periods or cultured under the indicated conditions using Sepasol I (Nacalai). Northern blotting was performed as described previously (12). The membrane was hybridized to the appropriately radiolabeled cDNA probes and exposed to an imaging plate (Fuji). Radiographic images were obtained using a BAS 2500 Bioimaging analyzer (Fuji). Quantification of the band intensity was performed on a Macintosh iBook G4 computer using the public domain NIH Image program (available from the Internet by anonymous ftp from zippy.nimh.nih.gov).

**Plasmids Construction**—BAC clones containing hZnTs genomic DNA were screened using the BAC Library Screening Service (Genome Systems Inc.). A 3.5-kilobase Sacl-Smal fragment that contained 5′-flanking and 5′-untranslated regions just upstream of start ATG of hZnTs gene was recovered from a BAC clone and subcloned into the Sacl and Smal sites upstream of luciferase reporter gene in pPGV-B2 plasmid (Toyobo Inc.). This plasmid was named −3.3K. The deletion construct containing 0.9 or 0.11 kilobases of a 5′-flanking region was generated by eliminating the fragment of −3.3 to −0.9 or −3.3 to −0.11 from −3.3K by digestion with the appropriate restriction enzyme and named −0.9K or −0.11K. The mutant plasmids in which TGACGTGG sequence (from −57 to −50, numbering is defined as the transcription starting site as +1) was substituted by TTCAATTGG was used for analysis (2). The deletion construct corresponding to the fragment (5′-GATGCTAGCTGCGAGTCAAGTCG-3′) that is complementary to the cap oligonucleotide sequence ligated to the cap site and reverse primer (571R, 5′-CGACGCAATCTCCTAGTG-3′, numbering is defined as the transcription starting site as +1) that anneals with cDNA of hZnTs. A nested PCR was performed using the amplified fragment as a template with the primers 5′-GGAGGGGTAAGGATCTACATTGG-3′ (forward) and 290R, 5′-GTGATTGATCTGGCTGGCTG-3′ (reverse), for

**Determination of the Transcription Start Site of hZnTs Gene**—The cap site hunting kit (Nippon Gene) was used for determination of the transcription start site. Briefly, the first PCR was performed using the human kidney cap site cDNA (Nippon Gene) as a template with the forward primer (5′-GATGCTAGCTGCGAGTCAAGTCG-3′) that is complementary to the cap oligonucleotide sequence ligated to the cap site and reverse primer (571R, 5′-CGACGCAATCTCCTAGTG-3′, numbering is defined as the transcription starting site as +1) that anneals with cDNA of hZnTs. A nested PCR was performed using the amplified fragment as a template with the primers 5′-GGAGGGGTAAGGATCTACATTGG-3′ (forward) and 290R, 5′-GTGATTGATCTGGCTGGCTG-3′ (reverse), for
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RESULTS

The hTNAP Expressed in ZnT5- ZnT7
t was degraded during the secretory process—We previously reported that the inactive placenta alkaline phosphatase expressed in ZnT5- ZnT7
t cells was transported to the plasma membrane as is the active placenta alkaline phosphatase expressed in wild-type cells despite having activity (12). To examine whether this is also the case for other zinc-requiring enzymes, we established ZnT5- ZnT7
t cells and wild-type cells that stably express hTNAP. The total TNAP activity (exogenous hTNAP plus endogenous chicken TNAP (cTNAP)) in wild-type cells expressing hTNAP was increased about 25-fold, but that in ZnT5- ZnT7
t cells expressing hTNAP was significantly reduced as in the case of cTNAP (Fig. 1A). Expression of hZnT5 in ZnT5- ZnT7
t cells expressing hTNAP restored the reduced activity to the level comparable with that in wild-type cells expressing hTNAP as described in endogenous cTNAP (Fig. 1A) (12). By immunoblot analysis, hTNAP was mostly detected as the ~80-kDa form in wild-type cells but mainly as the ~60-kDa form in ZnT5- ZnT7
t cells (Fig. 1B). Expression of hZnT5 increased the amount of the ~80-kDa form, although the ~60-kDa form still remained (Fig. 1B).

To investigate why hTNAP was detected in different molecular sizes in these cells, we performed a pulse-chase experiment using [35S]methionine/cysteine. The hTNAP in the wild-type cells was detected as the ~60-kDa form just after the 10-min pulse (at 0 h) and shifted to the ~80-kDa form with time up to 4 h (Fig. 2A, lanes 1–4). In contrast, the hTNAP in ZnT5- ZnT7
t cells were detected as the ~60-kDa form at 0 h to the

FIGURE 1. The hTNAP Expressed in wild-type DT40 cells and ZnT5- ZnT7
t cells. A, the hTNAP activity exogenously expressed in ZnT5- ZnT7
t cells was decreased like the endogenous cTNAP activity. The TNAP activity (sum of exogenously expressed hTNAP plus endogenous cTNAP) was measured using total cellular protein prepared from wild-type (WT) cells, WT cells expressing hTNAP, ZnT5- ZnT7
t cells, ZnT5- ZnT7
t cells expressing hTNAP, or ZnT5- ZnT7
t cells expressing both hTNAP and hZnT5. The TNAP activity is shown as milliunits (µM/mg of cellular protein. Each value is the mean ± S.D. of triplicate experiments. B, the molecular size of hTNAP was different between wild-type cells and ZnT5- ZnT7
t cells. The hTNAP was detected as the 60-kDa form in wild-type cells but mainly as the 80-kDa form in ZnT5- ZnT7
t cells. The Endo H digestion was carried out as described under “Experimental Procedures.” After immunoprecipitation with anti-hTNAP antibody/protein A-Sepharose beads, the immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. C, the subcellular localization of the hTNAP in wild-type cells and in ZnT5- ZnT7
t cells. Indirect immunofluorescence staining was performed using the anti-hTNAP antibody followed by secondary antibody conjugated with Alexa 488 without permeabilization. D, the degradation of hTNAP expressed in ZnT5- ZnT7
t cells was blocked by a proteasomal inhibitor, MG132. The pulse-labeled cells were chased for 4 h in the absence or presence of 25 or 100 µM MG132 and analyzed by SDS-PAGE/fluorography as in A. C, the position of the protein size marker is indicated on the left in B.

FIGURE 2. The hTNAP expressed in ZnT5- ZnT7
t cells is degraded by proteasome-mediated degradation. A, the behavior of hTNAP expressed in wild-type (WT) cells, ZnT5- ZnT7
t cells, and ZnT5- ZnT7
t cells expressing hZnT5. The cells were pulse-labeled with [35S]methionine/cysteine for 10 min and chased for the indicated time. After immunoprecipitation with anti-hTNAP antibody/protein A-Sepharose beads, the immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. B, the ~60-kDa form of hTNAP was sensitive to Endo H digestion, whereas the ~80-kDa form was resistant. The Endo H digestion was carried out as described under “Experimental Procedures.” After incubation with (+) or without (−) Endo H, the supernatant was analyzed by SDS-PAGE/fluorography as in A. C, the subcellular localization of the hTNAP in wild-type cells and in ZnT5- ZnT7
t cells. Indirect immunofluorescence staining was performed using the anti-hTNAP antibody followed by secondary antibody conjugated with Alexa 488 without permeabilization. D, the degradation of hTNAP expressed in ZnT5- ZnT7
t cells was blocked by a proteasomal inhibitor, MG132. The pulse-labeled cells were chased for 4 h in the absence or presence of 25 or 100 µM MG132 and analyzed by SDS-PAGE/fluorography as in A. The position of the protein size marker is indicated on the left in A, B, and D.
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FIGURE 3. The UPR is exacerbated in ZnT5−/− ZnT7−/− cells. A, glycosylation was unaffected in ZnT5−/− ZnT7−/− cells. Lectin blotting was performed using wheat germ agglutinin. The position of the protein size marker is indicated on the right. B, the GRP78 mRNA expression was increased in ZnT5−/− ZnT7−/− cells under the UPR condition. Total RNA was prepared from wild-type (WT) cells and ZnT5−/− ZnT7−/− cells cultured without or with 0.3 or 0.6 μg/mL Tm for 10 h. C, the increase of the UPR induction by ER stress in ZnT5−/− ZnT7−/− cells was confirmed by luciferase reporter analysis. Wild-type cells and ZnT5−/− ZnT7−/− cells were transfected with the luciferase reporter plasmid, p5xATF6GL3. After transfection, the cells were cultured with (+) or without (−) 250 μM DTT for 13 h. D, the increased GRP78 mRNA expression by Tm treatment was enhanced by zinc depletion. Total RNA was prepared from DT40 cells cultured in the indicated conditions (Tm; 0.5 μg/mL and/or TP; 5 μM TPEN) for 10 h. TP, TPEN, E, the increase of the UPR induction by DTT treatment was enhanced by zinc deficiency. Transfection was performed as in C. The cells were precultured for 24 h in zinc-free medium containing Chelex-treated FCS and then cultured for 13 h in the same medium with (+) or without (−) 250 μM DTT after transfection. In B and D, fold induction was calculated after normalizing the band intensity of GRP78 mRNA expression by that of β-actin and was shown relative to that of WT DT40 cells cultured without drug. The same blot was used sequentially (after stripping) for hybridization to GRP78 and β-actin cDNA probes in this order. Triplicate experiments were performed, and the panels show the representative results. In C and E, the luciferase activity in DT40 cells cultured without DTT is defined as 1, and the relative luciferase activities are shown. Each value is the mean ± S.D. of triplicate experiments.

extent similar to wild-type cells (Fig. 2A, lanes 1 and 5) but was only slightly detected as the ~80-kDa form at 4 h (Fig. 2A, lanes 4-8). Expression of hZnT5 restored the ~80-kDa form of hTNAP with time up to 4 h (Fig. 2A, lanes 9-12). The ~60-kDa form was sensitive, and the ~80-kDa was resistant to Endo H digestion, which indicated that the former corresponded to the N-linked high mannose-type hTNAP before being trafficked to the medial-Golgi apparatus, whereas the latter was the complex carbohydrate-type after that (Fig. 2B). A slight amount of the ~80-kDa form of hTNAP expressed in ZnT5−/− ZnT7−/− cells was hardly detected on the plasma membrane by indirect immunofluorescence staining without permeabilization (Fig. 2C), which indicated that the hTNAP expressed in ZnT5−/− ZnT7−/− cells was rapidly degraded intracellularly before being trafficked to the plasma membrane.

Several mutants of hTNAP associated with lethal hypophosphatasia, an inherited disorder featured by defective bone mineralization, shows properties similar to the hTNAP expressed in ZnT5−/− ZnT7−/− cells and are degraded in the proteasomes because of defective folding (17, 18). We considered whether or not this holds true in the case of hTNAP was degraded in the proteasomes because of defective folding (17, 18).

The UPR Was Exacerbated in ZnT5−/− ZnT7−/− Cells—The hTNAP was degraded during the secretory process in ZnT5−/− ZnT7−/− cells, which suggested that the homeostatic maintenance of secretory pathway function might be affected in the cells. To examine this possibility, we first examined whether or not the glycosylation pathway was affected in ZnT5−/− ZnT7−/− cells. Lectin blotting using wheat germ agglutinin showed no differences between wild-type cells and ZnT5−/− ZnT7−/− cells (Fig. 3A), which indicated that glycosylation was unaffected in ZnT5−/− ZnT7−/− cells. Then we examined whether or not UPR pathway was affected in ZnT5−/− ZnT7−/− cells by comparing the expression of GRP78 mRNA between wild-type cells and ZnT5−/− ZnT7−/− cells because the GRP78 is known to be one of the most inducible genes in response to the ER stress (19). As shown in Fig. 3B, the GRP78 mRNA expression in ZnT5−/− ZnT7−/− cells was not significantly changed under normal culture conditions but increased when increased the cell was treated with Tm, a potent ER stress inducer, which indicates that the UPR was exacerbated in ZnT5−/− ZnT7−/− cells. Furthermore, the exacerbation was ensured by reporter analysis using p5xATF6GL3 plasmid that has a sensitive synthetic ER stress-inducible promoter with five repeats of the ATF6 site upstream of the firefly luciferase gene (20); the luciferase activity in ZnT5−/− ZnT7−/− cells transiently transfected with p5xATF6GL3 plasmid was not significantly increased under normal culture condition but increased by treating the cell with DTT, another ER stress inducer (Fig. 3C).

We next examined whether or not zinc deficiency in the secretory pathway exacerbated the UPR. The increased GRP78 mRNA expression in wild-type DT40 cells by Tm treatment was enhanced in the presence of 5 μM concentrations of the zinc chelator N,N,N′,N′-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) (Fig. 3D), and the increased luciferase activity by DTT treatment was enhanced in the cells cultured in zinc-free medium containing Chelex-treated FCS (Fig. 3E). These results indicate that zinc transport complexes would have important contributions in homeostatic maintenance of secretory pathway function.

The Expression of ZnT5 mRNA Is Up-regulated by the ER Stress—Transcripts of many genes whose products are involved in the homeostatic maintenance of secretory pathway function are induced by the ER stress (21-23). Zinc deficiency in the secretory pathway increased the UPR, which raised the possibility that a feedback mechanism(s) facilitating zinc transport into the pathway may operate. To examine this possibility, we examined whether or not the expression of ZnT5, ZnT6, and ZnT7 mRNAs was up-regulated. The expression of ZnT5 mRNA was clearly up-regulated in the same UPR condition as in Fig. 3D, and that of ZnT6 and ZnT7 mRNA was also slightly up-regulated in this condition, although the degree of the effect was less than that of ZnT5 (Fig. 4A). Consequently, we examined the up-regulation of ZnT5 mRNA expression in more detail. The up-regulation of ZnT5 mRNA expression was dependent on the concentration of Tm and peaked at 4 h after Tm treatment (Fig. 4B). The pattern of the up-regulation was similar to that of the GRP78, although the degree of the effect was lower...
These results suggest that the up-regulation of the ZnT5 mRNA expression is directly regulated by UPR signaling in DT40 cells.

**Up-regulation of the ZnT5 mRNA Expression by the ER Stress Is Conserved among Vertebrate Cells**—We next examined whether or not up-regulation of the ZnT5 mRNA expression by the ER stress was conserved in mammalian cells. The up-regulation was observed in all tested mammalian cell lines in a manner similar to that in DT40 cells, but the degree of the effect was dependent on the cell types; in most cell lines the degree was relatively low, as in the case of HeLa cells (Fig. 5A, top) but it was higher (~3.7-fold) in some secretory cell lines like mouse insulinoma, MIN6 cells, or mouse embryonic stem cells, TT2 cells (Fig. 5A, middle and bottom). These results strongly suggest that the up-regulation of the ZnT5 mRNA expression by the ER stress would have an intimate involvement in the secretory functions.

Then we examined whether or not other stimuli up-regulated the ZnT5 mRNA expression. Because the mRNA expression of immediate-early response factors and heat-shock proteins was down-regulated in the heart of ZnT5<sup>−/−</sup> mouse in the microarray analysis (24), we investigated the effects of several stimuli that induce immediate-early response factors and heat-shock proteins on the ZnT5 mRNA expression. However, heavy metals (zinc), hypoxia, heat, and cAMP analog 8-bromo-cAMP failed to up-regulate the expression of ZnT5 mRNA in all tested cell lines (Fig. 5B). Thus, the ZnT5 gene may specifically respond to the ER stress in vertebrate cells.

**Up-regulation of hZnT5 Transcript by the ER Stress Is Mediated by XBP1 via the TGACGTGG Sequence in the hZnT5 Promoter Region**—Last, we examined whether or not the up-regulation of ZnT5 mRNA expression was transcriptionally regulated by UPR signaling. The comparison of 5′- and 3′-flanking sequence among human, mouse, and chicken ZnT5 genes showed that the 110-bp region from the transcription start site was the only region highly homologous among them (>80% or >60% identities between human and mouse or human and chicken, respectively) (Fig. 6A). By searching the consensus sequences for the binding sites of XBP1, ATF6, and ATF4 that are essential transcription factors for the UPR (19, 25–27), we found the TGACGTGG sequence in this region, which coincides with the consensus sequence for the binding site of XBP1 (the ACGT is the core sequence) (28, 29) (Fig. 6A). The TGACGTGG sequence was completely conserved between human and mouse and changed only in initial thymidine to guanidine in chicken (Fig. 6A), which strongly suggests that this

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**FIGURE 4.** The ZnT5 mRNA expression is up-regulated by the ER stress in DT40 cells. A, total RNA was prepared from DT40 cells cultured in the presence of 0.5 μg/ml Tm and/or 5 μM TPEN for 10 h. The same blot was used sequentially (after stripping) for hybridization to ZnT5, ZnT6, ZnT7, GRP78, and β-actin cDNA probes. TP, TPEN. B, fold induction was calculated after normalizing the band intensity of ZnT5, ZnT6, and ZnT7 mRNA expression by that of β-actin and was shown relative to that cultured without Tm or at 0 h. The same blot was used sequentially (after stripping) for hybridization to ZnT5, GRP78, and β-actin cDNA probes in this order. In A and C, triplicate experiments were performed, and the panels show the representative results.

**FIGURE 5.** Up-regulation of the ZnT5 mRNA expression by the ER stress is conserved among vertebrate cells. A, total RNA was prepared from HeLa, MIN6, and TT2 cells cultured in the presence of the indicated concentrations of Tm for 10 h (left panel) or 2 μg/ml Tm for the indicated period (right panel). B, total RNA was prepared from the same cell lines cultured in the indicated conditions (~, not treated; Zn, treated with 100 μM zinc; Hy, at 1% oxygen (hypoxia); He, at 42°C (heat shock); 8br, 8-bromo-cAMP) for 10 h. In all panels, fold induction was calculated after normalizing the band intensity of ZnT5 mRNA expression by that of β-actin and was shown relative to that cultured without Tm or at 0 h or without stimuli, respectively. The same blot was used sequentially (after stripping) for hybridization to ZnT5, GRP78, and β-actin cDNA probes in this order. Triplicate experiments were performed, and all panels show the representative results.

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sequence was functional as the ER stress response element in ZnT5 genes. To examine this possibility, we performed luciferase reporter analysis using HeLa cells. The luciferase activity in HeLa cells transiently transfected by hZnT5 promoters, where the luciferase gene is under control of respective 5'-flanking regions of hZnT5 gene, was all about 2-fold increased by Tm treatment (Fig. 6B), which indicated that the ER stress response element was located within the 110-bp region containing the TGACGTGG sequence. Because this 110-bp region was essential for the luciferase activity (data not shown), we hereafter refer to it as hZnT5 promoter. The null mutation in the TGACGTGG sequence (to TTCATTGG) abolished the increases (Fig. 6C), which indicated that the TGACGTGG sequence was indispensable to mediate the increases of luciferase activity by Tm treatment. In co-transfection experiments of the spliced formed XBP1 (constitutive active form) expression plasmid with wild-type hZnT5 promoter plasmid, the luciferase activity was increased dependent on the ratio of XBP1 expression plasmid to hZnT5 promoter plasmid in the presence or absence of Tm, and the increased activity was saturated to similar levels by co-expression with 50 ng of XBP1 expression plasmid (Fig. 6D). These results demonstrated that the ZnT5 mRNA expression was transcriptional regulation of hZnT5 transcript by the ER stress is mediated by XBP1 via the TGACGTGG sequence in its promoter. A, the ZnT5 promoter is highly homologous in 5'- and 3'-flanking regions of ZnT5 genes among human, mouse, and chicken genes. The sequence of hZnT5 promoter region (from −110 to +22) and the corresponding sequence in mouse and chicken ZnT5 genes are shown. Identical nucleotides are indicated by an asterisk, and the KGACGTGG sequence is indicated by bold letters. B, hZnT5 promoter contains the ER stress response element. HeLa cells were transfected with the luciferase reporter plasmids, −3.3K, −0.9K, and −0.11K. After transfection the cells were cultured with (+) or without (−) Tm (2 μg/ml) for 18 h. GRP78 promoter construct was used as a positive control. C, the TGACGTGG sequence in hZnT5 promoter mediates the response to Tm. HeLa cells were transiently transfected with wild-type −0.11K plasmid (shown as −0.11K(WT)) or the null mutated plasmid (shown as −0.11K(null)) and cultured as in B. Mutations are underlined. D, XBP1 increases the hZnT5 promoter activity via the TGACGTGG sequence. HeLa cells were transiently co-transfected with −0.11K(WT) or −0.11K(null) plasmids and XBP1 expression plasmid and cultured as in B. The amount of XBP1 expression plasmid is indicated. E, the TGACGTGG sequence in hZnT5 promoter mediates the response to Tm but not to forskolin. HeLa cells were transiently transfected with wild-type −0.11K plasmid (shown as −0.11K(WT)) or two mutated plasmids (shown as −0.11K(CRE) and −0.11K(null)). Mutations are underlined. After transfection the cells were cultured with Tm or forskolin or without drugs for 18 h. F, the TGACGTGG sequence mediates the UPR but not cAMP response. HeLa cells were transiently co-transfected with −0.11K(WT) or −0.11K(CRE) plasmids and 50 ng of the XBP1 expression plasmid or 50 ng of the constitutive active protein kinase A expression plasmid. In B–F, the luciferase activity by −0.11K(WT) plasmid without drug is defined as 1, and the relative luciferase activities are shown. Each value is the mean ± S.D. of triplicate experiments.
ally up-regulated by the ER stress and that XBP1 mediates the up-regulation via the TGACGTGG sequence in hZnT5 promoter.

The TGACGTGG sequence is similar to the cAMP-responsive element (CRE; TGGACGTC) and operates as the response element to protein kinase A signal in concert with the neighboring sequence (30). However, the luciferase activity in HeLa cells transfected by the −0.11K plasmid was not increased by forskolin, the activator of adenylyl cyclase (Fig. 6E) and by co-expression with the constitutive active protein kinase A expression plasmid (Fig. 6D). The substitution of the TGACGTGG sequence to the CRE (TGACGTCA) abolished the increases by Tm (Fig. 6E) and by co-expression with the XBP1 expression plasmid (Fig. 6F) but stimulated the increase by forskolin (Fig. 6G) and by co-expression with the constitutive active protein kinase A expression plasmid (Fig. 6F). These results clearly showed that the TGACGTGG sequence in hZnT5 promoter does not operate as the CRE, which is consistent with ZnT5 mRNA expression unchanged by 8-bromo-cAMP (see Fig. 5B). Thus, the XBP1 pathway will be important for ZnT5 to fulfill its functions.

**DISCUSSION**

In this study we showed that the inactive hTNAP expressed in ZnT5−/− cells was degraded by proteasome-mediated degradation without being transported to the plasma membrane and that the UPR was increased in ZnT5−/− cells. These results suggest that many other zinc-requiring secretory proteins may be degraded by proteasome-mediated degradation without being elicited in ZnT5−/− cells and that the increase of such proteins may be one reason for the exacerbation of the UPR in ZnT5−/− cells under the UPR condition. Alternatively, it may be possible that the activity of some zinc-requiring chaperone in the secretory pathway such as Scj1 and calreticulin in the ER is reduced in ZnT5−/− cells, which contributes to the UPR exacerbation in the cells.

The exacerbation of the UPR in ZnT5−/− cells and the up-regulation of ZnT5 mRNA expression by the ER stress indicate that ZnT5/ZnT6 hetero-oligomeric complexes is involved in the ER homeostasis, which somewhat conflicts with the fact that they are localized mainly to the Golgi apparatus, not to the ER (13, 24, 31). We have no data to explain this discrepancy at present but there are several possibilities not mutually exclusive. First, ZnT5/ZnT6 hetero-oligomeric complexes might transport zinc directly into the ER when they are newly synthesized. Second, they might transport zinc directly into the ER if they cycle between the Golgi apparatus and the ER although mainly localized to the Golgi apparatus. Third, they take zinc up into the Golgi lumen, and the zinc might be delivered by retrograde trafficking to the ER lumen. Recently, Ellis et al. (10, 11) showed that Msc2/Zrg17 hetero-oligomeric complexes are localized to the ER and required for correct ER functions in yeast and that Msc2/Zrg17 hetero-oligomeric complexes would be the yeast counterpart of ZnT5/ZnT6 hetero-oligomeric complexes because co-expression of ZnT5 and ZnT6 complements the growth defects of the msc2 and/or zrg17 mutants. This may support the first possibility for ZnT5/ZnT6 hetero-oligomeric complexes.

In yeast, UPR is increased in msc2 and/or zrg17 mutants under zinc-deficient conditions (10, 11). The Zrg17 transcript is up-regulated by zinc deficiency via zinc-responsive transcription factor Zap1 (32, 33), from which Ellis et al. (11) propose the feedback model that the transcriptional regulation of Zrg17 by zinc controls the zinc transport activity of Msc2/Zrg17 hetero-oligomeric complexes to maintain the ER homeostasis in yeast (11). Taking into account the fact that UPR is increased in ZnT5−/− cells or in wild-type cells under a zinc-deficient condition (See Fig. 3) with the fact that the expression of ZnT5 mRNA is reported to be up-regulated by zinc depletion induced by TmEN treatment in HeLa cells (34), analogous model may be possible in vertebrate cells. However, we showed here that the expression of ZnT5 mRNA is transcriptionally up-regulated by the ER stress via transcription factor XBP1, from which we propose another feedback model that the transcriptional regulation of ZnT5 by the ER stress controls the homeostatic maintenance of secretory pathway function by regulating the zinc transport activity of ZnT5/ZnT6 hetero-oligomeric complexes in vertebrate cells. Very recently, ZnT5 was reported to be a target gene of XBP1 in microarray analysis in nematode (the nematode ZnT homologue is designated as CDFS in the study) (23), which indicates that up-regulation of the ZnT5 transcript by XBP1 would be conserved among metazoan in the course of evolution.

XBP1 is suggested to coordinate the protein folding capacity via controlling the expression of a large set of genes whose products have important functions to maintain the homeostasis in the ER and the secretory pathway (22, 23, 35). The expression of ZnT5 mRNA is up-regulated by XBP1, which is well in accord with the fact that ZnT5 participates in the homeostatic maintenance in the secretory pathway. Our results indicated that the up-regulation of ZnT5 mRNA expression was higher in MIN6 cells and TT2 cells than in HeLa cells, which seems to be attributed to the expression level of XBP1 because the XBP1 mRNA expression is known to be high in the cells suitable for protein secretion like pancreatic cells (36) and antibody-secreting plasma cells (37). Interestingly, the XBP1 mRNA is highly expressed in osteoblasts in the developmental stage, and the expression pattern of XBP1 mRNA is found to be very similar to that of TmAP in the bone throughout development (38). ZnT5−/− mice show osteopenia due to impairment of osteoblast maturation along with the reduced TmAP activity (24), and similar defects are observed in TmAP−/− mice (39, 40) and in patients of hypophosphatasia caused by mutations of the TmAP gene (41, 42). Because TmAP mRNA has not been identified as a target gene of XBP1, it may be possible to raise the interesting hypothesis that the TmAP activity required for the bone formation is regulated by ZnT5 expression under control of XBP1.

Vertebrate cells produce numerous secretory proteins and, therefore, have evolved more complicated UPR signaling pathways than yeast (5, 23). Zinc plays a role in the homeostatic maintenance of secretory pathway function to maintain the correct conformation and activation of zinc-requiring proteins that pass through and reside there (10, 11). Thus, two oligomeric zinc transport complexes would exist in the secretory pathway in vertebrates, whereas lower eukaryotes such as fruit fly, nematode, and yeast have only one or the other (43). Moreover, for physiological activity, zinc transport into the secretory pathway would be strictly maintained in diverse ways. The up-regulation of the ZnT5 transcript by the ER stress would be one way. We have not elucidated whether or not expression of ZnT6 and ZnT7 mRNA is directly up-regulated by UPR signaling, but their regulation may also contribute to the homeostatic maintenance of secretory pathway function during the UPR.

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