YKE4 (YIL023C) Encodes a Bidirectional Zinc Transporter in the Endoplasmic Reticulum of Saccharomyces cerevisiae*

YIL023C encodes a member of the SLC39A, or ZIP, family, which we refer to as yeast KE4 (YKE4) after its mouse ortholog. Yke4p was localized to the endoplasmic reticulum (ER) membrane using Yke4p-specific antisera. YKE4 is not an essential gene; however, deletion of YKE4 resulted in a sensitivity to calcifiuor white and poor growth at 36 °C on respiratory substrates containing high zinc. Overexpression of transition metal transporters Zrc1p and Cot1p or the mouse orthologue mKe4 in Δyke4 suppressed the poor growth at 36 °C on respiratory substrates. We found that the role of Yke4p depends on the zinc status of the cells. In a zinc-adequate environment, Yke4p transports zinc into the secretory pathway, and the deletion of YKE4 leads to a zinc-suppressible cell wall defect. In high zinc medium, transport of zinc into the secretory pathway through Yke4p is a way to eliminate zinc from the cytosol, and deletion of YKE4 leads to toxic zinc accumulation in the cytosol. Under low cytosolic zinc conditions, however, Yke4p removes zinc from the secretory pathway, and deletion of YKE4 partially compensates for the loss of Msc2p, an ER zinc importer, and therefore helps to alleviate ER stress. In our model, Yke4p balances zinc levels between the cytosol and the secretory pathway, whereas the previously described Msc2p-Zrg17p ER zinc importer complex functions mainly in zinc-depleted conditions to ensure a ready supply of zinc essential for ER functions, such as phospholipid biosynthesis and unfolded protein response.

Zinc is not redox-active; however, its ability to form multiple bonds has made it an important structural component in hundreds of different enzymes; about 3% of human genes are thought to encode zinc-binding proteins (1). Eukaryotic zinc transporters are grouped into two families, the cation diffusion facilitator (CDF or SLC30) family (2, 3) and the zinc transporter (ZIP or SLC39) family (4, 5). Members of the CDF family include the human zinc transporter ZIP4, the fruit fly ZNF3, and the zebrafish FOI (6). The CDF family can be divided into four large families. One of these families, the LIV1/KE4 family, contains several transporters, such as the human and mouse ZIP4, the fruit fly FOI, and the zebrafish LIV1 (4). These transporters (ZIP4, FOI, and LIV1) form a subfamily, usually named after LIV1, which was the first gene of this group to be identified.

Although the Ke4-like proteins share many features with the LIV1 group, they form a separate subfamily that is present in all investigated eukaryotes. The human, mouse, zebrafish, Drosophila melanogaster, Arabidopsis thaliana, and Saccharomyces cerevisiae KE4-like genes are orthologous. It is of interest that the mammalian KE4 gene is located in the major histocompatibility complex. It is generally thought that the CDF and ZIP families play opposing roles in eukaryotic zinc transport; CDF proteins lower the cytosolic zinc content by transporting zinc out of the cell or into intracellular organelles, whereas the ZIP proteins increase cytosolic zinc by transporting zinc into the cells or out of intracellular organelles. Consistent with this view, a recent study has suggested that the mouse Ke4 is a Golgi zinc exporter (5). We have investigated the function of the yeast Ke4 protein. Here we provide genetic and biochemical evidence that shows that Yke4p is capable of transporting zinc in both directions. Since the mouse Ke4 gene can substitute for the yeast and A. thaliana genes, we suggest that bidirectional transport may be conserved in all eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Growth Media, and Plasmids—** Yeast strains used in this study are shown in Table 1. YKE4 and PSD1 (phosphatidylserine decarboxylase 1) were deleted in W303 wild type cells (DY150), utilizing the KanMX4 cassette. KanMX4 cassette-disrupted open reading frames were PCR-amplified from the genome-wide deletion collection (Invitrogen) using the following primers: YKE4 PR1032, 5′-AGT AGA ATT TCT GCG -3′; and PR1235, 5′-GG TAC GTA GCG CTT TAA GCC TCA -3′; and PSD1 PR1235, 5′-GG TAC GTA GCG CTT TAA GCC TCA -3′; and PSD1 PR1236, 5′-AAG GGG TAC ATG ACA TGG -3′. The PCR-amplified yke4::KanMX4 cassette was transformed into DY150 cells. psd1::KanMX4 cassette was transformed into both DY1457 (MATa) and DY150 (MATa) strains. DY1457psd1 was used to mate the DY150-based strains to create double and triple mutant cells. The double and triple deletion strains were confirmed by PCR analysis.

YKE4 was cloned and expressed with its own promoter (defined as a 1000-bp-long 5′ region of the start codon) into high copy episomal (pTF63) and low copy centromeric (YCP33) plasmids using PR1035 (5′-ACG TGG ATC CAG GCC TCA -3′) as the vector backbone.

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TABLE 1

Cell lines used in this study

| Strain     | Genotype                  | Source          |
|------------|---------------------------|-----------------|
| DY150      | MATa, ura3-52, leu2-3, 112, trpl1-1, his3-11, ade2-1, can1-100(oc) | W303 wild type  |
| Δyke4      | MATa, ura3-52, leu2-3, 112, trpl1-1, his3-11, ade2-1, can1-100(oc) | This study      |
| Δmsc2      | MATa, ura3-52, leu2-3, 112, trpl1-1, his3-11, ade2-1, can1-100(oc) | This study      |
| Δmsc2Δyke4 | MATa, ura3-52, leu2-3, 112, trpl1-1, his3-11, ade2-1, can1-100(oc) | This study      |
| Δpsd1      | MATa, ura3-52, leu2-3, 112, trpl1-1, his3-11, ade2-1, can1-100(oc) | This study      |

**High Copy Suppressor Screen**—To identify high copy suppressors of the high zinc sensitivity of Δyke4 cells, a genomic library cloned into YEp13 was transformed into Δyke4. About 18,000 colonies were replica-plated onto YPGE plates containing 5.5 mm zinc. The plates were incubated at 36 °C for 6 days. Twenty-seven colonies were identified. After repeated testing of these colonies, nine were found to grow on high zinc plates. The plasmids were rescued and transformed into *Escherichia coli* (DH10B; Invitrogen) by electroporation. The genomic inserts in the plasmids were sequenced using PR0324 (5′-CCA TTA TCG ACT ACG CGA-3′) and PR0325 (5′-ATG TCG GCG ATA TAG GCG-3′) and then subcloned to determine the specific genes responsible for the suppression.

**Antibody Generation and Western Blotting**—To make antibodies against Yke4p and mouse Ke4, we generated synthetic peptides of NRDHGHANHE (amino acids 25–36 of Yke4p) and GAVDSDVAG of mKe4 (amino acids 411–420) that are in hydrophilic regions of Ke4 proteins. The peptides were conjugated to bovine serum albumin using gluteraldehyde, and the conjugate was injected into rabbits using complete Freund’s adjuvant (Calbiochem). The anti-Ke4p antibodies were affinity-purified using ovalbumin-conjugated peptide linked to an AminoLink Plus resin according to the manufacturer’s protocol (Pierce).

**Immunofluorescence**—Yeast cells were processed for immunofluorescence as previously described (7). The affinity-purified rabbit anti-Yke4p antibody was used at a 1:20 dilution followed by a 1:750 dilution of Alexa594-conjugated goat anti-rabbit IgG (Molecular Probes). To detect Ke2p-HA, cells were incubated with a 1:100 dilution of mouse anti-HA (Covance) followed by a 1:750 dilution of Alexa594-conjugated goat anti-rabbit IgG (Molecular Probes). Anti-Dpm1 antibody (Molecular Probes) was used at a 1:50 dilution followed by a 1:750 dilution of Alexa594- or Alexa488-conjugated goat anti-rabbit IgG (Molecular Probes). Images were captured on an Olympus epifluorescent microscope. C57BL/6 mouse fibroblasts were grown on coverslips in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and processed for immunofluorescence as described previously (8). The rabbit anti-mKe4 serum was used at a 1:100 dilution followed by an Alexa488-conjugated goat anti-rabbit IgG (1:750) (Molecular Probes).
The abbreviations used are: ER, endoplasmic reticulum; CM, complete syn-
thetic medium; IAA, indole-3-acetic acid; UPR, unfolded protein response;
UPRE, unfolded protein response element; YPD, yeast extract-peptone-
dextrose; YPGE, yeast extract-peptone-glycerol-ethanol; ZRE, zinc
response element; GE, glycerol and ethanol.

**RESULTS**

Localization of Ke4 Protein to the ER/Golgi Apparatus—To identify
the cellular localization of Ke4, we generated anti-peptide antibodies
against both mouse and yeast Ke4 proteins. Western blots and
immunofluorescence microscopy were used to test the affinity-purified
antisera (Fig. 1). The specificity of anti-Yke4p antisera was tested on
lysates from wild type, Δyke4, and Δyke4 cells in which YKE4 was pro-
vided on a plasmid. Lysates from Δyke4 cells transformed with a plasmid
containing YKE4 expressed under the control of its endogenous
promoter showed a novel band migrating at ~33 kDa that was not
seen in lysates from Δyke4 cells (Fig. 1A). The addition of the immuno-
genic peptide to the antisera blocked the detection of the Yke4p
band. The predicted mass of the protein, based on the amino acid compo-
sition of YIL023C, is 33.7 kDa.

The localization of Yke4p was examined by subcellular fraction-
ation. Cells were spheroplasted and homogenized and organelles
were separated by fractionation on iodixanol (OptiPrep) gradients (0–25%),
and the fractions were analyzed by Western blotting. Western blots were probed
with antibodies to Kex2p-HA, a Golgi marker (1:1000; mouse
anti-HA (Covance)), Dpm1p, an ER2 marker (1:500; mouse
anti-Dpm1p (Molecular Probes)), Porin, a mitochondrial
marker (1:1000; mouse anti-porin (Molecular Probes)), and
alkaline phosphatase, a vacuole membrane marker (1:1000;
mouse anti-alkaline phosphatase (Molecular Probes)) followed
by peroxidase-conjugated goat anti-rabbit IgG or goat anti-
mouse IgG (Jackson ImmunoResearch). Western Lightning
Chemiluminescence Reagent (PerkinElmer Life Sciences) was
used for visualization.

β-Galactosidase Enzyme Assay—We used β-galactosidase
reporter gene constructs to indirectly estimate the ER and cyto-
plasmic zinc concentrations. The unfolded protein response ele-
ment (UPRE) containing the upstream activation element from
the promoter of the KAR2 gene (UPRE-lacz; pMCZY or
pMCZY-L) (9) and the zinc response element from
ZRT1 (ZRE-lacz; pDg2 or
pDg2-L) (10) were used to drive the expression of the lacZ gene in

FIGURE 1. The yeast and mouse Ke4 proteins localize in the secretory pathway. A, wild type (WT), Δyke4, and Δyke4 cells transformed with pYKE4 were grown to midlog phase, spheroplasted, and Dounce homogenized, and membrane fractions were analyzed by SDS-PAGE and Western analysis using a rabbit antisera against Yke4p. An equal amount of the membrane fraction of wild type cells was preincubated for 2 h with a 100-fold molar excess of the Yke4p peptide. B, wild type cells were spheroplasted and disrupted by Dounce homogenization. The membrane fraction of the cell homogenate was loaded onto iodixanol gradients and fractionated by centrifugation. The first fraction is the heaviest, and fraction 15 is the lightest. The fractions were subjected to Western blot and stained with antibodies against the indicated proteins. C, wild type cells transformed with pKEX2-HA were processed for immunofluorescence and stained with anti-Yke4p and anti-HA antibodies. D, wild type cells were grown overnight in normal, low (Bio 101) and high (250 mM) zinc media and processed for immunofluorescence using anti-Yke4p and anti-Dpm1p antibodies. The staining pattern indicates a mainly ER localization for both Dpm1p and Yke4p under all three conditions. E, C57BL/6 mouse fibroblasts were processed for immunofluorescence using a 1:100 dilution of the anti-mouse Ke4 antisera. The mKe4 localized primarily to the Golgi stacks with some punctate vesicle staining as well.

The localization of Yke4p was confirmed using immunoflu-
orescence microscopy. Wild type cells, expressing KEX2-HA on a plasmid,
were stained with the affinity-purified rabbit anti-Yke4p anti-
serum followed by an Alexa594-conjugated goat anti-rabbit IgG or
mouse anti-HA (Covance) antibody to detect Kex2p-HA
followed by Alexa594-conjugated goat-anti-mouse IgG. The
images showed a perinuclear localization typical of the ER in
*S. cerevisiae* for Yke4p and a punctate staining for the Golgi-
localized Kex2-HA (Fig. 1C). There was, however, co-localiza-
tion of Yke4p and the ER protein Dpm1p (Fig. 1D). These
results suggest that Yke4p is localized to the ER. The localiza-
tion of Yke4p was investigated under both high and low zinc
conditions and was unaffected by media zinc concentration.

The mouse Ke4 protein was localized by immunofluores-
cence. Mouse fibroblasts stained with a mouse-specific anti-
Ke4 antibody showed a robust staining of the Golgi apparatus

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2 The abbreviations used are: ER, endoplasmic reticulum; CM, complete syn-
thetic medium; IAA, indole-3-acetic acid; UPR, unfolded protein response;
UPRE, unfolded protein response element; YPD, yeast extract-peptone-
dextrose; YPGE, yeast extract-peptone-glycerol-ethanol; ZRE, zinc
response element; GE, glycerol and ethanol.
(Fig. 1E). In addition to the Golgi stacks, large numbers of vesicles, probably secretory vesicles, also stained positive for mKe4. Huang et al. (5), using an anti-peptide antiserum, observed a similar staining pattern in other cell lines, such as WI-38 (human lung fibroblast), WPE1 (human prostate epithelium), K-562 (human erythroblast leukemia), and MCF-7 (human mammary gland epithelium) cells. Ke4p localization in mouse cells was unaltered in zinc limiting or zinc replete conditions (data not shown). We conclude, therefore, that the Ke4 protein is mainly localized to the Golgi apparatus in mouse cells, whereas it mainly localizes to the ER in yeast.

**Δycle4 Cells Are Sensitive to High Zinc**—The YKE4 gene was disrupted in a W303 strain using a KanMX4 deletion cassette, and the gene deletion was confirmed by PCR. The Δycle4 strain was tested for common phenotypes, such as growth on various carbon sources, as well as sensitivities to temperature, osmotic changes, cell wall-damaging agents, and high and low transition metals. We found that Δycle4 cells did not grow at elevated temperatures (36–38 °C) on high zinc (3.5–5.5 mM ZnSO4) on media containing the respiratory substrates glycerol and ethanol (GE) as carbon sources. The high zinc sensitivity phenotype of Δycle4 cells was suppressed by providing the YKE4 gene on either a low or high copy plasmid using either its own promoter (Fig. 2) or the constitutively active ADH1 promoter (not shown). We also demonstrated that expression of mouse Ke4 in Δycle4 cells could suppress the high zinc-sensitive phenotype of these cells, suggesting that the function of yeast and mouse proteins is conserved (Fig. 2).

**Extragenic Suppression of the Zinc-sensitive Phenotype of Δycle4 Cells**—We took advantage of the zinc-sensitive phenotype of Δycle4 cells grown on YPGE medium at 36 °C to identify genes that could suppress that phenotype (Fig. 3A). A yeast genomic library cloned into a high copy plasmid vector (YEp13) was used to transform Δycle4 cells (18,000 colonies), resulting in nine colonies that were resistant to high zinc (5.5 mM) on GE medium at 36 °C. Five plasmids contained ZRC1, three contained PCL1, and one contained RPL23A. PCL1 encodes a cell cycle-regulated cyclin gene that is expressed in the G1 phase (12), RPL23A encodes a ribosomal protein that is part of the 60 S large subunit (13), and ZRC1 encodes a vacuolar zinc transporter that mediates the uptake of zinc from cytosol (14).

Zrc1p is a vacuolar zinc importer. Overexpression of Zrc1p decreases cytosolic zinc (14), suggesting that the cause of the high zinc sensitivity of the Δycle4 cell is increased cytosolic zinc. This hypothesis is further supported by overexpressing a second vacuolar zinc importer, Cot1p (14), in Δycle4 cells. Cot1p on a high copy plasmid also suppressed the zinc sensitivity of the deletion strain (Fig. 3B).

We tested the ability of Pcl1p and Rpl23p to suppress the zinc sensitivity of Δzrc1 cells and found that the high copy expression of PCL1 improved the growth of Δzrc1 cells on high zinc plates (3–4 mM zinc in CM) but the expression of RPL23A did not (data not shown).

**Overexpression of RPL23A and PCL1 Delays the Cell Cycle Arrest Caused by High Metal Concentrations**—Pcl1p is a cell cycle-regulated cyclin that binds to Pho85p. The Pho85 cyclin families are defined by sequence similarity and include 10 members, most of which are not regulated by the cell cycle but instead participate in the homeostasis of various metabolites (15). Evidence suggests that high levels of metals can cause cell cycle arrest. Cells expressing the constitutively active allele of the iron-sensitive Aft1p transcription factor, Aft1-1<sup>AP</sup>, accumulate iron due to inappropriately high induction of the elementiron uptake system. Incubation of aft1-1<sup>AP</sup> cells in iron-containing medium leads to arrest of the cell cycle at the G<sub>1</sub> phase. The iron-induced arrest can be bypassed by the overexpression of a mutant G<sub>1</sub> cyclin, cln3-2 (16). To investigate if Pcl1p is specific to zinc toxicity, we transformed the PCL1-containing high copy plasmid into Aft1<sup>−</sup> cells. Ccc1p is a vacuolar iron importer, and Δccl1 cells are sensitive to high iron, since they are unable to store cytosolic iron in their vacuoles (17). Overexpression of PCL1 was able to rescue the growth arrest of Δccl1 cells, as shown on Fig. 4A, suggesting that Pcl1p is not specific to zinc but protects cells from high cytosolic metal.

The connection between zinc and Rpl23ap is not known. Rpl23ap is a component of the large (60 S) ribosomal subunit and similar to the E. coli L14 and rat L23 ribosomal proteins (13). Many ribosomal proteins contain zinc, but Rpl23ap is not thought to be one of them, since it contains no predicted zinc ribon motif. In order to determine if Rpl23ap suppresses growth defects due to high cytosolic metals, we examined whether overexpression of Rpl23ap could also affect the high iron growth arrest of the Δccl1 strain. The effect of overexpression of RPL23A was not specific to zinc, since RPL23A was able to able rescue the growth arrest of Δccl1 cells (Fig. 4B). Although the mechanism is unclear, both Pcl1p and Rpl23ap can delay the cell cycle arrest caused by high cytosolic metal.
concentrations, further suggesting that the cause of cell growth defect of Δyke4 cells on high zinc GE plates is caused by zinc accumulation in the cytosol.

Deletion of YKE4 Is Synthetically Lethal with Deletion of MSC2 That Encodes an ER Zinc Importer—The above results indicate that Yke4p can lower cytosolic zinc by transporting zinc into the ER/Golgi system. To investigate the contribution of Yke4p to the secretory pathway zinc content, we generated an ER zinc importer, and cells with deletions in both genes. Cells with deletions in both Δyke4 and Δmsc2 show an increased ER stress response that can be suppressed by growth in high zinc medium. Cells with a deletion in Δmsc2 or Δyke4 or with a deletion in both genes (Δyke4Δmsc2) have no growth deficit in YPD at 30 °C (Fig. 5A). At elevated temperatures, Δyke4 cells grow like wild type cells, but there is growth retardation for Δmsc2. The double deletion strain does not grow at 38 °C, showing that there is a synthetic lethal effect when YKE4 and MSC2 are deleted. The addition of zinc (2.0 mM) suppresses the growth defect of the Δmsc2; however, the Δyke4Δmsc2 strain requires higher concentrations of zinc to suppress the growth defect.

The synthetic lethality resulting from deletion of Δyke4 and MSC2 under these conditions suggests that these transporters are importing zinc into the ER. To test this possibility, we examined the effect of overexpression of the Msc2p-Zrg17p complex on the growth of Δyke4 cells. We hypothesized that overexpression of the ER zinc-importer complex Msc2p-Zrg17p should improve the growth of Δyke4 cells on high zinc plates if Yke4p is an ER zinc importer. Wild type and Δyke4 cells were transformed with a plasmid containing ZRG17 under its own promoter (21) and a plasmid containing MSC2 under a methionine-regulated MET3 promoter (11). The cells were grown at 37 °C on zinc-containing CM + GE plates lacking methionine to induce the expression of both ZRG17 and MSC2. Overexpression of ZRG17 or MSC2 alone resulted in no change in the growth of the wild type and Δyke4 strains (Fig. 5B). Overexpression of ZRG17 and MSC2 together, however, led to growth arrest of wild type cells at 37 °C, and this growth arrest was not rescued by high zinc. Overexpression of ZRG17 and MSC2 together rescued the growth defect of Δyke4 cells on high zinc plates (Fig. 5B), supporting the idea that Yke4p may function as an ER zinc importer.

Yke4p Is Required for Normal Cell Wall Synthesis—We found that Δyke4 cells are sensitive to calcofluor white, a dye that binds to cell wall chitin, perturbing cell wall structure and inhibiting cell growth (Fig. 6A). Although it is a chitin-specific dye, mutations affecting other cell wall components can exacerbate the effect of calcofluor white (18). The calcofluor white phenotype is complemented by expression of plasmid-encoded YKE4 or mouse Ke4 (data not shown). We tested whether the sensitivity of Δyke4 to calcofluor white can be overcome by the addition of zinc. We used Δgas1 cells as a control to make sure that extracellular zinc does not interfere with the toxicity of calcofluor white. Gas1p is an enzyme, β-1,3-glucanosyltransferase, which is required for proper cell wall synthesis, and deletion of GAS1 leads to calcofluor white sensitivity (19). The missing enzymatic activity cannot be recovered by zinc. Whereas the calcofluor white sensitivity of Δyke4 can be suppressed by excess zinc, zinc did not suppress the calcofluor white sensitivity of Δgas1 cells.

We hypothesized that if zinc is required for proper cell wall synthesis in the secretory pathway, then Δmsc2 cells should also have a cell wall defect. We found that Δmsc2 cells are even more sensitive to calcofluor white than Δyke4 cells. As observed for Δyke4 cells, the calcofluor white sensitivity was suppressed by excess zinc in the medium, although the zinc suppression was only partial (Fig. 6B).

Zinc Requiring Biosynthetic Processes in the Secretory Pathway—There are no known zinc requiring proteins or enzymes in the yeast secretory pathway. However, Iwanyshyn...
et al. (20) showed that under low zinc conditions, the activities of the phospholipid biosynthetic enzymes are decreased by 25–50%. With the exception of phosphatidylserine decarboxylase 1 (Psd1p), which is a mitochondrial protein, the enzymes required for phospholipid biosynthesis are in the secretory pathway (Fig. 7). We generated a series of mutant strains in which PSD1 is deleted. PSD1 encodes about 95% of the cellular phosphatidylserine decarboxylase activity, and the remaining 5% is provided by the Golgi-localized Psd2p. Δpsd1 cells are viable on rich fermentable media, such as YPD, since Psd2p provides enough enzyme activity.

We hypothesized that Δpsd1 cells are sensitive to low zinc conditions. We tested this in two ways, by growing cells on low zinc medium and by deleting zinc transporters localized to the secretory pathway. We found that Δpsd1 cells do not grow on low zinc dextrose plates at 36 °C, but growth can be rescued by providing zinc in the medium (Fig. 8A). Psd1p catalyzes the formation of phosphatidylethanolamine from phosphatidylserine, and growth of the Δpsd1 strain can be rescued by providing ethanolamine in the growth medium; ethanolamine can be incorporated instead through the Kennedy pathway (20) (Fig. 7). If zinc is required for phospholipid biosynthesis in the secretory pathway, then the growth of Δpsd1 cells should be affected by deleting MSC2. The growth defect of Δpsd1Δmsc2 cells is more severe than the Δpsd1 cells, and ethanolamine can only partially rescue the growth of Δpsd1Δmsc2 cells (Fig. 8A). The reason for this partial rescue might be that the other three enzymatic steps in the phospholipid pathway in addition to Psd1p may also be zinc-dependent.

To identify the effect of Yke4p on zinc-sensitive phospholipid biosynthesis, we examined the effect of the deletion of YKE4 in Δpsd1 cells as well as in cells where other vesicular zinc transporters were deleted. We found that the Δpsd1Δmsc2Δyke4 cells grow better than the Δpsd1Δmsc2 cells (Fig. 8A). If Msc2p is a zinc importer and deletion of YKE4 improves cell growth, then Yke4p may be transporting zinc out of the secretory pathway.

We also expected that Δpsd1 cells would be sensitive to calcofluor white, since phospholipids are required for cell wall function. As shown in Fig. 8B, Δpsd1 cells are sensitive to calcofluor white, and providing zinc in the medium can restore cell growth. Cells with a deletion in both PSD1 and YKE4 (Δpsd1Δyke4) are slightly more sensitive to calcofluor white than either of the single deletions. Deletion of YKE4 in the Δpsd1Δmsc2 strain resulted in better growth on calcofluor white (Fig. 8B). Our conclusion from these experiments is that the apparent direction of Yke4p-
mediated zinc transport depends on the zinc status of the cytosol and vesicular compartments. Under low zinc conditions, Yke4p transports zinc out of the secretory apparatus, since deletion of YKE4 helps the growth of Δpsd1 and Δpsd1Δmsc2 cells on low zinc plates. On YPD + calcofluor white plates, however, deletion of YKE4 makes the calcofluor white sensitivity of Δpsd1 cells slightly worse. Deletion of YKE4 in Δpsd1Δmsc2 cells, however, helps growth on calcofluor white-containing YPD plates. This result suggests that ER zinc starvation caused by the deletion of MSC2 results in decreased ER zinc levels (9) that reversed the direction of zinc transport via Yke4p. Under this condition, Yke4p transports zinc out of the secretory pathway.

We confirmed this hypothesis using reporter genes to measure relative zinc levels in the cytosol and the secretory pathway. An indirect way to estimate the zinc levels is to use reporter constructs that reflect either cytosolic zinc (ZRE-lacZ) or ER zinc insufficiency (UPRE-lacZ). In zinc-depleted conditions, the Zap1 transcription factor is activated, and Zap1p binds to the zinc response element (ZRE)-containing promoter regions (2). We utilized a ZRE-lacZ construct that contains the ZREs from the promoter of Zrt1p, the high affinity plasma membrane zinc transporter. ZRE-lacZ-encoded β-galactosidase activity is, therefore, a measure of the cytosolic and nuclear zinc level. Ellis et al. (9) showed that when zinc is decreased in the ER, the ER stress response called unfolded protein response (UPR) is activated. Zinc limitation in the ER and activation of the UPR can be achieved by deleting MSC2 or ZRG17, which encodes its partner protein (21). We utilized a UPRE-lacZ reporter construct to measure the levels of zinc in the ER. We tested the ZRE-lacZ and UPRE-lacZ reporter constructs in wild type, Δyke4, Δmsc2, and Δyke4Δmsc2 cells. UPRE-lacZ activity showed a 2-fold increase in Δmsc2 and Δmsc2Δyke4 cells and no activity above baseline in Δyke4 cells (Fig. 9A). ZRE-lacZ activity, as expected, showed the opposite behavior: ~30% increase in Δyke4 cells and no significant change in Δmsc2 and Δmsc2Δyke4 cells. These results suggest that Yke4p appears to transport zinc out of the secretory pathway.

The measured differences between these strains were reproducible but modest; therefore, we employed strains that show more compelling differences in zinc concentration between the cytosol and the secretory pathway. We generated a series of mutant strains, in which the vacuolar zinc transporters ZRC1 and COT1 were deleted. Δzrc1Δcot1 cells cannot store zinc in the vacuole, and cells are highly sensitive to the concentration of zinc in the medium, since zinc accumulates in the cytosol. Zrc1p and Cot1p also contribute to the zinc supply in the ER, and deletion of these genes exacerbates the effect of an Msc2 deletion (9). Growth of Δzrc1Δcot1 cells is inhibited by ~200 μM zinc on YPD plates (22). At 30°C on YPD plates, Δyke4Δzrc1Δcot1 cells show similar zinc sensitivity as Δzrc1Δcot1 on YPD at 30°C (not shown). On YPG plates, however, Δyke4Δzrc1Δcot1 cells are slightly more resistant to high zinc than Δzrc1Δcot1 cells (Fig. 9B). At 36°C, Δzrc1Δcot1 cells grow slowly on YPD plates and not at all on YPG plates (data not shown). At the higher temperature, deletion of YKE4 in Δzrc1Δcot1 cells leads to growth on YPG plates (Fig. 9B).

We transformed ZRE-lacZ and UPRE-lacZ reporter plasmids into these deletion strains. The ZRE-lacZ reporter is expressed if cytosolic zinc concentration is low. The addition of 5 μM zinc to wild type cells reduced the expression of the reporter construct by about 80% (Fig. 9C). In Δzrc1Δcot1 cells, 1 μM zinc was required to achieve the same level of suppression, since these cells have increased cytosolic zinc content. ZRE-lacZ reporter activity was increased in Δzrc1Δcot1Δyke4 cells compared with Δzrc1Δcot1 cells, suggesting that Yke4p transports zinc into the cytosol, since its deletion lowers the cytosolic zinc content.

The UPR is activated in low zinc medium, as detected by the UPRE-lacZ reporter gene expression. Deletion of YKE4 in Δzrc1Δcot1 cells reduces the expression of UPRE-lacZ in low zinc medium, suggesting that under this condition Yke4p is an ER zinc exporter (Fig. 9C). Taken together, these experiments suggest that Yke4p transports zinc according to the relative zinc levels in the cytosol and ER.

**DISCUSSION**

The predicted protein product of the yeast open reading frame YIL023C, Yke4p, is a novel member of the ZIP, or SLC39, family and is suggested to be a transition metal transporter found in all eukaryotes. Phylogenetic studies suggest that all KE4-like genes are orthologous, as the mouse Ke4 can complement phenotypes due to both yeast (Fig. 2) and Arabidopsis KE4 mutations (23). Whereas genetic studies show that the genes are orthologous, the proteins appear to show differences in localization between yeast and mouse. Yke4p was localized to the ER in yeast, but as reported in this paper and by Huang et al. (5), the endogenously expressed mouse Ke4 is localized to the Golgi apparatus. There is a precedent for yeast and mammalian orthologues having different subcellular localizations. Msc2p, a zinc transporter that is a member of the cation diffusion facilitator family, is found in the ER in yeast (9, 11), but its mammalian orthologue Znt5 is found in the Golgi (24). The localization of both the mouse (5) and the yeast Ke4 (Fig. 1D) proteins was not changed by varying zinc conditions.

Yke4p has been speculated to be a transition metal transporter based on genetic studies. Mutant alleles for Ke4 have been reported in D. melanogaster and A. thaliana. Mutants of Ke4 in Drosophila, named “Catecholamines up” or Catsup, have significantly elevated tyrosine hydroxylase activity (25). It was proposed that Catsup protein down-regulates tyrosine hydroxylase activity. In Arabidopsis, KE4 alleles were discovered through a screen devised to find mutants resistant to the root elongation inhibitory effects of indole-3-acetic acid (IAA)-amino acid conjugates. IAA is a plant hormone critical for many developmental processes. In higher plants, IAA is usually conjugated to amino acids, sugars, or peptides, and hydrolytic enzymes are required to liberate the hormone from the hormone conjugates. These enzymes usually require manganese or cobalt for their function. iar1 (IAA-alanine-resistant 1) is a loss-of-function mutant of the Ke4-encoding gene, and manganese was found to suppress the IAA-Ala resistance of iar1 (23). It was suggested that iar1p might transport metals required for the IAA-amino acid conjugate hydrolase or may remove metals that inhibit enzyme activity. iar1p was thought not to be a manganese
transporter, since IAR1 expression did not rescue the manganese sensitivity of a mutation in the yeast Golgi manganese transporter mutant (23).

Recent studies in both yeast and mammalian cells suggest that Ke4p is a zinc exporter. Huang et al. (5) used zinquin, a zinc-sensitive fluorescent dye, to show that mammalian cells transiently transfected with a Ke4-containing plasmid have decreased zinquin staining compared with cells expressing Znt7, a Golgi zinc importer. The same authors expressed the mouse Ke4 gene (using a GAL1 promoter) in yeast cells in which the ZRT3 gene was disrupted. Zrt3p is thought to release zinc from the vacuolar storage, and deletion of ZRT3 leads to decreased cytosolic zinc. Expression of the mouse Ke4 protein reversed the cytosolic zinc deficiency as detected by the ZRE-lacZ reporter gene. Further, an overexpressed mouse Ke4 gene also decreased the total zinc content in Δzrt3 cells when exposed to excess zinc (100 μM) in the medium. Huang et al. (5) concluded from these experiments that mKe4 transports zinc from the Golgi into the cytoplasm.

This conclusion is consistent with some of the observations reported here: 1) deletion of YKE4 helps the growth of Δpsd1Δmsc2 cells on low zinc plates, and 2) deletion of YKE4 helps the growth of Δpsd1Δmsc2 cells on calcofluor white-containing plates. A Δpsd1 strain is sensitive to ER zinc depletion, since eliminating the ER zinc importer Msc2p in Δpsd1 cells resulted in a severe growth limitation. This growth limitation was alleviated by either increasing medium zinc or deleting YKE4. The calcofluor white sensitivity of Δpsd1Δmsc2 cells could also be suppressed by providing excess zinc in the medium or by deleting YKE4. Finally, 3) deletion of YKE4 also decreased the activation of the zinc-sensitive ER stress-response UPR. These results are consistent with the view that Yke4p is a secretory pathway zinc exporter.

There is clear evidence, however, that under certain conditions Yke4p and mouse Ke4 expressed in yeast can transport zinc into the secretory pathway. Evidence supporting this conclusion includes the following. 1) Deletion of YKE4 results in a sensitivity to zinc at high temperature in cells growing on respiratory substrates. 2) This sensitivity is caused by increased cytosolic zinc as suggested by the observations that increased expression of vacuolar zinc transporters (ZRC1 and COT1) suppresses this phenotype. Whereas the exact function(s) of the two other suppressors of the highly zinc-sensitive phenotype are unknown, our studies show that they can also suppress decreased growth resulting from high cytosolic iron. The inference is that they overcome growth retardation resulting from high concentrations of cytosolic transition metals. 3) Deletion of YKE4 results in a pronounced calcofluor white phenotype, which is suppressed by high zinc. Since calcofluor white sensitivity due to deletion of

**FIGURE 9. Effect of deletion of YKE4 on cytosolic and ER zinc.** A, the specified yeast strains were transformed with either pZRE-lacZ or pUPRE-lacZ. The strains were grown in either CM (gray bars) or CM made limited for zinc (black bars). After 16 h of growth, cells were harvested, and β-galactosidase activity and cell protein were determined. B, the specified yeast strains were grown on YPGE at either 30 or 36 °C with the designated concentration of zinc. C, the specified yeast strains were transformed with either pZRE-lacZ or pUPRE-lacZ. The strains were grown in low zinc medium supplemented with the indicated amount of zinc. After 16 h of growth, cells were harvested, and β-galactosidase activity and cell protein were determined. The results shown are the averages of three independent experiments and the S.E. values of the measurements. The β-galactosidase enzymatic activities are shown as the percentage of the reporter gene activity in wild type cells in medium without added zinc.
GAS1 is not affected by zinc, we surmise that deletion of YKE4 affects calcofluor white sensitivity by decreasing vesicular zinc. 4) Supporting this view is the observation that deletion of MSC2 also results in a zinc-suppressible calcofluor white phenotype. Double deletion of MSC2 and YKE4 leads to a more severe sensitivity to high temperature than any of the single mutants, and overexpression of MSC2 and ZRG17 can rescue the growth defect of Δyke4 cells on high zinc-containing respiratory media at elevated temperatures.

Our conclusion is that YKe4p can function bidirectionally, transporting zinc both into and out of the vesicular apparatus. Whereas the simplest explanation is that Yke4p responds to the zinc concentration gradient between cytosol and vesicular lumen as a zinc exchanger, alternate explanations are possible. One explanation that we are currently considering is that the direction of transport does not depend solely on the zinc gradient but is a consequence of a stress response. Examination of microarray data sets shows increased expression of YKE4 mRNA in response to stress conditions that lead to activation of Pkc1p (protein kinase C1) (26). Indeed, overexpression of Pkc1p through a regulatable PKC1 construct leads to a 50-fold induction of YKE4 mRNA (27). Based on these results, we hypothesize that the directionality of Yke4p-mediated transport may be defined not by the zinc gradient but by cellular stress responses. Increased stress might require increased concentrations of vesicular zinc, which would be facilitated by Yke4p import.

Acknowledgments—We thank Kirsten Fischer Lindahl for support to start this project and Bruce F. Horazdovsky, Robert E. Hammer, József Pál, Carla G. Simmons, Jiang Qiaorong, and the members of the Kaplan laboratory for advice and help.

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