Heteromeric Protein Complexes Mediate Zinc Transport into the Secretary Pathway of Eukaryotic Cells*

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The cation diffusion facilitator (CDF) family of metal ion transporters plays important roles in zinc transport at all phylogenetic levels. In this report, we describe a novel interaction between two members of the CDF family in Saccharomyces cerevisiae. One CDF member in yeast, Msc2p, was shown recently to be involved in zinc transport into the endoplasmic reticulum (ER) and required for ER function. We describe here a newly recognized CDF family member in yeast, Zrg17p. ZRG17 was previously identified as a zinc-regulated gene controlled by the zinc-responsive Zap1p transcription factor. A zrg17 mutant exhibits the same zinc-suppressible phenotypes as an msc2 mutant, including an induction of the unfolded protein response in low zinc. Moreover, a significant fraction of the total Zrg17p protein appears to localize to the ER. Their common phenotypes and localization suggested that these two proteins function together to mediate zinc transport into the ER. Consistent with this hypothesis, Msc2p and Zrg17p physically interact with each other, as determined by co-immunoprecipitation. Therefore, we propose that Msc2p and Zrg17p form a heteromeric zinc transport complex in the ER membrane. We also demonstrate that ZnT5 and ZnT6, mammalian homologues of Msc2p and Zrg17p, functionally interact as well. These results suggest that heteromeric complexes formed by different CDF members may be a common phenomenon for this ubiquitous family of metal ion transporters.

Zinc is required by all organisms because of the essential roles this metal plays in cells. For example, zinc is important for the activity of many proteins that reside in or move through the secretory pathway. In pancreatic β cells, zinc is needed for the assembly of proinsulin into homohexamers in the endoplasmic reticulum (ER) and/or Golgi (1, 2). The subsequent packaging of insulin into crystalline structures within secretory granules also requires zinc (1). Killer T cells present antigen peptides on their cell surface bound to major histocompatibility complex class I molecules. The processing of some of these peptides takes place in the ER by the ER-associated aminopeptidase, which utilizes zinc in its active site (3, 4). Zinc metalloenzymes responsible for attaching phosphoethanolamine groups to glycosylphosphatidylinositol (GPI) anchors are resident ER proteins (5, 6). As a last example, the Scj1p protein chaperone in the ER of yeast is a DnaJ homologue needed for protein folding and ER-associated degradation (7, 8). Scj1p has two zinc finger motifs and probably requires zinc for its function (9, 10).

Because of these and the many other needs for zinc, eukaryotic cells must utilize zinc transport proteins in their plasma membranes and intracellular organelles for the uptake and intracellular distribution of zinc. The activities of these transporters are often regulated to maintain consistent intracellular zinc levels in the face of changing zinc availability. The budding yeast Saccharomyces cerevisiae has been an excellent model system for studying zinc transport and regulation. The initial uptake of zinc into the cytoplasm of yeast is accomplished by three transporters on the plasma membrane: Zrt1p, Zrt2p, and Fet4p (11–13). In times of zinc deficiency, zinc stores in the vacuole are mobilized by the Zrt3p transporter protein to supply zinc to the cytoplasm and other organelles (14). Alternatively, when zinc levels in the cytoplasm become replete or excessive, the Zrc1p protein is involved in transporting extra zinc into the vacuole to be stored (15). The genes for these zinc transporter proteins are regulated by the zinc-responsive transcription factor, Zap1p. Zap1p up-regulates genes in zinc-deficient conditions by binding to an 11-base pair consensus sequence, a zinc-responsive element (ZRE), found in one or more copies in the promoters of its target genes (16). Lyons et al. (17) performed microarray analysis on the yeast transcriptome and estimated that as many as 46 genes in the yeast genome are directly regulated by Zap1p.

Whereas many of the yeast zinc transporters known to date have been shown to be Zap1p targets, the Msc2p protein is not. Msc2p is a member of the cation diffusion facilitator (CDF) family of metal ion transporters (18, 19). Many members of the CDF family transport metal ions such as zinc either out of the cell or into intracellular organelles (18, 20). Msc2p was recently shown to be localized to the ER and involved with zinc transport into this compartment (10, 21). Under low zinc conditions, an msc2 mutant exhibits an up-regulation of the unfolded protein response (UPR) and has defects in ER-associated protein degradation, suggesting that Msc2p and zinc are needed for proper ER function (10). The UPR and ER-associated degradation defects as well as other phenotypes of the msc2 mutant are all suppressible by elevated zinc. These observations suggested that there are other zinc transporters active in the ER/secretory pathway. Two vacuolar zinc transporters, Zrc1p and Cot1p, were found to potentially contribute to ER zinc levels; an msc2 zrc1 cot1 triple mutant caused an up-regulation.

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1 The abbreviations used are: ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; ZRE, zinc-responsive element; CDF, cation diffusion facilitator; UPR, unfolded protein response; TAP, tandem affinity purification.
and regulated by zinc at the mRNA level (regulated in low zinc and down-regulated in high zinc) (17, 22).

Table I
Plasmids used in this study

| Plasmid | Relevant genotype | Reference/Source |
|---------|-------------------|------------------|
| pTF63   | High copy vector  | Ref. 46         |
| YEpmSc2-myc (pMSc2ox) | MSC2-myc in pTF63 | Ref. 21         |
| pRS316GAL1 | Low copy vector | Ref. 47         |
| pRS316GAL1LEU2 | Low copy expression vector (LEU2-marked) | This work         |
| pGAL-ZRG17HA (pZRG17ox) | ZRG17-3X-HA driven by GAL1 promoter | This work         |
| YEp24   | High copy vector  | Ref. 48         |
| pHSIP150 | HSP150 in YEp24 | This work         |
| pMCZ-Y (UPRE-lacZ) | UPRE-lacZ reporter | Ref. 49         |
| pDg2L (ZRE-lacZ) | ZRE-lacZ reporter | Ref. 14         |
| pRg215   | PMA1-HA driven by GAL1 promoter | Ref. 50         |
| pGEV-TRP1 | GAL4/estrogen receptorVP16 hybrid activator | Ref. 28         |
| pZRG17   | ZRG17 in low copy vector | This work         |
| pZRG17HA | ZRG17-3X-HA in low copy vector | This work         |
| pMSc2    | MSC2 in low copy vector | Ref. 10         |
| pMSc2HA  | MSC2-3X-HA in low copy vector | Ref. 10         |
| pYES2    | High copy expression vector | Invitrogen       |
| pYES2L   | High copy expression vector (LEU2-marked) | Ref. 24         |
| pYES2ZnT5 (pZnT5) | ZnT5 driven by GAL1 promoter | Ref. 24         |
| pZnT5L   | ZnT5 driven by GAL1 promoter (LEU2-marked) | This work         |
| pYES2ZnT6 (pZnT6) | ZnT6 driven by GAL1 promoter | Ref. 25         |

of the UPR in low zinc even higher than that seen in an msc2 mutant alone. However, even this triple mutant was suppressed by elevated zinc (10). These observations led us to look for more potential zinc transporters in the secretory pathway. One candidate for such a protein was Zrg17p.

Previous studies of ZRG17 determined it to be a Zap1p target and regulated by zinc at the mRNA level (i.e. ZRG17 is up-regulated in low zinc and down-regulated in high zinc) (17, 22). Zrg17p is a probable membrane protein with multiple transmembrane domains. It was previously proposed that Zrg17p was involved in zinc uptake, perhaps under environmental conditions where Zrt1p was less effective (22). However, zrg17 mutants have an abnormal large cell morphology, which is suppressed by the addition of higher amounts of zinc in the medium (22). An msc2 mutant also exhibits this zinc-suppressible large cell phenotype (21). This suggested that Zrg17p may be involved in ER zinc transport. In this report, we determined that this is the case and that Zrg17p and Msc2p physically interact to form a complex that transports zinc into the ER.

Last, we suggest that Zrg17p is a distant member of the CDF family and present evidence that other CDF family members interact to form heteromeric complexes for metal ion transport.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—Media used wereYPD, SD + 2% glucose or galactose, YPGE, and L2M, as described previously (23). We also used rich YPD medium supplemented with 2% glycerol, 2% ethanol, and 2% galactose (YPGEgal) to induce expression of the GAL1 promoter in cells growing on poorly fermentable and nonfermentable substrates. Yeast strainsDY150 (MATa ade2 can1 his3 leu2 trpl ura3) andDY150 msc2 (DY150 msc2::HIS3) (21), BY4741 (MATa his3 leu2 met15 trpl ura3) (Research Genetics), ZRG17-TAP (BY4741 ZRG17::TAP), andMSC2-TAP (BY4741 MSC2::TAP) (Open Biosystems) have been described previously. To generate mutant strains,CEY9 (DY150 zrg17::KanMX) and CEY11 (DY150 msc2::HIS3 zrg17::KanMX), the KanMX cassette with 500 μg flanking the ZRG17 open reading frame was amplified by PCR from the zrg17 yeast deletion mutant (Research Genetics). The PCR fragment was then transformed into DY150 or DY150 msc2 to generate CEY9 and CEY11, respectively.

Plasmids—Plasmids used in this study are described in Table I. pZnT5 was a gift from Taiho Kambe (Kyoto University) (24), and pYES2ZnT5 was a gift from Leping Huang (University of California, Davis, CA) (25). pRS316GAL1LEU2, pYES2L, and pZnT5L were generated by swapping theURA3 gene on pRS316GAL1, pYES2, and pZnT5, respectively, with theLEU2 gene using the marker swap plasmid pUL9 (26). pZRG17 and pZRG17HA were constructed the same way as pMSC2 and pMSC2HA (10). Briefly, PCR products containing the ZRG17 promoter and open reading frame (and terminator in the case of pZRG17) were put into pFL38 or YcpZRC1-HA by homologous recombination to generate pZRG17 and pZRG17HA, respectively (27). To generate thepGAL-ZRG17HA construct, the ZRG17 open reading frame starting at the fourth in-frame ATG plus 3× hemagglutinin antigen (HA) tags and termination sequence from pZRG17HA was amplified by PCR. This fragment was then inserted into theSacI site of the vector pRS316GAL1LEU2 by homologous recombination. We previously determined that the annotation in theSaccharomyces Genome Data Base is likely to be incorrect regarding the ATG translation start site of the ZRG17 gene (17). All of the above plasmids encoding ZRG17 constructs were confirmed to be functional by complementation of the zrg17 mutant growth phenotype on YPGE plates at 37 °C. pGEO-TRP1 was co-transformed with pRg215, pZnT5L, or pZnT6 so that expression levels ofPrna1p-HA, ZnT5, and ZnT6 could be controlled by the addition of β-estradiol (Sigma) (10−7 to 10−5 M) (28).

Isolation of pHSIP150—In a screen to identify suppressors of themsc2 37 °C growth defect, the msc2 mutant was transformed with a yeast genomic library in the high copy vector YEp24. After initial selection for the plasmids, transformed cells were harvested and subsequently plated onto YPGE plates at a concentration of ~10,000 cells/plate. YPGE plates were incubated at 37 °C for 3 or 4 days and screened for colonies. Plasmids were isolated from these colonies and sequenced. One of the plasmids isolated was pHSIP150, whose insert consists of a genomic fragment from yeast chromosome X and contains theHSP150 gene.

Growth Assays—The desired strains were grown overnight inYPD, SD galactose, or SD glucose with the appropriate auxotrophies and β-estradiol where indicated. These cultures were subsequently diluted into the same medium, and 5 μl of diluted culture, yielding 104 or 105 cells, were spotted onto YPGE or YPGEgal plates. Where indicated, different concentrations ofZnCl2 and/or β-estradiol were added to the plates. The plates were incubated 3–4 days and photographed.

β-Galactosidase Assays and Subcellular Fractionation—β-Galacto- sidase assays, with specific activity normalized to protein content, and subcellular fractionation, where protein extracts were fractionated on sucrose gradients with or without Mg2+ , were performed as described previously (10).

Protein Lyases, Co-immunoprecipitation, and Immunoblotting—Protein lyases for co-immunoprecipitation were obtained as follows. Yeast cultures were grown to an A600 = ~ 1.0 in L2M medium containing 1 or 1000 μM zinc. The cells were harvested, washed once with water, and resuspended in 50 mM NaCl. Protein lyases were obtained using glass beads in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM EDTA (all from Sigma), mini-EDTA-free protease inhibitor mixture pellets (Roche Applied Science)) and vortexing 10 × 30 s, with 30 s on ice between pulses. The cell debris was pelleted by centrifuging for 2 min at 500 × g at 4 °C. The resulting supernatant was the total protein lysate. The lysates were then solubilized with 3% LM (n-dodecyl-β-D-maltoside) (MP Biomedical) on ice for 2 h. The insoluble proteins were pelleted by centrifuging for 15 min at 15,000 × g at 4 °C. The detergent-
beads were rotated at 4 °C for 30 min, after which the beads were added to each lysate. The lysates plus boxed using ClustalW. The fungal proteins closely related to Zrg17p (Zrg17p with other CDF members. The dendrogram was generated topology of the Zrg17p protein. The histidine-rich domain between DAG1p, Cot1p, Mmt1p, Mmt2p, Msc2p, and Zrc1p) and humans (ZnT1 to 9) were also included in the analysis.
soluble protein lysates were diluted 1:3 into IPP150 buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl), and then 50 μl of IgG-Sepharose beads (Amersham Biosciences) were added to each lysate. The lysates plus beads were rotated at 4 °C for 30 min, after which the beads were washed four times with IPP150 buffer plus 0.1% LM. The beads were resuspended in elution buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% SDS) and incubated at 65 °C for 30 min to elute the proteins off of the beads. Equal volumes of eluted protein lysates were loaded onto SDS-PAGE for immunoblotting. When denatured by boiling, epitope-tagged Zrg17p exhibited high molecular weight aggregates on immunoblots (data not shown). Therefore, lysates containing epitope-tagged Zrg17p were always denatured for 30 min at either 37 °C or 65 °C prior to SDS-PAGE. Immunoblots were done by standard techniques (29). Blots were visualized with POD (Roche Applied Science). Band quantitation to determine -fold difference in protein levels was performed using NIH Image 1.61. Antibodies used were rabbit anti-HA (Sigma), mouse anti-Dpm1p (Molecular Probes, Inc., Eugene, OR), rabbit anti-Kex2p (gift of Steven Nothwehr, University of Missouri-Columbia), goat anti-mouse horseradish peroxidase-conjugated secondary (Pierce), and goat anti-rabbit horseradish peroxidase-conjugated secondary (Pierce).

RESULTS

Zrg17p Is a Distant Member of the CDF Family of Metal Ion Transporters—The Zrg17p protein is 504 amino acids in length. While originally estimated to have seven transmembrane domains (22), the consensus of several algorithms (e.g., TOPPRED2, SOSUI, DAS, HMMTOP, and TMPRED) predicted only six such domains in the Zrg17p protein (Fig. 1A). Membrane topology predictions of Zrg17p consistently placed both the amino- and carboxyl-terminal ends on the cytosolic surface of cellular membranes. This topology is common among many members of the CDF family of metal ion transporters (18). Sequence data base comparisons indicated that Zrg17p is closely related to gene products found in a number of other fungal species, including Aspergillus nidulans, Gibberella zeae, Neurospora crassa, Schizosaccharomyces pombe and Ashbya gossypii. When the amino acid sequences of these orthologs were used to identify other related proteins in the sequence data bases using PSI-BLAST, several members of the CDF family were identified in the first iteration. For example, the human ZnT3 and ZnT6 CDF proteins were detected with E-values of 1 × 10^{-6} and 8 × 10^{-6}, respectively. These results suggested that Zrg17p and its fungal orthologs are distant members of the CDF family of metal transporters. Zrg17p and its closely related proteins appear to form a distinct subfamily of CDF proteins, as indicated by a multiple sequence alignment/tree-building analysis depicted in Fig. 1B. Unlike many other CDF proteins, however, Zrg17p has a histidine-rich domain (... HDHDEINEQIPHSH ... ) located between predicted transmembrane domains III and IV (Fig. 1A). Similar domains are found between transmembrane domains IV and V in most other eukaryotic CDF proteins.

Studies Implicating Zrg17p in ER Zinc Transport—Zrg17p was previously proposed to be involved in zinc uptake across the plasma membrane (22). However, the apparent relationship of Zrg17p to members of the CDF family suggested that this protein was more likely to transport zinc either outside of the cell or into an intracellular compartment. Furthermore, the zinc-suppressible large cell phenotype previously observed for zrg17 mutants was similar to that seen with msc2 mutants. This suggested that Zrg17p may also be involved in transporting zinc into the ER lumen. If so, we predicted that zrg17 mutants would share other phenotypes with msc2 mutants. We generated an isogenic strain where the ZRG17 gene was deleted from the genome. We also generated an msc2 zrg17 double deletion strain. If Msc2p and Zrg17p functioned separately to supply zinc to the ER, we predicted that these phenotypes would be more severe in the double mutant than in either single mutant. However, if Msc2p and Zrg17p functioned in the same pathway, no additivity of their effects would be expected.

Mutant msc2 cells grow well at 30 °C on rich YPGE medium plates containing the respired carbon sources glycerol and ethanol but grow poorly at 37 °C (10). This temperature-sensitive growth phenotype is suppressible by high zinc added to the medium (21). As seen in Fig. 2A, the zrg17 mutant also exhibited a temperature-sensitive growth defect on YPGE, and it was also suppressible by added zinc. To determine whether the msc2 and zrg17 single mutations differed in their severity, we assessed suppression of the growth defect over a range of zinc concentrations added to these plates. The single mutants each showed partial suppression with the addition of 100–250 μM zinc and full suppression with 500 μM zinc added. Thus, msc2 and zrg17 mutants show very similar zinc-suppressible growth defects at 37 °C. As expected, the msc2 zrg17 double mutant also showed poor growth on YPGE at the higher temperature. Similar concentrations of added zinc suppressed the double mutant to the same degree as the single mutants, indicating that the two mutations are not additive for this phenotype.

Another previously observed phenotype of the msc2 mutant is the up-regulation of the UPR in zinc-limiting conditions (10). The UPR is a response to misfolded proteins in the ER of cells whereby protein chaperones and degradation systems are up-regulated to refold or degrade the aberrant proteins. The increased level of UPR in msc2 mutants probably reflects, at least
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in part, the zinc requirement of luminal protein chaperones (i.e., Scj1p) (10). We introduced a UPR reporter construct (UPRE-lacZ) into the msc2, zrg17, and msc2 zrg17 mutants and determined the activity of this reporter over a range of zinc concentrations. As observed previously, wild type cells show a small induction in UPRE-lacZ expression under low zinc conditions, and the msc2 mutation exacerbates this effect (Fig. 2B). Like the msc2 mutant, the zrg17 mutant also exhibited elevated UPRE-lacZ activity in low zinc (LZM + 0.3–10 μM ZnCl2) compared with wild type cells. The double mutant showed an increase similar to that seen in both single mutants, again demonstrating that these mutations are not additive with respect to this phenotype. We have shown previously that activity of a HIS4-lacZ reporter, which is not responsive to either zinc or unfolded proteins, showed high expression in both wild type and mutant strains at all zinc concentrations (10). This indicates that the results presented here are specific to the UPRE-lacZ reporter and not a general effect on lacZ activity. Therefore, the UPRE-lacZ results suggest that Zrg17p, like Msc2p, is needed to maintain protein folding in the ER.

Previous studies suggested that loss of Msc2p function results in an increased level of labile cytosolic/nuclear zinc, and this is consistent with the role of Msc2p in transporting cytosolic zinc into the ER lumen (10, 21). This phenotype was observed using a Zap1p-regulated ZRE-lacZ reporter construct. Zap1p activity and ZRE-lacZ expression is high when labile zinc levels decrease and is reduced when labile zinc levels rise. If Msc2p and Zrg17p are involved in the same process, we predicted that the zrg17 mutant would also have altered Zap1p-mediated regulation. The ZRE-lacZ reporter was transformed into the msc2, zrg17, and msc2 zrg17 mutants to determine whether these strains have altered zinc homeostasis. In wild type cells, Zap1p is more active in low zinc, resulting in high expression of the ZRE-lacZ reporter (Fig. 2C). With increasing medium zinc concentrations, Zap1p becomes less active, resulting in decreased expression of the reporter. The reporter expression profile in the msc2, zrg17, and msc2 zrg17 mutants was quite different from the wild type profile. All three mutants had reporter activity in LZM plus 1–30 μM ZnCl2 lower than that seen in wild type cells. These results suggest that Zap1p is sensing higher amounts of zinc in the cytosol of these mutants and are consistent with Zap1p, like Msc2p, being needed to transport zinc into an intracellular compartment. Once more, the phenotypes of the single mutants were not additive in the double mutant.

Finally, we tested whether zrg17 mutants also show a genetic interaction that we discovered for msc2. We performed a genetic screen for genes that, when overexpressed from a high copy plasmid, could suppress the msc2 temperature-sensitive growth defect (see “Materials and Methods”). One gene identified in this screen was HSP150. Hsp150p is a cell wall protein whose exact function is unknown. However, overexpression of Hsp150p completely suppresses the temperature-sensitive msc2 growth defect. As seen in Fig. 3, HSP150 overexpression from a high copy plasmid (pHSP150) also suppressed the growth defect of the zrg17 mutant as well as the msc2 zrg17 double mutant. Thus, the zrg17 and msc2 mutant defects are alleviated by the same overexpression suppressor. These re-
results, combined with shared and nonadditive phenotypes of the zrg17 and msc2 mutants described in Fig. 2 argue that both proteins are involved in the same pathway supplying zinc to the endoplasmic reticulum of yeast.

Zrg17p and Msc2p Co-localize to the ER — We next addressed the question of whether Msc2p and Zrg17p were functioning in the same subcellular compartment. Msc2p was previously localized to the ER (10, 21). Zrg17p was tentatively localized to the ER as part of a genome-wide protein localization study (30). However, the green fluorescent protein-tagged construct used in that study was not shown to be functional, leaving this localization tentative. We determined whether Zrg17p was indeed localized to the ER when expressed at normal levels as a functional epitope-tagged protein. We generated a construct of ZRG17 on a single-copy plasmid where Zrg17p and Msc2p Physically Interact and Are Both Required to Form a Functional Complex — If Msc2p and Zrg17p separately perform the same function in the cell (i.e. as parallel pathways of ER zinc transport), then overexpression of one gene might be able to compensate for the loss of the other. Therefore, we generated a C-terminal Myc-tagged construct on a high copy plasmid. Li and Kaplan (21) showed previously that this increased gene dosage results in Msc2p overexpression.

To determine whether overexpression of one gene could compensate for the loss of the other, we introduced the Msc2p and Zrg17 overexpression plasmids into the msc2 and zrg17 mutant strains. Overexpression of one gene conferred partial suppression of the mutant phenotypes, a range of zinc was added to the plates, and growth was scored. At each concentration, overexpressed Zrg17p did not show any suppression of the msc2 mutant phenotype. Similarly, overexpression of Msc2p did not show any ability to suppress the zrg17 mutation. These results suggested either of two models. First, Msc2p and Zrg17p may perform separate steps in the same pathway. Alternatively, these proteins may interact to form a functional complex.
complex for ER zinc transport. With Msc2p and Zrg17p both being integral membrane proteins and co-localizing to the ER, we favored the second model and determined whether Msc2p and Zrg17p form a complex. A genome-wide yeast two-hybrid screen looking for potential interactions between proteins identified a possible interaction between Msc2p and Zrg17p (32). Although how this two-hybrid approach would succeed for two integral membrane proteins was puzzling (see “Discussion”), these results were sufficiently intriguing to warrant further assessment of this interaction. To do this, we performed co-immunoprecipitation of HA-tagged Msc2p and “tandem affinity purification” (TAP)-tagged Zrg17p. The TAP tag consists of a calmodulin-binding protein followed by a tobacco etch virus protease cleavage site and ends with protein A, which can bind to IgG proteins (33). We obtained a strain where a TAP tag was fused to the C terminus of ZRG17 in the genome, and we verified function of the ZRG17-TAP allele in this strain by its wild type levels of UPR induction in low zinc (data not shown).

Protein lysates for co-immunoprecipitation were generated from cells grown in low zinc (LZM plus 1 μM ZnCl2) for co-immunoprecipitation. Lysates were solubilized with detergent, diluted with buffer, and added to IgG-Sepharose beads. After washing of the beads, bound proteins were eluted and subjected to immunoblotting, probing with anti-HA antibody, which recognizes both HA and TAP tags. The asterisk denotes a background band, presumably IgG lost from the Sepharose beads, observed in all co-immunoprecipitation samples regardless of the presence or absence of tagged proteins. Shown are representative immunoblots from three independent experiments. A, co-immunoprecipitation of wild type (BY4741) and ZRG17-TAP cells bearing pMSC2 or pMSC2HA. B, co-immunoprecipitation of wild type (BY4741) and MSC2-TAP cells bearing pZRG17 or pZRG17HA.

**Fig. 6.** Msc2p and Zrg17p physically interact. Lysates were prepared from cells grown in LZM plus 1 μM ZnCl2 for co-immunoprecipitation. Lysates were solubilized with detergent, diluted with buffer, and added to IgG-Sepharose beads. After washing of the beads, bound proteins were eluted and subjected to immunoblotting, probing with anti-HA antibody, which recognizes both HA and TAP tags. The asterisk denotes a background band, presumably IgG lost from the Sepharose beads, observed in all co-immunoprecipitation samples regardless of the presence or absence of tagged proteins. Shown are representative immunoblots from three independent experiments. A, co-immunoprecipitation of wild type (BY4741) and ZRG17-TAP cells bearing pMSC2 or pMSC2HA. B, co-immunoprecipitation of wild type (BY4741) and MSC2-TAP cells bearing pZRG17 or pZRG17HA.

**DISCUSSION**

Zrg17p was previously proposed to be involved in zinc uptake (22). Several lines of evidence now indicate that this is not the case and that Zrg17p is involved in supplying zinc to the endoplasmic reticulum. First, we found that Zrg17p is likely to be a distant member of the CDF family of metal ion transporters. Although this relationship was not readily detected using single pass database search methods such as FASTA and BLAST, significant similarities were detected using the PSI-BLAST iterative profile search method. In addition, the CDF-like predicted topology of Zrg17p also argues that this protein is indeed a CDF family member. This relationship is important in identifying the function of Zrg17p because most of the members of this family are responsible for zinc transport from the cytoplasm to either outside of the cell or into intracellular organelles. Second, we found that zrg17 mutants share several phenotypes with msc2 mutants. Msc2p was shown previously to be involved in zinc transport into the ER lumen. Like msc2 mutants, zrg17 mutants show a zinc-suppressible cell morphol-
might succeed for integral membrane proteins like Zrg17p and Msc2p. One explanation is that because of their normal ER localization, these two proteins can form a complex on the inner membrane of the nuclear envelope, resulting in a functional membrane-tethered hybrid transcriptional activator.

We predict that Zrg17p and Msc2p come together to form a functional transporter complex. Several CDF members have been postulated or shown to form homodimers or other higher order homo-oligomeric species (34–37). However, we present here the first evidence for a physical interaction between different CDF proteins. The stoichiometry of subunits in the Msc2p-Zrg17p complex is still unknown, but they may form either heterodimers or higher order structures. Addressing these questions will require further experiments. An alternative model consistent with our results is that one protein (e.g. Zrg17p) interacts with Msc2p transiently to direct the proper localization of the Msc2p protein. This scenario is analogous to the role of Shr3p in conferring the proper localization of amino acid permeases such as Gap1p through a transient interaction that occurs in the ER. Shr3p and Gap1p have been shown to directly interact using precipitation experiments similar to those used here, and this interaction is essential to proper Gap1p localization (38). However, arguing against this hypothesis, we found that mutation of ZRG17 does not alter the ER localization of Msc2p, and an msc2 mutation does not affect the distribution of Zrg17p in the cell (data not shown).

One intriguing aspect of the heteromeric transporter complex model is how its transport activity may be regulated by zinc status. It was previously demonstrated that the ZRG17 gene is a direct target of Zap1p gene regulation (17, 22). ZRG17 mRNA levels increase in zinc-deficient cells, and Zrg17p protein levels increase to a similar degree. In contrast, the Msc2p gene is not a target of Zap1p regulation, and its mRNA levels are unaffected by zinc status (17). These observations suggest that Zrg17p is the rate-limiting subunit, and its transcriptional regulation by zinc controls the activity of the transporter complex. This model provides a clear explanation for why ZRG17 is a Zap1p target gene (i.e. to maintain zinc status in the ER by up-regulating the transport activity of the complex in zinc-limited cells through modulation of Zrg17p levels). In vitro assays of ER zinc transport are currently being developed to address these hypotheses directly.

The effects of msc2 and zrg17 mutations on UPRE-lacZ activity are also readily explained by our model. Failure to efficiently transport zinc into that compartment results in ER dysfunction. The large cell phenotype and temperature-sensitive growth defects on resired carbon sources are more enigmatic. One clue to the underlying molecular causes of these phenotypes comes from our identification of HSP150 as an overexpression suppressor of the zrg17 and msc2 mutant phenotypes. HSP150 gene expression is strongly induced by heat treatment (39) (hence its designation as a heat shock protein), but its gene product is not a protein chaperone. Rather, Hsp150p is a highly glycosylated secreted protein that is found covalently attached to the cell wall (39, 40). This protein is somehow involved in cell wall biogenesis and is a member of a family of four related proteins in yeast with partially redundant functions in conferring cell wall stability. We found that HSP150 overexpression suppresses both the morphological and temperature-sensitive growth defects of zrg17 and msc2 mutants (Fig. 3 and data not shown). This observation suggests that these phenotypes may be related to defects in cell wall formation. Many cell wall biosynthesis proteins are linked to the cell surface via GPI anchors, and GPI anchor synthesis is not affected by these mutations.

C. Ellis, unpublished observation.
known to be a zinc-dependent process (6). Interestingly, HSP150 was also isolated as an overexpression suppressor of a las21 mutation (41). Las21p is a GPI-phosphoethanolamine transferase involved in GPI anchor synthesis and one of the zinc-dependent enzymes in this pathway. Thus, HSP150 overexpression may somehow suppress defects in GPI anchor synthesis resulting from ER zinc deficiency. Alternatively, the defect associated with zrg17 and msc2 mutations may be directly linked to Hsp150p activity per se. Reduced ER zinc may lead to reduced Hsp150p function, and this can be alleviated by HSP150 overexpression. Consistent with this hypothesis, hsp150 mutants have a large cell phenotype and are temperature-sensitive for growth (40). It should be noted, however, that HSP150 overexpression does not suppress the UPR hyperinduction-phenotype, indicating that HSP150 actually be a more common phenomenon among CDF members.

Although we present evidence here for a novel interaction between two different CDF family members in yeast, this may actually be a more common phenomenon among CDF members. CDF proteins are found in bacteria, fungi, plants, invertebrates, insects, and mammals (18). One mammalian protein that is very similar to Msc2p is ZnT5, and Zrg17p is related to ZnT6. We found that the expression of both ZnT5 and ZnT6 can suppress the 37 °C growth phenotype of the msc2, zrg17, and msc2 zrg17 mutants (Fig. 7). This suppression only took place when both ZnT5 and ZnT6 were expressed in the same cell. This result suggests that ZnT5 and ZnT6 interact to form a complex that can transport zinc into the secretory pathway. It also argues that neither mammalian protein can interact with the analogous yeast protein to form a functional unit. Additional evidence supporting a ZnT5-ZnT6 interaction includes the observation that ZnT5 and ZnT6 are both localized in the trans-Golgi network in mammalian cells (24, 25). In addition, the SLC30A5 (ZnT5) and SLC30A6 (ZnT6) genes are expressed in many of the same tissues (42). ZnT6 may also form functional complexes with ZnT7, the mammalian CDF most closely related to ZnT5, in some tissues. ZnT7 is also localized to the trans-Golgi network in mammalian cells (43).

We proposed above that the transcriptional regulation of Zrg17p controls the transport activity of the yeast complex in response to zinc status. The activity of the mammalian complex may also be regulated by zinc. ZnT5 mRNA was shown to be up-regulated by TPEF treatment, an inducer of zinc deficiency, in mammalian cells (44), analogous to the zinc regulation of ZRG17 in yeast.

The exact function of ZnT5 and ZnT6 in transporting zinc into the trans-Golgi network of mammalian cells is still unknown. However, a recent report suggested that ZnT5 is needed to supply zinc to GPI-anchored alkaline phosphatases, which transit through the secretory pathway to the plasma membrane (45). Because ZnT5 and ZnT6 can complement the 37 °C growth defect of the msc2 and zrg17 mutants (Fig. 7), these results suggest that ZnT5 and ZnT6 do indeed perform the same function as Msc2p and Zrg17p (i.e., supplying zinc to the secretory pathway to provide the metal cofactor for zinc-dependent proteins). We have recently shown that zinc is required for ER function in mammalian cells (10). Heteromeric complexes of CDF proteins may supply zinc to the secretory pathway in the cells of many eukaryotic organisms.

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