The ZRT2 Gene Encodes the Low Affinity Zinc Transporter in *Saccharomyces cerevisiae*<sup>†</sup>

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Zinc accumulation in *Saccharomyces cerevisiae* occurs through either of two uptake systems. A high affinity system is active in zinc-limited cells, and the ZRT1 gene encodes the transporter protein of this system. In this study, we characterized the low affinity system that is active in zinc-replete cells. The low affinity system is time-, temperature-, and concentration-dependent and prefers zinc over other metals as its substrate. Our results suggest that the ZRT2 gene encodes the transporter of this system. The amino acid sequence of Zrt2p is remarkably similar to those of Zrt1p and Irt1p, an Fe<sup>2+</sup> transporter from *Arabidopsis thaliana*. Overexpressing ZRT2 increased low affinity uptake, whereas disrupting this gene eliminated that activity, but had little effect on the high affinity system. Therefore, the high and low affinity systems are separate uptake pathways. Analysis of the zinc levels required for growth of *zrt2* mutant strains as well as the effects of the *zrt2* mutation on the regulation of the high affinity system demonstrated that the low affinity system is a biologically relevant mechanism of zinc accumulation. Finally, a *zrt1zrt2* mutant was viable, indicating the existence of additional zinc uptake pathways.

How the cells of all organisms acquire metal ions from their extracellular environment is one of the central unresolved questions in the biochemistry of these important nutrients. Zinc is essential because it is an integral cofactor of many proteins and is a critical determinant of their catalytic activity and/or structural stability (1). Moreover, zinc is an important component of many transcription factors, the zinc finger proteins, that regulate gene expression (2). Biochemical assays of zinc uptake in yeast indicated that this process is transporter-mediated, i.e. zinc uptake is time-, temperature-, and concentration-dependent and requires metabolic energy (3–5). Recent studies suggested the presence of two separate uptake systems (6). One system has high affinity for zinc with an apparent *K<sub>m</sub>* of 0.5–1 μM and is required for zinc-limited growth. The ZRT1 (for zinc-regulated transporter) gene appears to encode the transporter protein of this system. ZRT1 is a member of a closely related family of transporter genes found in organisms as diverse as fungi, plants, nematodes, and humans. This family includes the *IRT1* gene from *Arabidopsis thaliana*, which encodes an Fe<sup>2+</sup> transporter expressed in plant roots (7). Like the other members of this family, Zrt1p is predicted to be an integral membrane protein containing eight potential transmembrane domains.

The level of ZRT1 expression correlated with activity of the high affinity system; overexpression of ZRT1 increased high affinity uptake, whereas a *zrt1* mutation eliminated high affinity activity and resulted in poor growth of the mutant on zinc-limited media (6). The high affinity system was induced in activity >100-fold in response to zinc-limiting growth conditions. When cells were grown in media containing different zinc concentrations, high affinity uptake and ZRT1 mRNA levels were closely correlated, as was the β-galactosidase activity generated by a reporter gene in which the ZRT1 promoter was fused to the *Escherichia coli lacZ* gene. The ZRT1-lacZ fusion gene showed a similar pattern of regulation in response to cell-associated zinc levels in both wild-type and *zrt1* mutant cells despite the 75-fold higher extracellular zinc level required to down-regulate the promoter in the mutant. These results indicate that the activity of the high affinity system is controlled, at least in part, by transcriptional regulation of the ZRT1 gene in response to a regulatory pool of intracellular zinc.

The second system for zinc uptake in yeast has a lower affinity for substrate (apparent *K<sub>m</sub>* = 10 μM), and it is active in zinc-replete cells. Low affinity uptake was unaffected by the *zrt1* mutation, suggesting that this system is a separate uptake pathway for zinc. As described in this report, an initial characterization was conducted to determine some of the biochemical properties of the low affinity system. We also report the analysis of another member of the IRT/ZRT gene family, ZRT2. ZRT2 was identified in the sequence data bases because of the close sequence similarity of its product to Irt1p and Zrt1p (6, 7). Our analysis of ZRT2 suggests that this gene encodes the transporter protein of the low affinity system.

**EXPERIMENTAL PROCEDURES**

*Strains and Culture Methods—* Strains used were DY1457 (*MATa ade6 can1 his3 leu2 trp1 ura3*), ZHY1 (*MATa ade6 can1 his3 leu2 trp2 trp1 ura3 zrt1::LEU2*), ZHY2 (*MATa ade6 can1 his3 leu2 trp1 ura3 zrt2::HIS3*), and ZHY3 (*MATa ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*). Yeast were grown in YP or SD medium (8) supplemented with necessary auxotrophic requirements and either 2% glucose or 2% galactose. Zinc-limited YP and SD agar plates contained either bathophenanthroline disulfonate (200 μM) or EDTA (1 mM), respectively. A liquid zinc-limited medium (low zinc medium (LZM))<sup>1</sup> was prepared in the same manner as low iron medium (9) except that the ZnSO<sub>4</sub> in low iron medium was replaced with 10 μM FeCl<sub>3</sub> in LZM. LZM is similar in composition to SD medium with two modifications essential to controlling zinc availability. First, 1 mM EDTA is added to provide buffering for the concentration of free metal ions. Second, the medium is pH-buffered at 4.2 with 20 mM citrate to prevent pH changes that could alter the metal binding ability of EDTA. LZM was also prepared without EDTA (LZM–EDTA), which is less zinc-limiting be-

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‡ The abbreviations used are: LZM, low zinc medium; ORF, open reading frame; [Zn]γ, total zinc concentration; [Zn]δ, free zinc concentration.

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cause the predominant chelator in this medium, citrate, binds zinc with less affinity than does EDTA. The concentrations of free (i.e. unchelated) zinc were calculated using MAXCHELATOR software (Chris Patton, Stanford University). Cell number in liquid cultures was determined by measuring the absorbance of cell suspensions at 600 nm (A600) and converting to cell number with a standard curve.

**Zinc Uptake and β-Galactosidase Assays—** Zinc uptake assays were performed as described previously for iron uptake (10) except that 65ZnCl₂ (Amersham Corp.) and LZM–EDTA were substituted for 56FeCl₃ and low iron medium without EDTA, respectively. Cells were incubated for 5 min in LZM–EDTA plus the indicated concentration of 65Zn, collected on glass fiber filters (Schleicher & Schuell), and washed with 10 ml of ice-cold SSW (1 mM EDTA, 20 mM trisodium citrate, 1 mM KH₂PO₄, 1 mM CaCl₂, 5 mM MgSO₄, and 1 mM NaCl, pH 4.2), and cell-associated radioactivity was measured by liquid scintillation counting. Cells were starved for glucose by incubating them in LZM–EDTA prepared without glucose for 1 h at 30°C prior to assay. Michaelis-Menten kinetic values were determined using KINETASYST software (IntelliKinetics, Princeton, NJ). Stock solutions of the chloride salts of cobalt, copper, magnesium, manganese, and nickel were prepared in 0.02 N HCl, and the FeCl₃ stock solution was prepared at 50 mM in 0.1 N HCl. The statistical significance of the differences of values relative to controls was determined with one-way analysis of variance, followed by a Dunnett multiple comparison test. β-Galactosidase activity was assayed in cells harvested at an A600 of 0.5–2.0 as described by Guarente (11), and activity is expressed as the change in absorbance at 420 nm x 1000 divided by (min × ml of culture used × A600 of the culture). Cell-associated zinc was measured in parallel cultures supplemented with tracer amounts of 65Zn (10 μM) and nonradioactive zinc to the indicated final concentration. Aliquots (0.5 ml) were filtered and washed with 10 ml of ice-cold SSW, and radioactivity was measured by liquid scintillation.

**Isolation of the ZRT2 Gene and DNA Manipulations—** E. coli and yeast transformations were performed using standard methods (12, 13). To screen for multicopy suppressors of the zrt1 mutation, ZHY1 cells were transformed with a genomic library constructed in the multicopy vector YEp24 (14). Approximately 40,000 Úra⁺ transformants were isolated and replated onto zinc-limited YP glucose + bathophenanthroline disulfonate agar plates. Three independent transformants were isolated that formed larger colonies on this medium than the untransformed parent strain. Plasmid dependence was verified by selectively removing the plasmids from each transformant with 5-fluoroorotic acid (15), followed by replating onto YP glucose + bathophenanthroline disulfonate. DNA was prepared from each transformant, and the plasmids were then transformed into E. coli TOP10F' (Invitrogen). Plasmid DNA was prepared and restricted, and the ends of the inserts were sequenced as described by Borson et al. (16). This analysis demonstrated that two of the plasmids (pMC1 and pMC5) have cDNA inserts containing ZRT1. The third plasmid (pMC4) contains the ZRT2 gene. Computer data base comparisons were performed using BLAST (17); potential transmembrane domains were identified using TOP-PREDII (18); and multiple sequence alignment was performed using PILEUP ( Genetics Computer Group) (19).

A fragment bearing the ZRT2 open reading frame was prepared from pMC4 by the polymerase chain reaction using primers derived from the ZRT2 sequence with either SacI (Primer 1, 5'-ACCGGTGCTGACAGTGTGATCTTATATAGGCGAG-3') or SacI (Primer 2, 5'-CCCGAGCTCTCCTATGCCATTTTCCCTAG-3') restriction sites added to their 5'-ends. The resulting fragment was inserted into Bluescript SK⁺ (Stratagene) to generate pSK·ZRT2. A BomHI fragment containing the HIS3 gene was prepared from YCP407 (20) and inserted into pSK·ZRT2 to generate pZHD. This plasmid contains the zrt2 disruption mutation (zrt2::HIS3). Plasmid pZHD was digested with SacI and SacI to liberate the zrt2::HIS3 fragment and transformed into DY1457 and ZHY1 to replace the chromosomal locus by single-step gene transplacement (21). The resulting strains, ZHY2 and ZHY3, were confirmed to contain the zrt2::HIS3 allele by Southern blot analysis (data not shown). The SacI-SacI polymerase chain reaction fragment generated with Primers 1 and 2 was also cloned into pRS316-GAL1 (22) to generate pOEZ. Plasmid pG1 (6), containing a fusion between the ZRT1 promoter and the E. coli lacZ gene, was digested with NcoI and transformed into DY1457, ZHY1, ZHY2, and ZHY3 to integrate the plasmid at the URA3 locus (21).

**RESULTS**

**Low Affinity Zinc Uptake—** Zinc accumulation by the low affinity system was assayed in zrt1 mutant cells in which the high affinity system has been eliminated. When incubated at 0°C, these cells accumulated little zinc (Fig. 1A). At 30°C, cell-associated zinc levels increased linearly with time for up to 40 min. Similar results were obtained with wild-type cells grown under zinc-replete conditions in which the high affinity system is not expressed (data not shown). Thus, zinc accumulation by the low affinity system is time- and temperature-dependent. This accumulation was also dependent on glucose; after 5 min at 30°C in 10 μM 65Zn, glucose-starved zrt1 cells had no detectable zinc accumulation, whereas the same cells incubated with glucose accumulated 3.7 pmol/10⁶ cells. Taken
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Zinc reduced the uptake rate of radioactive zinc to the control rate. Copper and Fe$^{2+}$ was unaffected by cobalt, Fe$^{3+}$ less extent than nonradioactive zinc (low affinity system (32 and 79% of the control rate), but to a lesser extent than nonradioactive zinc (p < 0.05). Cobalt, Fe$^{3+}$, magnesium, manganese, and nickel did not diminish zinc uptake by the low affinity system. These results demonstrate that while copper and Fe$^{2+}$ could potentially be substrates, the low affinity system prefers zinc over other metals.

To compare the substrate specificities of the low and high affinity uptake systems, we determined if these metals could inhibit uptake by the high affinity system under similar conditions (Fig. 1B). Again, the concentration of added metal was 10-fold higher (20 μM) than the radioactive zinc concentration (2 μM). As with the low affinity system, the high affinity system was unaffected by cobalt, Fe$^{3+}$, magnesium, manganese, and nickel, whereas Fe$^{2+}$, copper, and, to a far greater extent, zinc were inhibitory of high affinity uptake (p < 0.05). In fact, the only significant difference between these systems was that copper was more inhibitory to the low affinity system than it was to the high affinity system. These results suggested that the high and low affinity systems are closely related, and this conclusion is supported by the high degree of sequence similarity between the Zrt1p high affinity transporter and the product of the ZRT2 gene. As described below, our studies suggest that ZRT2 encodes the low affinity zinc transporter.

Identification of the ZRT2 Gene—The ZRT2 gene was identified as an open reading frame (ORF) of unknown function during sequence analysis of the yeast genome (GenBank accession number X91258). The hypothesis that ZRT2 encodes the low affinity zinc transporter was suggested by the close similarity of its predicted amino acid sequence to that of Zrt1p (6). This hypothesis was further supported by our isolating ZRT2 as a multicopy suppressor of the zinc-limited growth defect of a zrt1 mutant (see “Experimental Procedures”). Multicopy suppressors are genes that, when overexpressed due to the increased gene dosage provided by a multicopy plasmid vector, reduce the phenotypic effects of a mutation in another gene (23). Overexpression of the low affinity transporter could suppress the zinc-limited growth defect of the zrt1 mutant, and a multicopy plasmid containing the ZRT2 gene (pMC4) was isolated in this way. This plasmid is a weaker suppressor of the zrt1 mutant than a multicopy plasmid containing a genomic copy of ZRT1 (pMC5), i.e. pMC4 restored the ability of the zrt1 mutant to grow under moderately zinc-limiting conditions, but not on severely zinc-limited media, where pMC5 could still complement the growth defect (Fig. 2). This result is consistent with the 10-fold difference in apparent $K_m$ values of the high and low affinity systems.

Plasmid pMC4 contains a 9-kilobase insert derived from the chromosome XII, immediately adjacent to the ACE2 gene (24). This fragment contains four ORFs originally designated L3120, L3116, L3111, and L3105 (Fig. 3). ORF L3120 is the gene that we have named ZRT2. The amino acid sequence of Zrt2p contains a total of 26 acidic residues in its 142-amino acid variable region, i.e. 18%, and Zrt1p contains 14 acidic residues in its 72-amino acid variable region (19%). These acidic residues could also contribute to metal binding. The membrane topologies of all three proteins, as predicted by the “positive-inside” rule (18), suggest that these domains show the greatest degree of sequence similarity among these proteins. The sequence alignment shown in Fig. 4 also indicates that transmembrane domains III and IV are separated by a region of variable length and sequence. The different lengths of this “variable region” largely account for the different overall sizes of these three proteins. Both Irt1p and Zrt1p contain a cluster of 3 to 4 histidine residues in the variable region that we have proposed previously to be a metal-binding domain (6, 7), and these histidines are also found in Zrt2p. Moreover, the variable regions of Zrt2p and Zrt1p carry a highly negative net charge. Zrt2p contains a total of 26 acidic residues in its 142-amino acid variable region (i.e. 18%), and Zrt1p contains 14 acidic residues in its 72-amino acid variable region (19%). These acidic residues could also contribute to metal binding. The membrane topologies of all three proteins, as predicted by the “positive-inside” rule (18), suggest that their variable regions are located on the cytoplasmic surface of the membrane.

ZRT2 Overexpression Increases Low Affinity Uptake—Plasmid pMC4 suppresses the growth defect of a zrt1 mutant on zinc-limited media. Given the high degree of similarity between Zrt1p and Zrt2p, this suppression was likely to result from increased expression of the ZRT2 gene and a concomitant increase in zinc uptake. To test this hypothesis, we assayed zinc uptake with yeast transformed with either pMC4 or the vector YEp24. At all concentrations tested, pMC4 transformants had a ~15-fold higher rate of zinc uptake than the corresponding vector control (Fig. 5A). To determine if the pMC4-dependent increase in uptake rate is due to overexpression of the ZRT2 gene rather than overexpression of one of the three other ORFs

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similarity located. The predicted transmembrane domains for Zrt1p and Irt1p are
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ZRT2
is required for the low affinity system to function, we
overexpression increases the zinc uptake rate. A, effect of pMC4. ZHY1 (zrt1) cells transformed with either pMC4 (closed squares) or the vector YEp24 (open squares) were grown to exponential phase in SD glucose medium and assayed for zinc uptake rate over a range of 65Zn concentrations. B, effect of ZRT2 expression from pOE2. ZHY1 (zrt1) cells transformed with pOE2 (closed circles) or the vector pRSS316-GAL1 (open circles) were grown to exponential phase in SD galactose medium and assayed for zinc uptake over a range of 65Zn concentrations. The insets show Lineweaver-Burk reciprocal plots of the corresponding data. Each point represents the mean of two separate experiments, each performed in duplicate. The standard deviation of each point was <15% of the corresponding mean.

The higher uptake rate observed in ZRT2-overexpressing cells could result from increased activity of the low affinity system or increased activity of a third, previously unknown, zinc uptake system. To address this question, we determined the Michaelis-Menten kinetic properties of the data presented in Fig. 5 using Lineweaver-Burk reciprocal plots (Fig. 5, insets). Although the Vmax values are much higher in the ZRT2-overexpressing strains, the apparent Km values are very similar to those of the low affinity system measured in the corresponding vector-only controls (Table I, part A). These results suggest that ZRT2 overexpression increases the activity of the low affinity system. Also consistent with this hypothesis, pMC4- and pOE2-dependent uptake activity was inhibited by copper and Fe3+ to the same degree that these metals inhibited the low affinity system, but not by any of the metals that do not inhibit the low affinity activity (data not shown). The lower apparent Km observed in the pOE2-overexpressing strains was very reproducible, and the underlying mechanism for this difference is not yet known. It is possible that other proteins are expressed under these conditions that alter the apparent Km of this system.

ZRT2 Is Required for Low Affinity Uptake—To determine if ZRT2 is required for the low affinity system to function, we

constructed a disruption mutation in this gene. This allele, designated zrt2::HIS3, was constructed by inserting the wild-type HIS3 gene into the center of the ZRT2 ORF (Fig. 4). The disruption allele was transformed by gene transplacement into wild-type and zrt1 haploid strains, and viable zrt2::HIS3 mutants were obtained in both. These results indicate that ZRT2 is not an essential gene, even in a zrt1 mutant strain where the high affinity uptake system has been eliminated.

Zinc uptake assays were performed on wild-type, zrt1, zrt2, and zrt1zrt2 mutant strains to determine if the zrt2 mutation altered the activity of either the low or high affinity zinc uptake system. In zinc-limited cells, no difference in the activity of the high affinity system was observed in the zrt2 mutant relative to the wild-type strain (Fig. 6A). Calculations of the apparent Km and Vmax from these data confirmed the conclusion that the zrt2 mutation does not affect the high affinity system (Table I, part B). Zinc-replete wild-type and zrt1 mutant cells had similar levels of low affinity activity (Fig. 6B and Table I, part B). In the zrt2 single mutant, however, the low affinity system was eliminated and apparently replaced by increased activity of the high affinity system (Fig. 6B). The apparent Km of uptake in zrt2 cells was similar to the apparent Km of the high affinity system (Table I, part B). Furthermore, neither low nor high affinity activity was detected in the zrt1zrt2 mutant. These
results demonstrate that the ZRT2 gene is required for function of the low affinity uptake system, but is not necessary for high affinity activity.

The Low Affinity System Is a Relevant Source of Zinc—The presence of high affinity uptake activity in zrt2 mutants grown in a zinc-rich medium suggests that the low affinity system is a relevant source of zinc; these cells have increased the activity of their high affinity system to compensate for the loss of the low affinity system. The relevance of the low affinity system as a source of zinc was also indicated by the observation that the activity of this system is zinc-regulated. zrt1 mutant cells grown in a zinc-replete medium (SD glucose medium) had a zinc uptake rate of 1.7 pmol/min/10^6 cells when assayed at 10 μM ^{65}Zn. However, cells grown in the same medium supplemented with extremely high levels of ZnCl_2 (2 mM) had an uptake rate of 28 ± 1 pmol/min/10^6 cells. The apparent Km (in terms of [Zn]_p) and V_max values were calculated from Lineweaver-Burk reciprocal plots. Values are means ± S.E.

| Plasmid | Km (μM) | V_max (pmol/min/10^6 cells) |
|---------|---------|-----------------------------|
| A. pMC4 | 8.0 ± 0.4 | 28 ± 1 |
| Vector  | 9.5 ± 0.8 | 2.2 ± 0.1 |
| pOE2    | 3.6 ± 0.1 | 17 ± 2 |
| Vector  | 10 ± 1   | 2.0 ± 0.1 |

a ND, uptake not detectable.

To further assess the role of the low affinity system as a source of zinc, we examined growth of wild-type and zrt2 cells in a zinc-limited medium (LZM) supplemented with increasing amounts of zinc. We have shown previously that the zrt1 mutant requires >500 μM total zinc ([Zn]_p) in LZM to undergo its maximum number of cell divisions (6), and this value corresponds to a calculated free (i.e. unchelated) zinc concentration ([Zn]_f) of ~500 μM. However, no difference in zinc requirement was observed between the wild-type and zrt2 strains, where as little as 10 μM total zinc ([Zn]_p = ~6 μM) was sufficient for maximum growth yield (Fig. 7A). This result was expected given that the high affinity system, which would be more important than the low affinity system for zinc-limited growth, is not reduced in activity by the zrt2 mutation.

LZM is zinc-limiting because of the presence of 1 mM EDTA, a high affinity zinc chelator. The zinc requirement of the zrt1 and zrt1zrt2 strains was determined in LZM–EDTA. LZM–EDTA is less zinc-limiting than LZM at a given concentration of total zinc because citrate, the predominant chelator in LZM–EDTA, binds the metal with lower affinity than EDTA. While the zrt1 single mutant divided its maximum number of times in LZM–EDTA with as little as 0.5 μM total zinc ([Zn]_p = ~6 nM), the zrt1zrt2 mutant required 500 μM total zinc ([Zn]_p = ~6 μM) to do so (Fig. 7B). Thus, zrt1zrt2 mutants are hypersensitive to zinc limitation and require at least 1000-fold more zinc for growth than the zrt1 strain. Given that the zrt1 mutant already requires 100-fold more zinc than the wild-type strain for optimal growth (6), this result indicates that the zrt1zrt2 mutant requires >10^7-fold more zinc in the medium than the wild-type strain.

We also examined the effects of the zrt2 mutation on the regulation of the ZRT1 gene. Our previous studies demonstrated that ZRT1 is regulated at the transcriptional level by a regulatory pool of intracellular zinc and that ZRT1 expression increases when this pool level is low (6). Furthermore, cell-associated zinc levels are much lower in the zrt1 mutant grown under zinc-limiting conditions. At higher concentrations of extracellular zinc, however, these levels increased to the wild-type levels. We proposed that this accumulation was the result of zinc uptake by the low affinity system. To test this hypothesis and to determine the effect of the zrt2 mutation on the pool of intracellular zinc that regulates ZRT1 gene expression, we measured β-galactosidase activity from the ZRT1-lacZ fusion in wild-type, zrt1, zrt2, and zrt1zrt2 mutant strains grown in...
media supplemented with a broad range of zinc concentrations. ZRT1-lacZ β-galactosidase activity in the zrt2 mutant was indistinguishable from the activity in wild-type cells (Fig. 8A). This result indicates that ZRT1 regulation in response to the regulatory pool of intracellular zinc is not greatly altered by the zrt2 mutation. As noted previously, the high affinity system is induced in zrt2 mutants growing in zinc-rich media (Fig. 6B), yet no increase in β-galactosidase activity was observed in this experiment. This apparent contradiction can be explained by the observation that the high affinity activity observed in the zrt2 mutant is very low (i.e. only 1–2% of the maximum activity), and β-galactosidase assays may be too insensitive to reliably detect this slight increase in expression.

ZRT1-lacZ expression was greatly altered in the zrt1zrt2 strain. While β-galactosidase activity in the zrt1 mutant decreased to its minimal level with as little as 10 μM total zinc ([Zn]₀ = −0.12 μM), expression in the zrt1zrt2 mutant was down-regulated only at total zinc concentrations of 200 μM ([Zn]₀ = −2.4 μM) or higher (Fig. 8B). These results suggest that the regulatory pool of intracellular zinc is at a lower level in the zrt1zrt2 strain grown under these conditions than in the zrt1 single mutant. This conclusion was supported by measurements of cell-associated zinc in these strains. At 10 μM total zinc, cell-associated zinc in the zrt1 strain was 133 ± 12 pmol/10⁶ cells, compared with 5 ± 0.6 pmol/10⁶ cells in the zrt1zrt2 strain. At 1000 μM total zinc, the zrt1 strain had a cell-associated zinc level of 168 ± 14 pmol/10⁶ cells, and the zrt1zrt2 level rose to 86 ± 21 pmol/10⁶ cells. Taken together, these results demonstrate that Zrt2p and the low affinity system contribute to the accumulation of zinc in the intracellular zinc pool that controls ZRT1 expression.

DISCUSSION

Our previous studies (6) suggested that at least two zinc uptake systems are present in Saccharomyces cerevisiae. The high affinity system has an apparent Km of 1 μM total zinc, which corresponds to a calculated free zinc concentration of ~10 nM. The low affinity system has an apparent Km of 10 μM total zinc, which corresponds to ~100 nM free zinc. Although other roles are also possible, we propose that ZRT2 encodes the transporter of the low affinity system. Consistent with this hypothesis, the ZRT2 gene was isolated as a multicopy suppressor of the zinc-limited growth defect of a zrt1 mutant.
Furthermore, the level of ZRT2 expression correlated with low affinity uptake activity. ZRT2 overexpression increased the activity of a system biochemically indistinguishable from the low affinity system. Conversely, disruption of the ZRT2 gene eliminated low affinity uptake. Thus, ZRT2 expression is both necessary and sufficient for low affinity activity. The predicted amino acid sequence of Zrt2p also suggests that this protein plays a direct role in the transport of zinc. Zrt2p shares remarkable similarity with Zrt1p (6) and Irt1p, an Fe$^{3+}$ transporter from A. thaliana (7). The distribution of hydrophobic amino acids suggests that all three gene products are integral membrane proteins with eight transmembrane domains. It is possible that Zrt2p is only one subunit of a heteromeric transport complex, but this hypothesis seems unlikely given that overexpression of ZRT2 alone increases zinc uptake activity.

ZRT2 is a member of a new and rapidly growing gene family of putative metal transporters. We have identified closely related genes in organisms as diverse as fungi, plants, nematodes, and humans (6, 7). Given that three members of this family, IRT1, ZRT1, and now ZRT2, have been implicated in metal transport, it seems likely that the other genes in this family play similar roles in metal metabolism. The structural similarity of these different gene products suggests that they may use a similar mechanism to transport their substrates. Zinc uptake in yeast requires metabolic energy (5). What then is the driving force for zinc uptake by Zrt2p? Like the other members of this family, Zrt2p does not contain ATP-binding domains, nor does the protein bear any significant similarity to the ubiquitous P-type ATPase family of transport proteins. This observation suggests that uptake may be driven by indirect coupling to energy metabolism, perhaps through the electrical potential generated across the plasma membrane by the plasma membrane ATPase. Alternatively, uptake may be driven by a transmembrane gradient of another ion. Uptake of zinc by the low affinity system was not inhibited by high extracellular K$^+$ (100 mM), indicating that a zinc/K$^+$ antiporter mechanism, as has been previously proposed (3, 25), is unlikely.

A cluster of histidines in Zrt2p is also found in Zrt1p, Irt1p, and the other members of this gene family. In Zrt2p and Zrt1p, these histidines are located in a region with a highly negative net charge due to the abundance of acidic amino acids. Imidazole ring nitrogens and carboxylate groups frequently serve as coordinating ligands for zinc (1), so these amino acids may be responsible for binding the metal substrate. In all of these proteins, the histidines are found in a region between two transmembrane domains that is predicted to be exposed on the cytoplasmic face of the membrane. Given this location, these amino acids may act in a late step in the uptake process by binding the metal after it has been transported across the membrane. Alternatively, these histidines may serve as part of a feedback regulation system. High intracellular zinc levels could result in binding of zinc to Zrt2p and, by some mechanism, reduce the activity of the transporter. Whatever their role, the conservation of these histidine residues within the IRT/ZRT gene family suggests that they are critical to the function of these proteins. This conclusion is further supported by the observation that similar histidine-rich domains are found in the sequences of four transport proteins implicated in zinc detoxification, i.e. Zrc1p and Cot1p from yeast and the mammalian ZnT-1p and ZnT-2p proteins (26–30). These proteins are apparently efflux transporters that transport metal ions from the cytoplasm either into an intracellular compartment or outside of the cell and, aside from a histidine-rich domain, share no significant similarity with the IRT/ZRT gene family. In each case, the histidine-rich domain is predicted to be cytoplasmically located. The functional importance of the conserved histidines in the IRT/ZRT gene family is currently under investigation. Furthermore, the interplay between zinc uptake transporters like Zrt1p and Zrt2p and efflux transporters like ZnT-1p and ZnT-2p will play an important role in cellular zinc homeostasis and merits further study.

Our results demonstrate that the high and low affinity systems are genetically and biochemically separate uptake pathways. We have also shown that the low affinity system is a relevant source of zinc for growing yeast cells. First, metal inhibition studies indicate that the low affinity system is very similar to the high affinity system in its specificity for zinc over other metals. While copper and Fe$^{3+}$ were capable of inhibiting zinc uptake by both the low and high affinity systems, further experiments will be required to determine if these metals are actually transported substrates. Second, the low affinity system is the major pathway for zinc uptake in wild-type cells grown under zinc-replete conditions (e.g. cells grown in SD glucose medium); no high affinity activity is detectable in these cells. Third, a zrt2 mutant strain that lacks the low affinity system has increased high affinity activity. This increased activity is presumably to compensate for loss of low affinity activity. In addition, the zrt1zrt2 mutant requires >1000-fold more zinc in the medium to grow and to supply the regulatory pool of intracellular zinc and to down-regulate the zinc-responsive ZRT1 promoter than does the zrt1 single mutant. These results indicate that the low affinity system is a major contributor to zinc accumulation in the zrt1 strain, and we infer that it also contributes to wild-type zinc accumulation under the same growth conditions.

Additional evidence that the low affinity system is a relevant source of zinc is provided by the observation that this system is regulated by zinc. Low affinity activity was diminished in cells grown in a medium containing extremely high levels of zinc (200 μM). The high affinity system and ZRT1 mRNA levels are regulated by zinc, and this regulation is mediated at the transcriptional level in response to an intracellular zinc pool (6). The analysis of low affinity system described here does not distinguish between transcriptional and post-transcriptional mechanisms. One possible mechanism, as discussed above, is dorn-regulation of the low affinity system by feedback inhibition of transporter activity. What is clear is that the regulatory systems that control high and low affinity uptake are responsive to different levels of cell-associated zinc. A decrease in ZRT1 expression and high affinity activity was apparent when cell-associated zinc levels rose to as little as 30 pmol/10⁶ cells (6). In that same analysis, we found that cells with a cell-associated zinc level of 120 pmol/10⁶ cells still had maximum low affinity activity (V_max = 2 pmol/min/10⁶ cells). Therefore, down-regulation of the low affinity system requires much higher levels of cell-associated zinc than is needed to repress the high affinity system. These observations pose an interesting regulatory question as to how these two systems respond to different levels of presumably the same signal, intracellular zinc.

We demonstrated previously that zrt1 mutant cells are not more resistant to higher levels of extracellular zinc than are wild-type cells (6). Neither zrt2 nor zrt1zrt2 strains are more resistant to extracellular zinc than are the wild-type or zrt1 strains. This observation is consistent with the low level of both high and low affinity activity observed in cells treated with extremely high levels of zinc and demonstrates that neither of these two systems plays a major role in zinc toxicity. Toxicity may result from zinc accumulation by one or more

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3 H. Zhao and D. Eide, unpublished result.
additional uptake pathways. The existence of this pathway(s) is demonstrated by the observation that a strain lacking both the high and low affinity systems, the \textit{zrt1zrt2} mutant, is still viable. Undoubtedly, these cells are obtaining zinc, and this uptake may represent the activity of a third system for zinc accumulation. The identity of this third system is suggested by earlier studies in which zinc uptake in yeast was attributed to a "divalent cation uptake system" that was also capable of transporting magnesium, cobalt, manganese, and nickel (3). The apparent $K_m$ of zinc uptake by this system was estimated to be ~500 $\mu$M total zinc, i.e. 50- and 500-fold higher than the \textit{ZRT2}- and \textit{ZRT1}-dependent systems, respectively. This apparent $K_m$ is consistent with the high concentration of zinc required to confer maximum growth to the \textit{zrt1zrt2} mutant. Whatever the mechanism, given the 105-fold greater zinc requirement of the \textit{zrt1zrt2} mutant strain compared with the wild-type strain, it is unlikely that this third pathway plays a significant role in zinc accumulation under any but the most zinc-rich conditions.

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