The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation

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Communicated by Richard Palmieri, University of Washington School of Medicine, Seattle, WA, December 8, 1995 (received for review October 31, 1995)

ABSTRACT  The yeast Saccharomyces cerevisiae has two separate systems for zinc uptake. One system has high affinity for substrate and is induced in zinc-deficient cells. The second system has lower affinity and is not highly regulated by zinc status. The ZRT1 gene encodes the transporter for the high-affinity system, called Zrt1p. The predicted amino acid sequence of Zrt1p is similar to that of Irl1p, a probable Fe(II) transporter from Arabidopsis thaliana. Like Irl1p, Zrt1p contains eight potential transmembrane domains and a possible metal-binding domain. Consistent with the proposed role of ZRT1 in zinc uptake, overexpressing this gene increased high-affinity uptake activity, whereas disrupting it eliminated that activity and resulted in poor growth of the mutant in zinc-limited media. Furthermore, ZRT1 mRNA levels and uptake activity were closely correlated, as was zinc-limited induction of a ZRT1-lacZ fusion. These results suggest that ZRT1 is regulated at the transcriptional level by the intracellular concentration of zinc. ZRT1 is an additional member of a growing family of metal transport proteins.

Zinc is an integral cofactor of many proteins and is indispensable to their catalytic activity and/or structural stability (1). Moreover, zinc is a ubiquitous component of enzymes involved in transcription and of accessory transcription factors, the zinc finger proteins, that regulate gene expression (2). Because of the many roles this metal plays in cellular biochemistry, zinc is an essential nutrient for all organisms. Despite this importance, very little is known about the molecular mechanisms cells use to obtain zinc. No transporter genes involved in zinc uptake (i.e., influx transporters) have been isolated from any organism. Recently, genes have been identified whose products are responsible for detoxifying intracellular zinc by transporting the metal from the cytoplasm to the cell exterior or into intracellular compartments (i.e., efflux transporters). These genes include the closely related euarkaryotic genes, COT1, ZRC1, and Zrt1 (3–5). While important for zinc detoxification, these genes do not appear to play a role in zinc uptake.

The yeast Saccharomyces cerevisiae provides an excellent model system in which to study zinc uptake in a eukaryotic cell. Biochemical assays of zinc uptake in yeast indicated that this process was transporter-mediated—i.e., uptake was dependent on time, temperature, and concentration and required metabolic energy (6–8). In this report, the presence of two separate zinc uptake systems in S. cerevisiae is demonstrated. One system has high affinity for zinc, and its activity markedly increases in zinc-limited cells. The second system has a lower affinity for zinc and is not highly regulated by zinc availability. We have characterized a gene, ZRT1 (for zinc-regulated transporter), identified because of its significant similarity to IRT1, an Fe(II) transporter gene from the plant Arabidopsis thaliana (9). Our results indicate that Zrt1p is the zinc transporter protein of the high-affinity uptake system. To our knowledge, ZRT1 is the first influx zinc transporter gene from any organism to be characterized at the molecular level, and it is a member of a growing family of metal transport proteins identified in fungi, nematodes, plants, and humans.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions. Strains used were DY1457 (MATa ade6 can1 his3 leu2 trp1 ura3) and ZHY1 (MATa ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2). Yeast were grown in standard culture media (SD and YPD; see ref. 10) supplemented with necessary auxotrophic requirements and either 2% glucose or 2% galactose. A zinc-limiting medium (low-zinc medium, L2M) was prepared in the same manner as low-iron medium (LIM) (11) except that ZnSO4 in LIM was replaced with 10 μM FeCl3 in L2M. Cell number in liquid cultures was determined by measuring the optical density of cell suspensions at 600 nm (OD600) and converting to cell number with a standard curve.

Plasmids and DNA Manipulations. Escherichia coli and yeast transformations were performed by using standard methods (12, 13). Plasmids constructed are diagrammed in Fig. 1. A fragment bearing the ZRT1 open reading frame was prepared by the polymerase chain reaction (PCR), using primers derived from the ZRT1 sequence with either BamHI (primer 3) or SalI restriction sites (primer 4) added to their 5’ ends (Fig. 1; primer 3, 5’-CGGATCC/ATGAGCAACGTTACG-3’; and primer 4, 5’-TACGCGGATCC/TAGACGTTACG-3’; the slash indicates the beginning of the ZRT1 sequence in each primer). The resulting fragment was inserted into pBluescript II SK+(+) (Stratagene, La Jolla, CA) to generate pSK-ZRT1. A PstI fragment containing the LEU2 gene was prepared as described (14) and inserted into pSK-ZRT1 to generate pZH2. This plasmid contains the zrt1 disruption mutation zrt1::LEU2. Plasmid pZH2 was digested with BamHI and SalI and used to transform DY1457 to replace the chromosomal locus by single-step gene transplacement (15). The resulting strain, ZHY1, was confirmed to contain the zrt1::LEU2 mutation by Southern blot analysis (data not shown). Because ZHY1 grows more slowly than the wild-type strain on media containing metal chelators, a plasmid (pMC5) containing a genomic ZRT1 fragment was isolated from a genomic library (16) by complementation (17) of the growth defect displayed by ZHY1 on YPD + 200 μM bathophenanthroline disulfonate (Sigma). The 2.2-kb SacI-HindIII fragment from pMCs containing the genomic ZRT1 gene was subcloned in pRS316 (18) to generate pMCs-HS. The BamHI–SalI fragment generated with primers 3 and 4 was also inserted into pRS316-GAL1 (19) to generate pOE1. A PCR fragment containing bases −706 to +3 of ZRT1 (the first base of the ATG initiation codon is designated as position +1) was generated with primers 1 and 2 (Fig. 1; primer 1, 5’-GCGCAT/GAAGGGATTCGTAATTTCCAGC-3’; primer 2, 5’-TCTACGCGGATCC/TAGACGTTACG-3’;)

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acid sequence against the current sequence data bases indicated that *IRT1* belongs to a family of closely related genes of unknown function, including two additional genes in *A. thaliana* and genes in rice, *Caenorhabditis elegans*, and humans. This comparison also identified two closely related open reading frames of unknown function from *S. cerevisiae*. We have designated one of these two yeast genes *ZRT1* for zinc-regulated transporter. The sequence of the open reading frame corresponding to *ZRT1* (GenBank accession no. P32804) was originally obtained during sequence analysis of a portion of the yeast genome (27). In this analysis, it was determined that *ZRT1* is located on chromosome VII immediately adjacent to the *FZF1* gene (Fig. 1) and is predicted to encode a protein of 376 amino acids. We have found that *Zrt1p* is 30% identical and 50% similar (i.e., identities plus conservative substitutions) to *Irt1p* (Fig. 2). A model of *Zrt1p* membrane topology suggested the presence of eight potential transmembrane domains located in positions in the amino acid sequence nearly identical to those predicted for *Irt1p*.

*Irt1p* contains an amino acid sequence, 

\[(\text{HG})_4\text{-protein}\]

that could be a metal-binding domain (9). A similar sequence was also found in *Zrt1p* (Fig. 2), in which three of the four histidines are conserved but the fourth potential ligand is unclear. A histidine located approximately 30 amino acids toward the carboxyl terminus may contribute to metal binding. In both *Irt1p* and *Zrt1p*, this histidine-rich domain is found in a large loop between transmembrane domains 3 and 4. Topological predictions based on the "positive-inside" rule (23) suggested that in both proteins this loop is located on the cytoplasmic surface of the membrane.

**ZRT1 Is Required for Zinc-Limited Growth.** To examine the function of *ZRT1*, we constructed a disruption mutation, *zrt1::LEU2*, by inserting the *LEU2* gene into the center of *ZRT1* (Fig. 1). This *zrt1* disruption allele was then introduced

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\[\text{ZRT1~VTFTGQK~MQSPEYL~LTYD}~...\]

**RESULTS**

**Identification of ZRT1.** *ZRT1* was identified fortuitously in our recent studies of iron uptake in plants. An Fe(II) transporter gene, *IRT1*, was cloned from *A. thaliana* by functional expression in yeast (9). Comparing the predicted *Irt1p* amino acid sequence against the current sequence data bases identified that *IRT1* belongs to a family of closely related genes of unknown function, including two additional genes in *A. thaliana* and genes in rice, *Caenorhabditis elegans*, and humans. This comparison also identified two closely related open reading frames of unknown function from *S. cerevisiae*. We have designated one of these two yeast genes *ZRT1* for zinc-regulated transporter. The sequence of the open reading frame corresponding to *ZRT1* (GenBank accession no. P32804) was originally obtained during sequence analysis of a portion of the yeast genome (27). In this analysis, it was determined that *ZRT1* is located on chromosome VII immediately adjacent to the *FZF1* gene (Fig. 1) and is predicted to encode a protein of 376 amino acids. We have found that *Zrt1p* is 30% identical and 50% similar (i.e., identities plus conservative substitutions) to *Irt1p* (Fig. 2). A model of *Zrt1p* membrane topology suggested the presence of eight potential transmembrane domains located in positions in the amino acid sequence nearly identical to those predicted for *Irt1p*.

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ZRT1 is required for high-affinity zinc uptake. Wild-type (DY1457, squares) and zrt1 mutant (ZHY1, circles) cells were grown to exponential phase in zinc-limited (open symbols) and zinc-replete (closed symbols) media and assayed for zinc uptake rate over a range of ZnSO₄ concentrations. Zinc-limited media were LzM + 10 μM zinc for the wild type and LzM + 500 μM zinc for the mutant. Zinc-replete conditions were LzM + 1000 μM for both strains. These conditions were selected on the basis of the experiment described in Fig. 6. ZHY1(pOE1) cells (triangles) were grown in zinc-replete SD/ galactose medium. Shown are the mean values of two experiments each performed in duplicate; error bars indicate ±1 SD.

apparent Kₘ and higher Vₘₐₓ than the activity observed in zinc-replete cells. These results suggest the presence of two zinc uptake systems in yeast, a high-affinity system induced by zinc limitation and a low-affinity system active in zinc-replete cells.

Zinc uptake assayed in zrt1 mutant cells grown in zinc-limiting and zinc-replete media displayed only low affinity activity (Fig. 4A, ○ and ●, respectively). The apparent Kₘ in each case was 10 ± 1 μM and the Vₘₐₓ was 1–2 pmol/min per 10⁶ cells. Expressing ZRT1 from the GAL1 promoter (pOE1; Fig. 1) in zinc-replete cells resulted in high-affinity uptake activity (apparent Kₘ of 0.6 ± 0.1 μM) with a Vₘₐₓ of 30 pmol/min per 10⁶ cells (Fig. 4B). No high-affinity activity was observed in these cells grown in glucose, in which the GAL1 promoter is not expressed, or in vector-only control cells grown in galactose or glucose (data not shown). These results demonstrate that the ZRT1 gene is both necessary and sufficient for high-affinity system activity but is not required for low-affinity system activity.

Regulation of ZRT1 mRNA Levels by Zinc. The observation that zinc-limited wild-type cells possess ZRT1-dependent uptake activity absent from zinc-replete cells suggested that the ZRT1 gene could be regulated by zinc. To test this hypothesis, we measured ZRT1 mRNA levels and zinc uptake activity in cells grown in a range of zinc concentrations. To provide a simpler means of assessing ZRT1 expression, we also constructed a fusion between the ZRT1 promoter and 5′ untranslated region and the E. coli lacZ gene, which encodes β-galactosidase (pG11; Fig. 1). ZRT1 mRNA was regulated in a
zinc-dependent manner; zinc-limited cells had 10-fold more ZRT1 mRNA than did zinc-replete cells (Fig. 5A). Uptake activity of the high-affinity system closely correlated with ZRT1 mRNA levels, and the ZRT1–lacZ fusion was regulated in an identical manner (Fig. 5B). The close correlation between ZRT1 expression levels and zinc uptake activity supports the hypothesis that ZRT1 encodes the high-affinity transporter. Furthermore, these results suggest that the ZRT1 gene is regulated at the transcriptional level by zinc and that the ZRT1–lacZ fusion accurately reflects that regulation.

The ZRT1–lacZ fusion allowed us to compare ZRT1 regulation in wild-type and zrtl mutant cells grown over a range of zinc concentrations. In the wild-type strain, β-galactosidase activity was highest in zinc-limited cells and decreased with increasing zinc concentrations in the medium (Fig. 6A). To test if zinc status alters β-galactosidase activity per se, cells bearing a HIS4–lacZ fusion were also assayed. HIS4 encodes a histidine biosynthetic enzyme and is dependent on the GCN4 leucine zipper protein for expression (30). This promoter fusion in wild-type cells generated β-galactosidase activity that correlated closely with the strain’s growth response to zinc (Fig. 3). Therefore, the repressive effects of zinc on β-galactosidase activity were not caused by zinc toxicity or negative effects of zinc excess on the activity of this enzyme. To estimate the size of the intracellular zinc pool in these cells and determine its relationship to ZRT1 expression, we measured the cell-associated zinc levels in cells grown in LZW containing 65Zn. The decrease in ZRT1-dependent β-galactosidase activity coincided with an increase in cell-associated zinc.

In the zrtl mutant strain, ZRT1–lacZ expression remained at its maximum level in cells grown with much higher concentrations of zinc in the medium than wild type (Fig. 6B). Thus, the zrtl mutant required more zinc in the medium to repress ZRT1 expression than did wild type cells. HIS4-dependent β-galactosidase activity was similar to the growth response of this strain to zinc as well. Finally, although the response of the ZRT1–lacZ fusion to extracellular zinc levels was very different in the wild type and mutant, the response to cell-associated zinc levels was unaffected. For example, approximately equal levels of cell-associated zinc were present in wild-type cells grown in LZW + 50 μM zinc and zrtl mutant cells grown in LZW + 750 μM zinc, and these cells also had similar levels of ZRT1 expression. These data suggest that the ZRT1 gene is regulated by intracellular zinc pools and that, although the amount of zinc required in the medium to supply these pools is greatly altered in the mutant, the regulatory system that controls ZRT1 expression in response to pool size is unaffected.

DISCUSSION

Our analyses demonstrate that yeast has two zinc uptake systems. One system has a high affinity for substrate, is induced by zinc limitation, and is necessary for growth in zinc-limiting conditions. Although other roles are formally possible, we propose that the ZRT1 gene encodes the transporter of this high-affinity system, and several lines of evidence support this hypothesis. First is the similarity between Zrt1p and Irt1p; Irt1p has been demonstrated to be an Fe(II) transporter and may also be capable of transporting zinc (9). Second, a mutation in the ZRT1 gene eliminated high-affinity uptake activity and inhibited growth on zinc-limiting media. Third, overexpressing ZRT1 increased activity of an uptake system that had an apparent Km almost identical to that of the high-affinity system. These results indicate that ZRT1 expression is both necessary and sufficient for high-affinity system
activity. We also found that high-affinity activity and ZRT1 expression were closely correlated across a wide range of extracellular zinc concentrations. It is possible that Zrt1p is only one subunit of a heteromeric transporter complex, but this is unlikely, given that overexpression of ZRT1 alone increased high-affinity activity. We have designated the second IRT1 homolog in yeast ZRT2 because of its similarity to ZRT1. Preliminary studies suggest that ZRT2 encodes the low-affinity transporter.

ZRT1 is, to our knowledge, the first influx zinc transporter gene from any organism to be characterized at the molecular level. Neither Irt1p nor Zrt1p contain ATP-binding domains, suggesting that uptake is driven by indirect coupling to energy metabolism, perhaps through a gradient of another ion such as K+ (6, 31). A group of histidine residues found in Irt1p was conserved in Zrt1p. This region may be a metal-binding domain, given that the imidazole ring nitrogens of histidine may serve as coordinating ligands for metal ions. In both proteins, this sequence is found in a loop region predicted to be on the cytoplasmic surface of the membrane. Similar histidine-rich sequences are also found in the three eukaryotic proteins implicated in zinc detoxification—i.e., Zrc1p, Cdt1p, and Zrt1p (3–5). In each case, the domain is predicted to be cytoplasmically located. This conservation suggests that the domain plays an important functional role in Irt1p and Zrt1p. For example, these histidines may serve as a means of feedback regulation of zinc transport. High intracellular zinc levels could result in binding of zinc by Zrt1p and reduce the activity of the transporter.

Zinc limitation induces activity of the high-affinity system. Because our results suggest that this system is regulated at the transcriptional level, it is tempting to speculate that a zinc finger DNA-binding protein may sense intracellular zinc levels to regulate ZRT1 expression. However, we cannot rule out a mechanism that controls mRNA stability through sequence elements located in the 5′ untranslated region of the mRNA. Whatever the mechanism, the high-affinity system is clearly regulated in response to the intracellular zinc content. This is demonstrated by the fact that the ZRT1–lacZ fusion gene shows similar responses to cell-associated zinc levels in wild-type and zrt1 mutants despite a 15-fold difference in their response to external levels of zinc. Thus, the regulatory system that controls ZRT1 expression in response to intracellular zinc pools is unaffected in the zrt1 mutant. We have also found that the zrt1 mutant is not any more resistant to high extracellular zinc levels than are wild-type cells (unpublished result). This result is consistent with the low level of ZRT1 expression observed in zinc-replete cells and demonstrates that the high-affinity uptake system does not play an important role in zinc toxicity.

All organisms require transition metals such as zinc and iron. Accumulation of these metals is transporter-mediated, yet little is known about these transporters or their molecular mechanisms of action. We have identified a zinc transporter gene from yeast that is a member of a growing family of similar genes found in organisms as diverse as fungi, plants, nematodes, and humans. The two members that have been examined experimentally, IRT1 and ZRT1, have been shown to encode metal transport proteins. Therefore, it seems likely that other genes in this family play similar roles in metal uptake in the organisms in which they are found.

We thank M. L. Guerinot for topology analysis and M. L. Guerinot and A. Thering for critical reading of the manuscript. This work was supported by National Science Foundation Grant MCB-9543435 to D.E.