Induction of the ZRC1 Metal Tolerance Gene in Zinc-limited Yeast Confers Resistance to Zinc Shock*

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Zinc is an essential nutrient but toxic to cells with overaccumulation. For this reason, intracellular zinc levels are tightly controlled. In the yeast Saccharomyces cerevisiae, the Zrc1 and Cot1 proteins have been implicated in the storage and detoxification of excess zinc in the vacuole. Surprisingly, transcription of ZRC1 is induced in zinc-limited cells by the zinc-responsive transcription factor Zap1. We show here that this increase in ZRC1 expression is a novel mechanism of zinc homeostasis and stress tolerance. Zinc-limited cells also express high levels of the plasma membrane zinc uptake transporters. As a consequence, when zinc-limited cells are resupplied with small amounts of zinc, large quantities quickly accumulate in the cell, a condition we refer to as “zinc shock.” We show here that ZRC1 and its induction in zinc-limited cells are required for resistance to this zinc shock. Experiments using the zinc-responsive fluorophore FuraZin-1 as an indicator of vacuolar zinc levels indicated that Zrc1 is required for the rapid transport of zinc into the vacuole during zinc shock. We also present evidence that cytosolic zinc rises to higher levels in cells unable to sequester this excess zinc. Thus, the increase in ZRC1 expression occurs prior to the zinc shock stress for which this induction is important. We propose that this “proactive” strategy of homeostatic regulation, such as we document here for ZRC1, may represent a common but largely unrecognized phenomenon.

All organisms face constantly changing nutrient availability and environmental stresses. As a consequence, organisms have regulatory mechanisms that maintain nutrient homeostasis and deal with damage caused by stress. For example, deficiency of an essential nutrient often induces expression of the genes involved in acquiring that nutrient. Stresses such as heat shock or reactive oxygen species increase expression of genes whose products reverse the resultant cellular damage. As a rule, these regulatory systems are reactionary in nature, responding to the stress to alter levels of gene expression and/or protein activity. An alternative strategy would be to increase expression of the required genes prior to, rather than in response to the specific stress. We designate this latter strategy as “proactive” regulation to distinguish it from the commonly recognized “reactive” responses. In this report, we describe an apparent proactive mechanism of zinc homeostasis in the yeast Saccharomyces cerevisiae, the up-regulation of a metal tolerance gene in zinc-deficient cells as protection against “zinc shock.”

Zinc is an essential nutrient required for many processes. Its chemical properties make this metal a useful catalytic and/or structural cofactor in many proteins. Despite its importance however, excess zinc is toxic. Zinc toxicity may involve competition with other metal ions for the active sites of enzymes or intracellular transport proteins. For this reason, organisms have evolved with mechanisms of zinc homeostasis to tightly control the intracellular level of zinc as extracellular concentrations change. An indication of the exquisite precision of this control was recently obtained in studies of Escherichia coli, where regulatory systems controlling zinc uptake and efflux apparently strive to maintain the free intracellular zinc concentration at less than one atom per cell (1). Several reports suggest that eukaryotic cells also maintain very low levels of cytoplasmic labile zinc under steady-state conditions (2–4).

Studies of yeast have revealed several components of zinc homeostasis in this organism (5). Among these, the ZRC1 gene encodes a potential transporter protein of the cation diffusion facilitator (CDF) family (6). This family also includes bacterial, plant, and mammalian proteins involved in zinc efflux and compartmentalization. ZRC1 is known to contribute to zinc tolerance (7, 8), and the Zrc1 protein was localized to the yeast vacuole membrane (9–11). Our recent in vitro studies provided evidence that Zrc1 directly mediates vacuolar zinc transport, most likely via a zinc/H+ antiport mechanism (11). The COT1 gene encodes a related protein that may act analogously to Zrc1 in cobalt detoxification (8, 12). Cot1 also contributes to zinc detoxification (13) and both Zrc1 and Cot1 appear to be required for sequestration of zinc in an intracellular storage compartment for later use under zinc-limiting conditions (14).

During the transition from zinc-replete to zinc-limiting conditions, many genes are induced to maintain adequate supplies of zinc for cell growth. Three such genes, ZRT1, ZRT2, and FET4 encode plasma membrane transporters responsible for zinc uptake under deficient conditions (15–17). All three genes are targets of the Zap1 transcriptional activator (18), a central player in zinc homeostasis. Zap1 is active in zinc-limited cells and is repressed by high cytoplasmic/nuclear zinc (19). When active, Zap1p binds to an 11-bp sequence, the zinc response element (ZRE),1 in the promoters of its target genes (20, 21).

DNA microarray analysis has suggested that at least 46 genes in yeast are direct targets of Zap1 regulation (21). Surprisingly, this group and others (22) noted ZRC1 among these Zap1 target genes. These data raise an intriguing question. Why would a transporter involved in zinc storage and detoxi-

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1 The abbreviations used are: ZRE, zinc response element; MES, 4-morpholineethanesulfonic acid; S. D., synthetic-defined medium or standard deviation; CSD, chelax-treated synthetic-defined medium; LZM, low zinc medium; WT, wild type.
ZRC1 Regulation and Zinc Shock Resistance

Yeast Strains and Growth Conditions—Four different media were used for yeast cultures. YPD and synthetic-defined (S.D.) medium with 2% glucose (23) are zinc-replete and contain no strong chelators. Low zinc medium (LZM) and chelex-treated synthetic-defined (CSM) medium were prepared as previously described (14, 21). LZM is zinc limiting because of the inclusion of 1 mM EDTA and 20 mM citrate as divalent cation chelators. CSM is zinc limiting because zinc is removed from the medium with the chel-ex-100 ion exchange resin. Zinc supplemented into CSM is much more bioavailable than in LZM due to the absence of strong chelators in CSM. Cell density determinations and β-galactosidase assays were performed as previously described (14). S. cerevisiae strains CM100, 102, 103, 104, and 142 were also previously described (14). All newly constructed strains are isogenic to CM100. CM141 (MATa zrc1::LEU2 zrc1::HIS3) was derived from a cross of ZHY1 (15) and CM102. The CM146 (MATα zrc1::zeo) strain was constructed by integrating the insert of YCp101::zeo at the chromosomal ZRC1 locus. Transformants were selected by complementation of the zinc-sensitive phenotype of a zrc1 cot1 double mutant strain (CM104), and the presence of the mutation was verified using PCR. The cot1 mutation was then removed by backcrossing to a wild-type strain.

DNA and Protein Manipulations—To construct the Yepzrc1::mzr plasmid, overlap PCR was used to generate transversion mutations in the ZRC1 promoter (24). The resulting fragment was inserted into Bip1-, BstXI-digested YCpZRC1 (11) by gap repair. Construction of YepZRC1::laczZ was previously described (21). Yepzrc1::mzr-laczZ was constructed by amplifying the ZRC1 promoter from Yepzrc1::mzr and inserting the fragment into Yep353 by gap repair. All plasmid constructs were verified by DNA sequencing. Total protein was extracted from yeast, and immunoblot analysis was performed as previously described (25). Anti-Vma1 antibody was obtained from Molecular Probes.

Zinc Uptake and Accumulation Assays—Zinc uptake was assayed as described (15). Standard zinc uptake assays were performed in LZM lacking EDTA (LZM-EDTA) to increase the bioavailability of the zinc for uptake into cells. In order to determine zinc-associated growth after growth in \( ^{65}Zn \)-containing medium, 1-ml aliquots of cells were collected on glass fiber filters (Whatman) and washed twice with 5 ml of wash buffer (20 mM sodium citrate, pH 4, 1 mM EDTA). Radioactivity retained on the filters was quantitated with a gamma counter. \( ^{65}Zn \) uptake by cells loaded with FuraZin-1 (see below) was assayed in the same buffer used for assays of fluorophore fluorescence to allow direct comparison of cell total and vacuolar zinc accumulation under these conditions.

Measurement of Vacuole Zinc Content with FuraZin—Yeast cultures (250 ml) were grown to log phase in LZM + 2 μM zinc. Cells were harvested, washed twice with phosphate-buffered saline and resuspended in phosphate-buffered saline at a final density of 5 \( \times \) 10⁶ cells/ml. 50 μg of FuraZin-1 acetoxyethyl (AM) ester was dissolved in 16.6 μl of a 20% fluorophore solution (both obtained from Molecular Probes) and the solution diluted 4-fold with MES-Tris to give a stock solution of 1.25 mM fluorophore and 5% Pluronic. Fluorophore was added to a 1-ml aliquot of the cell suspensions to give a final concentration of 25 μM. Another 1-ml aliquot of each strain was treated in parallel without exposure to fluorophore. The cell suspensions were incubated at 30 °C in the dark for 1 h with agitation. The cells were recovered by centrifugation, washed three times with 5 ml of chilled zinc uptake buffer (10 mM MES-Tris, pH 6.5, 4 mM MgCl₂, 2% glucose) and 1 mM EDTA and then incubated at 30 °C for another 30 min. This step allowed the cells to redistribute cytoplasmic fluorophore to the vacuole and complete its hydrolysis. The cells were then chilled and washed twice with MES-Tris uptake buffer (EDTA). Cell pellets were resuspended in 2 ml of uptake buffer and maintained on ice prior to zinc uptake assays. Fluorometric assays of fluorophore speciation were performed in a Hitachi F3010 spectrofluorimeter. To start the assay, an aliquot of loaded cells (100 μl) was added to 4 ml of MES-Tris uptake buffer and 100 μM zinc. The temperature of the cuvette was maintained at 30 °C using a recirculating water bath. With the instrument set at maximum scan speed, the excitation wavelength was varied from 250 to 450 nm, and the intensity of emission at 500 nm was recorded. Spectra were recorded at the start of the assay and at 2-min intervals for up to 6 min. To correct for the effects of fluorophore leakage during the experiment, immediately before each measurement a 1-ml aliquot of the assay was removed and filtered through a 0.45-μm syringe filter. After completion of the experiment, unloaded cells were added to samples of the filtered buffer to give the same cell density as present in the initial suspensions. Spectra of these samples were immediately recorded and the curves subtracted from the original spectra. This procedure provided a one-step correction for both cellular autofluorescence and leakage of fluorophore from the cells during the experiment. The corrected traces were scanned to obtain emission intensities at the excitation wavelengths of 325 and 380 nm.

RESULTS

ZRC1 and Cot1 Are Functionally Redundant for Steady-state Zinc Tolerance—A previous study indicated that both Zrc1 and Cot1 contribute to tolerance of excess zinc (13). In the yeast strain INVSC2, wild-type and cot1 cells were resistant to zinc concentrations up to ~5 mM. Mutant zrc1 cells tolerated up to 1 mM, but the zrc1 cot1 mutant was not viable at this zinc concentration. In our strain background (W303), we obtained qualitatively similar results (Fig. 1). Wild-type cells showed tolerance to up to 6 mM zinc. The zrc1 mutation caused a slight defect in zinc tolerance, reducing the inhibitory concentration by 1–3 mM. The cot1 mutation had little effect, only lowering the maximum tolerable concentration to 5 mM. In contrast to these minor effects, the zrc1 cot1 double mutant strain showed a ~50-fold reduction in the tolerable zinc concentration (0.07 mM) relative to the single mutants. These observations demonstrated that Zrc1 and Cot1 are functionally redundant for protection against high levels of zinc under steady-state conditions. However, even with the loss of both genes, yeast was still tolerant of a relatively high concentration of zinc (70 μM).

ZRC1 Is Induced in Low Zinc by Zap1—Previous studies suggested a role for Zrc1 in zinc-limited cells. Specifically, expression of the ZRC1 gene is induced in zinc-limited cells in a Zap1-dependent manner (21, 22). Furthermore, the ZRC1 promoter contains a potential ZRE that is functional when inserted into a heterologous promoter (21). To further examine this regulation, a mutant ZRC1 promoter-lacZ fusion was generated in which all nucleotides in the putative ZRE were altered by transversion mutations. Strains bearing wild-type or ZRE mutant reporter plasmids were cultured in LZM supplemented with a range of zinc levels and then assayed for β-galactosidase activity (LZM is zinc limiting because it contains EDTA, which chelates most zinc in the medium rendering it unavailable to cells). The wild-type promoter was strongly induced under zinc-limited conditions (Fig. 2A). Added zinc reduced expression to ~20% of the maximal level. This basal expression of ZRC1 is Zap1-independent (21, 22). Consistent

FIG. 1. Zinc sensitivity of zrc1 and cot1 mutant strains. CM100 (Wild-type), CM102 (zrc1), CM103 (cot1), and CM104 (zrc1 cot1) strains were grown in zinc-replete S.D. medium to stationary phase. 5-μl aliquots (2 × 10⁶ cells) were applied to S.D. medium plates supplemented with the indicated concentration of zinc. Plates were incubated at 30 °C for 2 days before photography.
with this conclusion, mutation of the ZRE completely eliminated zinc-responsive regulation without affecting basal expression. This result supports the contention that ZRC1 is transcriptionally regulated by Zap1 through this ZRE. Furthermore, the ZRE mutant promoter provided a useful reagent for subsequent studies (see below).

Transcriptional control of ZRC1 was also reflected in altered protein levels. We examined the accumulation of protein expressed from a functional (data not shown) epitope-tagged allele of ZRC1 regulated by its own promoter and integrated into its native chromosomal location. Immunoblot analysis showed that two closely spaced bands representing forms of the Zrc1 protein accumulated to higher levels in zinc-deficient cells (LZM + 0.3–10 μM zinc) compared with zinc-replete cells (Fig. 2B). No bands of this size were detected in protein from a presser from a functional (data not shown) epitope-tagged allele of Zrc1 and anti-Vma1 monoclonal antibodies (Molecular Probes), followed by secondary horseradish peroxidase-labeled anti-mouse antibody (Pierce). Signal was detected using ECL (Amersham Biosciences).

ZRC1 and COT1 Are Not Required for Steady-state Zinc-limited Growth—The Zap1-dependent regulation of ZRC1 suggested that the Zrc1 protein is required during zinc deficiency. Supporting this hypothesis, it was previously reported that zrc1 mutants grew poorly compared with wild type under zinc-limiting conditions (22). Contrary to that previous result, however, we found no defect in zinc-limited growth (LZM + 0.3–10 μM zinc) for zrc1, cot1, or zrc1 cot1 cells (Fig. 3A). The explanation for the apparent discrepancy between our results and the prior study will be presented under “Discussion.” The sensitivity of the zrc1 cot1 mutant to high zinc (LZM + 30–1000 μM zinc) is consistent with the redundancy of Zrc1 and Cot1 in steady-state zinc tolerance. To determine the effect of these mutations on zinc accumulation, we measured the total zinc content of wild-type and mutant strains after growth over a range of added zinc. Again, at zinc concentrations of 10 μM or less, these mutations had no effect on cell zinc content (Fig. 3B). In medium supplemented with 30 μM zinc or more, all three mutant strains accumulated less zinc than wild type, and the zrc1 cot1 double mutant accumulated less than either single mutant. Notably, these effects of zrc1 and cot1 mutations on growth and zinc accumulation were observed only at zinc concentrations in LZM of greater than 10 μM. This concentration was previously identified as the transition point between zinc deficiency and repletion (14). This can also be seen as such here because for the wild-type strain, the maximum growth rate is observed at 10 μM zinc and higher. Thus, we found no evidence that Zrc1 or Cot1 are required for zinc-limited growth or alter zinc homeostasis in zinc-limited cells.

To test for an effect of high ZRC1 activity on zinc homeostasis in zinc-deficient cells, we overexpressed the gene from a multicopy plasmid and measured the effect on total cellular zinc accumulation. We confirmed an increase in Zrc1 protein level due to overexpression in both high and low zinc media (data not shown). As shown in Fig. 4A, overexpression of ZRC1 significantly increased cellular zinc content when cells were grown under zinc-replete conditions. Importantly, it had no effect in zinc-deficient conditions (Fig. 4A, inset). The effect of this increased zinc accumulation on cytoplasmic zinc availability was then determined. Methods to directly measure cytoplasmic labile zinc are not currently available, but we have previously shown that an indirect assessment of this parameter can be made using a Zap1-regulated reporter gene (14). ZRE-lacZ reporter activity was significantly increased in cells that overexpressed ZRC1, indicating reduced cytoplasmic labile zinc. This observation is consistent with a model whereby Zrc1 expression results in zinc transport from the cytoplasmic/nuclear compartment into an organelle. Again however, an effect of Zrc1 was only seen in cells that were zinc-replete. In summary, the experiments shown in Figs. 3 and 4 revealed no evidence for an effect of Zrc1 on zinc homeostasis in yeast cells during growth under steady-state zinc-deficient conditions. Thus, while it is not possible to rule out a more subtle role not revealed by these assays, these experiments did not provide an obvious explanation for why ZRC1 is up-regulated in zinc-deficient cells.

ZRC1 and COT1 Protect Zinc-deficient Cells from Zinc Shock—In the experiments shown in Fig. 1, zinc-replete cells were used to inoculate plates. Zinc-replete cells have repressed

FIG. 2. Zinc-regulated expression of ZRC1. A, ZRC1 promoter activity over a range of zinc concentrations. Wild-type cells (DY1457) carrying YEprZRC1-lacZ or YEprzrc1<sup>r<sub>zrc1</sub></sup>-lacZ were grown to late log phase in LZM with the indicated zinc concentration and assayed for β-galactosidase activity. Symbols are the means of three replicates, with one standard deviation less than the width of the symbol in all cases. B, immunoblot analysis of CM142, which expresses hemagglutinin epitope-tagged Zrc1 from its own promoter. Protein was extracted from cells grown in LZM supplemented with zinc at the indicated concentration. 5 μg of protein was fractionated on a 10% acrylamide gel and blotted to nitrocellulose. The blot was probed with anti-hemagglutinin (Zrc1) and anti-Vma1 monoclonal antibodies (Molecular Probes), followed by secondary horseradish peroxidase-labeled anti-mouse antibodies (Pierce). Signal was detected using ECL (Amersham Biosciences).

FIG. 3. Effects of zinc availability on growth and zinc accumulation by zrc1 and cot1 mutants. A, effect of zinc availability on growth. WT, zrc1, cot1, and zrc1 cot1 strains (see Fig. 1) were grown to saturation in zinc-replete S.D. medium. LZM containing the indicated zinc concentration was inoculated at an initial absorbance at 600 nm (A<sub>600</sub>) of 0.01. Cultures were incubated for 18 h and final A<sub>600</sub> determined. B, effects on cell-associated zinc. S.D. cultures of the above strains were used to inoculate LZM containing <sup>65</sup>Zn<sup>2+</sup>-isotope and non-radioactive zinc to give the total zinc concentration indicated. Cultures were grown to late log phase and cell-associated zinc was quantified. Data points are the means of three replicates, and error bars represent ±1 S.D.
high-affinity zinc uptake systems and do not accumulate excess zinc when inoculated into fresh medium. In contrast, when zinc-limited cells are resupplied with zinc, they rapidly accumulate large quantities because of the high activity of the plasma membrane zinc transporters (15). We refer to this condition as zinc shock. During zinc shock, newly acquired zinc rapidly enters the cytoplasm where it can accumulate to high levels. We hypothesized that yeast detoxify this excess zinc by rapid transport into an intracellular compartment. If this is true, the high expression of the ZRC1 zinc tolerance gene in zinc-deficient cells may be required to mediate the rapid sequestration of excess cytoplasmic zinc during zinc shock. This model predicts that a zrc1 mutant will be hypersensitive to zinc shock; a prediction we confirmed (Fig. 5A). The medium used in this experiment, CSD, is made zinc limiting by treatment with a chelating resin. Because CSD contains no strong chelators, the availability of added zinc is many orders of magnitude higher than for equivalent concentrations of total zinc in LZM. When zinc-limited zrc1 mutants (i.e., pregrown in LZM + 1 μM ZnCl₂) were inoculated into CSD medium supplemented with as little as 1 μM zinc, zrc1 cells failed to grow. In contrast, neither the wild-type nor the cot1 mutant showed any growth defect up to 10 μM zinc (the increase in growth yield observed for the wild-type and cot1 strains with increased zinc is due to zinc-limitation in CSD with less than 1 μM added zinc). The zrc1 cot1 mutant was even more sensitive to zinc shock than the zrc1 mutant, indicating Cot1 also contributes to zinc shock tolerance. None of these cell types were zinc sensitive when inoculated from zinc-replete cultures (i.e. pregrown in LZM + 1 mM ZnCl₂) (Fig. 5B). Thus, the sensitivity is a consequence of the transition from zinc-limited to zinc-replete conditions. The retarded growth of the zrc1 cot1 mutant when inoculated from zinc-replete under zinc-limiting conditions does not correspond to zinc toxicity (because growth at higher zinc concentrations is similar to wild-type), and may be due to the low vacuolar zinc stores in this mutant (14).

While the Zrt1, Zrt2, and Fet4 plasma membrane uptake transporters are induced in zinc-limited cells, Zrt1 is the major pathway of zinc uptake; mutation of ZRT1 reduces zinc uptake in zinc-deficient cells by at least 80% (16). Therefore, we predicted that zinc shock results largely from the high activity of Zrt1 in deficient cells. To test this prediction, we examined the effect of a zrt1 mutation on the zinc sensitivity of a zrc1 mutant. As before, zinc-deficient cells were inoculated into CSD medium containing no zinc or 1 μM added zinc. The zrt1 mutation completely suppressed the zinc sensitivity associated with the zrc1 mutation (Fig. 5C). These data indicate that the high level of zinc accumulation mediated by Zrt1 in zinc-limited cells is responsible for the zinc sensitivity of zrc1 mutants undergoing zinc shock. Poor growth of the zrt1 and zrt1 zrc1 mutants without added zinc is likely due to the impaired zinc uptake in these strains.

**Effect of zrc1 and cot1 Mutations on Intracellular Zinc Compartmentalization**—To assess if zinc sequestration is altered in zrc1 and cot1 mutants during zinc shock, we first assayed the effects of these mutations on zinc accumulation. Accumulation of substrate on the cytoplasmic side of the plasma membrane can directly inhibit the transporters responsible for uptake, for example via trans-inhibition (26, 27). Data from zinc uptake experiments (15) predicted that in the absence of any intracellular compartmentalization during zinc shock, cytoplasmic zinc levels would rise into the millimolar range within minutes. Under these conditions, mutant strains unable to sequester zinc might exhibit impaired zinc uptake due to trans-inhibition. To test this prediction, zinc accumulation by wild-type, zrc1, cot1, and zrc1 cot1 mutants under zinc shock conditions was compared; i.e. zinc-deficient cells were transferred to an uptake buffer that contained 1 μM ⁶⁵Zn²⁺ and lacked strong metal ion chelators. In each strain, the initial rates of zinc accumulation over the first 1 min were indistinguishable (Fig. 6A). Thus, zrc1 and cot1 mutations do not affect zinc uptake activity in cells maintained under zinc-limiting conditions. A time course of uptake indicated that, although accumulation in wild-type and cot1 mutant cells continued almost linearly for 20 min, accumulation by zrc1 and zrc1 cot1 mutants began to plateau before 5 min (Fig. 6B). This observation was consistent with a failure of the zrc1 mutants to properly sequester zinc,
leaving to the direct inhibition of plasma membrane transporters by high cytoplasmic zinc concentrations. Subsequent experiments to directly assess vacuolar zinc compartmentation during zinc shock supported this interpretation (see below). No contribution of Cot1 was observed under the conditions described in the legend to Fig. 6B (growth of cells in LZM + 1 \( \mu \)M zinc). To further examine the potential role of \( \text{cot}1 \) during zinc shock, we used this impairment of zinc accumulation as an indirect assay to examine the contribution of \( \text{zrc}1 \) and \( \text{cot}1 \) to zinc homeostasis in cells grown over a range of zinc concentrations. Again, the mutations had no affect on the initial rate of zinc uptake as measured over 1 min (data not shown). Over a longer period (5 min), the \( \text{zrc}1 \) mutation reduced zinc accumulation by cells grown in zinc-limiting media (LZM + 0.3–10 \( \mu \)M zinc) (Fig. 6C). An effect of the \( \text{cot}1 \) mutation on zinc accumulation was observed, but only in the absence of \( \text{zrc}1 \) and in cells grown at zinc concentrations greater than 10 \( \mu \)M. Therefore, both \( \text{zrc}1 \) and \( \text{cot}1 \) appear to contribute equally to zinc storage and detoxification in zinc-replete cells, but in zinc-deficient cells undergoing zinc shock, \( \text{zrc}1 \) alone plays the dominant role.

We suspected that the decreased zinc uptake and growth inhibition phenotypes of \( \text{zrc}1 \) strains subjected to zinc shock were a consequence of a defect in vacuolar zinc uptake. To test this prediction directly, we used the ratiometric fluorescent indicator FuraZin-1 to non-invasively assay changes in vacuolar zinc content in living cells during zinc shock. When cells were loaded with the acetoxyethyl (AM) ester of FuraZin-1 and then treated with glucose (as described under “Materials and Methods”), the fluorophore accumulated predominantly in the vacuole (Fig. 7A). Counts of randomly selected cell fields indicated that >98% of the cells that had taken up FuraZin-1 had sequestered the fluorophore in the vacuole, and this distribution was independent of strain genotype (Fig. 7A and data not shown).
not shown). This location of FuraZin-1 in yeast allowed us to use simple fluorimetric assays of fluorophore speciation in cell suspensions to assay the vacuole zinc content during zinc shock. In cells grown in zinc-deficient medium, excitation wavelength scans showed that the fluorophore was predominantly in the zinc-free form, with an excitation maximum at 380 nm and a fluorescence ratio \(F_{325\text{ nm}}/F_{380\text{ nm}}\) of \(-0.2\). Under conditions of zinc shock, there was a rapid increase in the zinc-bound form (with an excitation maximum at 325 nm) of the fluorophore as indicated by the increase in the \(F_{325\text{ nm}}/F_{380\text{ nm}}\) ratio (Fig. 7B). No change in this signal was observed during incubation in the absence of zinc (not shown), and the zinc-induced ratiometric change was reversed by the addition of TPEN, a cell-permeant zinc chelator (Fig. 7D). A rapid increase in signal could also be induced by treating cells with the zinc ionophore pyrithione to allow free diffusion of zinc into the yeast cell (Fig. 7E). Taken together, these observations indicated that the ratiometric change in fluorescence observed during zinc shock resulted from an increase in the zinc content of the vacuole compartment.

When zinc-deficient \(zrc1\) mutant cells were loaded with FuraZin-1 and subjected to zinc shock, an increase in the fluorophore signal was also observed but at a much reduced rate in comparison to the wild-type (Fig. 7B). This difference was not due to an inability of the fluorophore within the \(zrc1\) cells to respond to zinc, as exposure to zinc-pyridine eliminated the difference between the strains (Fig. 7E). The observed difference in fluorophore signal was also not attributable to a difference in zinc uptake by the cells (Fig. 6B); under the conditions used in this experiment (cell growth in LZM + 2 \(\mu\)M zinc and transfer to 100 \(\mu\)M zinc), the initial rate of zinc uptake was the same (Fig. 7C). Therefore, these data provide direct evidence that during zinc shock, \(zrc1\) is required for sequestering zinc into the vacuole compartment. In addition, these data strongly suggest that during zinc shock, the \(zrc1\) mutant accumulated a larger proportion of zinc in the cytoplasm. This overaccumulation of zinc in the cytoplasm, and perhaps a resultant increase in zinc accumulated by other compartments (e.g. the mitochondria) may be responsible for the growth defect observed in the \(zrc1\) mutant following zinc shock (Fig. 5A).

**ZRC1 Induction by Zap1 Is Required for Zinc Shock Tolerance**—The observation that \(zrc1\) was largely responsible for zinc sequestration during zinc shock suggested that the Zap1-mediated induction of this gene might be important for zinc shock tolerance. To test this hypothesis, we generated a strain in which the \(ZRC1\) promoter with an inactivated ZRE (Fig. 2A) controlled expression of the chromosomal \(ZRC1\) locus. This allele, designated \(zrc1^{\text{mZRE}}\), did not alter the zinc tolerance of zinc-replete cells grown on high zinc (data not shown). This observation is consistent with the lack of an effect of the ZRE mutation on basal, Zap1-independent expression of the promoter (Fig. 2A). When the effect of the \(zrc1^{\text{mZRE}}\) mutant gene on zinc shock was tested, the results showed that the loss of \(ZRC1\) induction by low zinc substantially decreased tolerance (Fig. 8). In fact, the \(zrc1^{\text{mZRE}}\) mutant was almost as sensitive to zinc shock as the complete \(zrc1\) deletion mutant. Moreover, mutation of the ZRE impaired zinc accumulation during zinc shock (Fig. 6B), suggesting that the absence of \(ZRC1\) induction also impaired the compartmentalization of cytoplasmic zinc. Consistent with this interpretation, a \(zrc1^{\text{mZRE}}\) mutant strain was also observed to accumulate less vacuolar zinc during zinc shock, as measured by FuraZin-1 fluorescence (Fig. 7B). Therefore, the induction of \(ZRC1\) by Zap1 under zinc-deficient conditions is essential to protect cells against zinc shock.

**DISCUSSION**

\(ZRC1\) and \(COT1\) encode putative zinc transporters of the CDF family that are required for metal ion tolerance. Whereas previous reports suggested that \(ZRC1\) and \(COT1\) primarily confer tolerance to zinc and cobalt ions respectively (8, 12), it is now clear that under certain conditions, these genes are functionally redundant with respect to zinc. \(zrc1\) and \(cot1\) are both important determinants of zinc detoxification in zinc-replete yeast grown in high zinc, and both genes were implicated in the vacuolar storage of zinc in zinc-replete cells (13, 14). Given these roles of \(zrc1\) in zinc-replete or excess conditions, it was surprising to find that \(ZRC1\) is a Zap1 target gene and induced under zinc-limiting conditions (21, 22). This regulation is inconsistent with either of the previously recognized functions of \(zrc1\) in zinc homeostasis. One possible explanation is that zinc-limited cells require more \(zrc1\) activity to maintain a flux of zinc through the vacuole, perhaps to provide for zinc-dependent processes within that compartment (14, 22). As yet, we have found no evidence to support this hypothesis. Cells mutant in \(ZRC1\) (and/or \(COT1\)) showed no defect in growth (see below) or zinc accumulation under zinc-limiting conditions. This result was also obtained in time course studies comparing growth of these mutants and wild-type cells throughout lag and exponential growth phases (data not shown). It is conceivable that the deleterious effects of these mutations in zinc-limited cells may be too subtle to detect by these assays. However, additional assays also found no evidence that \(zrc1\) is capable of zinc transport when cytosolic zinc levels are low. Overexpressing \(zrc1\) increased zinc accumulation and reduced labile cytosolic zinc levels in zinc-replete cells but had no impact on cells grown in low zinc (i.e. LZM + 10 \(\mu\)M zinc, Fig. 4). This lack of activity may be due to an insufficient affinity of \(zrc1\) for the available substrate in zinc-limited cells. Given that expression of \(zrc1\) protein is maximal in zinc-limited cells, the absence of a detectable effect of \(zrc1\) under these conditions is remarkable.

If not active in zinc-limited cells, why then is \(ZRC1\) a Zap1 target gene? In this report, we have clearly shown the crucial importance of \(Zrc1\) activity and its regulation by Zap1 in tolerance to zinc shock. Zinc shock occurs when zinc-limited cells, which have a high latent capacity for zinc uptake, are resupplied with zinc. Large amounts of zinc then pass across the plasma membrane and accumulate in the cytoplasm. The cell responds to this zinc excess by down-regulating the transcript-
tion of ZRT1, ZRT2, and Fet4 (15, 16), and by inactivating the Zrt1 protein via zinc-induced endocytosis (28). However, these processes occur too slowly to prevent uptake of large quantities of zinc; we calculate that in the absence of any organelar sequestration, the cytosolic zinc levels would rise into the millimolar range within minutes. If cytosolic zinc is not rapidly sequestered into the vacuole, growth arrest may result from which the cells are unable to recover. Several lines of evidence are presented here to support this model. First, zrc1 and zrc1 cot1 mutants are extremely sensitive to conditions of zinc shock. Second, our data indicate that zinc sequestration in the vacuole was disrupted in zrc1 and cot1 mutants undergoing zinc shock. Third, zinc-limited zrc1 mutant cells are protected from zinc shock if they are unable to express the major inducible zinc uptake system, Zrt1. The induction of ZRC1 expression by Zap1 is a critical part of zinc shock resistance. Mutating the ZRE element in the promoter disrupted induction in zinc-deficient cells but did not affect basal, zinc-replete expression. Mutation of the ZRE also rendered the mutant cells almost as incapable of tolerating zinc shock as a full deletion of the ZRC1 gene, and this sensitivity was associated with a defect in vacuolar zinc uptake during zinc shock.

We suggest that tolerance to zinc shock is the major role of Zrc1 in zinc homeostasis, and not resistance to high steady-state extracellular zinc levels per se. This is evident when the levels of zinc required to elicit zinc toxicity under these conditions are compared. Under steady-state conditions of high zinc, Zrc1 is required for resistance to more than 4 mM zinc. However, under conditions of zinc shock, Zrc1 is required to tolerate as little as 1 μM zinc, i.e. a 4000-fold lower concentration. Transitions from zinc-deficient to moderately zinc-replete conditions are likely to occur frequently in nature, while millimolar concentrations of free zinc would be rarely encountered.

In this work we used a novel zinc indicator (FuraZin-1) to non-invasively determine the effect of Zrc1 on zinc homeostasis in living cells. Under appropriate conditions, ratiometric probes such as FuraZin-1 can be used to estimate in vivo ion concentrations with a standard equation (29). However, this equation is not applicable in the present case because the high concentration of fluorophore required in the vacuole to detect changes in zinc levels perturbs the labile vacuolar zinc pool (30). A more accurate approach to estimate vacuolar zinc levels considers the total concentration of fluorophore loaded into that organelle. By measuring indicator fluorescence in vitro and comparing with values obtained in vivo, we calculated that FuraZin-1 routinely reached 1 μM in the vacuole of loaded cells. Saturation of this concentration of fluorophore with zinc-pyridthione resulted in a maximum F325/F380 ratio of 2 (Fig. 7E). Given that the fluorophore was ~50% saturated (F325/F380 ratio of 1) in wild-type cells within 6 min of zinc treatment (Fig. 7B), these results indicate that vacuolar zinc levels quickly rise to the millimolar range during zinc shock. These data suggest that vacuolar sequestration during zinc shock is very efficient in removing cytosolic labile zinc.

In contrast to our results, Miyabe et al. (22) presented data that suggested that Zrc1 was required for growth under steady-state zinc-limited conditions. These authors grew wild-type and zrc1 mutant cells in a low zinc medium for a period of time and then plated those cells onto agar plates to determine the number of viable cells from the colonies that formed. Fewer colonies formed for the zrc1 mutant than the wild type suggesting that growth in the zinc-limited medium was impaired. However, we have determined that the reduced plating efficiency of the zrc1 mutant in these experiments was due to zinc shock; the medium used for cell counting, YPD, is zinc-rich (~10 μM total zinc (data not shown) with no strong chelators present). When we replicated their experiments, we found that zinc-deficient zrc1 mutant cells did not grow when plated on YPD, but that these cells were viable when plated on a zinc-limiting medium (data not shown). Thus, the results of the previous study suggesting Zrc1 was essential for growth under steady-state zinc-deficient conditions are in fact explained by the zinc shock effect.

While Zrc1 and Cot1 are closely related proteins, and both affect zinc homeostasis, Zrc1 clearly plays the major role in zinc shock tolerance. The minor tolerance that Cot1 provides is perhaps explained by its lack of regulation by Zap1 (21). It is intriguing then that the Cot1 gene may be a target of the Aft1 transcription factor (31). Aft1 activates transcription of genes in yeast in response to iron deficiency. Aft1 targets include the high affinity iron uptake system that is responsible for iron accumulation under limiting conditions. By analogy to Zrc1, perhaps Cot1 levels increase in iron-limited cells to protect those cells from the high influx of iron that occurs upon iron repletion. However, we found no effect of the cot1 mutation on tolerance to such “iron shock” conditions. Alternatively, increased Cot1 activity could help suppress the toxicity of other metal ions that may be accumulated by relatively nonspecific cation uptake systems induced in iron-limited cells (10, 17, 32).

To our knowledge, this report represents the first well documented example of a proactive mechanism of homeostasis. However, we note other reports of potential proactive adaptation to environmental change. For example, the Phm genes of yeast are required for synthesis of polyphosphate, the vacuolar storage form of inorganic phosphate (P_i) (33). The Phm genes are co-induced with Pho84, the high affinity phosphate uptake transporter, under phosphate-deficient conditions. However, yeast cells do not accumulate polyphosphate under steady-state phosphate-deficient conditions (34), so the reason for Phm gene induction under these conditions is not clear. We note that most phm mutations impair P_i uptake by deficient cells (33), a phenotype that could be attributed to trans-inhibition of Pho84 following the accumulation of cytoplasmic P_i. If this is true, then the induction of the Phm genes may be required for the rapid sequestration of P_i in the vacuole during the transition from phosphate-deficient to replete conditions. Phm mutants do show impaired polyphosphate synthesis under these conditions, but it is not known if this phenotype is associated with sensitivity to “phosphate shock” conditions. The CopB gene of Enterococcus hirae is another potential example of the proactive induction of a metal ion detoxification system. CopB and CopA encode Cu^2+-transporting P-type ATPases. Whereas both genes are present in the same operon, their products have different roles: CopA mediates Cu^2+ uptake, while CopB mediates efflux. Expression of both CopA and CopB is induced under copper-deficient conditions. Analogous to zinc shock, it was speculated that CopB induction by Cu^2+ deficiency is a proactive adaptation to the potential for a rapid increase in Cu^2+ availability (35). In plants, the induction of many genes conferring tolerance to UV irradiation and low temperature is linked to the circadian clock. It has been proposed that this regulation allows the anticipation of cyclically predictable stresses, such as cold nights or increased light intensity during daylight hours (36, 37). Similarly, zinc shock is a predictable stress faced by zinc-deficient yeast. These few examples suggest that the phenomenon of proactive homeostatic regulation is not restricted to zinc homeostasis in yeast, but may in fact represent a common and largely unrecognized feature of gene expression in many organisms.

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Induction of the ZRC1 Metal Tolerance Gene in Zinc-limited Yeast Confers Resistance to Zinc Shock

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