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Surplus Zinc Is Handled by Zym1 Metallothionein and Zhf Endoplasmic Reticulum Transporter in *Schizosaccharomyces pombe**

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Homeostatic mechanisms prevent the accumulation of free zinc in the cytoplasm, raising questions regarding where surplus zinc is stored and how it is delivered to and from these stores. A genetic screen for zinc hypersensitivity in *Schizosaccharomyces pombe* identified a missense mutation truncating Zhf, an endoplasmic reticulum transporter. These cells were ~5-fold more zinc-sensitive than other independent mutants. The targeted disruption of *zhf* prevented growth on low zinc medium and caused hypersensitivity to elevated zinc/cobalt but resistance to cadmium. The exposure to elevated zinc but not copper also promotes the accumulation of transcripts encoding a metallothionein designated Zym1. The *Sty*1 pathway is required for maximal *zym1* expression but is not obligatory for zinc perception. The targeted disruption of *zym1* impaired cadmium tolerance but only slightly impaired zinc tolerance, whereas *zym1* overexpression substantially rescued zinc hypersensitivity of *zhf⁻* cells. Four equivalents of zinc were displaced from Zym1 by up to 12 equivalents of *p*-(hydroxymercuri)phenylsulphonate. Zym1 thiols react rapidly with 5,5′-dithiobis-(2-nitrobenzoic acid) compared with bacterial zinc metallothionein (6.8 and 0.2 × 10^{-4} s^{-1}, respectively). Zym1 is unlike known fungal metallothioneins that are induced by and sequester copper but not zinc. Less zinc but normal cadmium was accumulated by *zym1*Δ, consistent with zinc sequestration by Zym1 *in vivo*.

Proteins that require zinc for catalysis pervade metabolism, and zinc is a common element of protein structure (1). However, excess zinc is toxic, and cells restrict the availability of zinc. In mammalian cells, cytosolic-free zinc content has been estimated as picomolar (2), and the sensitivity of cellular zinc sensors suggests that there is also effectively no free zinc in *Escherichia coli* (3). Surplus zinc must be sequestered and stored until it is required for the incorporation into nascent proteins. It remains to be fully defined how zinc is delivered to intracellular stores and mobilized from them.

In higher eukaryotes but not *Saccharomyces cerevisiae*, surplus zinc can be sequestered in small cysteine-rich metal-binding proteins called metallothioneins (4). The exposure to zinc and a diversity of other factors promotes a rapid increase in both metallothionein mRNA and protein (5, 6). Metallothioneins are capable of binding a range of metals, but in mammals they predominantly coordinate zinc with the exception of cells challenged with supraoptimal concentrations of metals that can displace zinc from metallothionein clusters (7). Mice that lack MT-I[1] and MT-II exhibit a small increase in sensitivity to zinc (8), but in cell lines that lack functional zinc transporters, metallothioneins confer some residual resistance (9). A number of roles have been proposed for MT-I and MT-II, but uncertainty remains around the evolutionary advantage that has selected for so many metallothionein genes in such a wide diversity of organisms (4). Importantly, in *S. cerevisiae*, the metallothioneins Cup1 and Crs5 coordinate copper are induced in response to elevated copper concentrations by the transcriptional regulator Ace1 and function predominantly in the detoxification of excess copper (10, 11). There is no evidence for any role for either protein in zinc homeostasis.

In mammalian cells, surplus intracellular zinc is also sequestered to compartments such as vacuoles and specialized vesicles, and this is also true of *S. cerevisiae* (12–15). The mutants of *S. cerevisiae* that are defective in either vacuolar biogenesis or acidification consequently exhibit increased sensitivity to metals including zinc (16, 17). Two important determinants of zinc tolerance in *S. cerevisiae* are the vacuolar metal transporters Zrc1 and Cot1 (18). The overexpression of either of these transporters increases zinc tolerance, whereas deletion reduces zinc tolerance (19–21). Furthermore, a loss of these proteins severely impairs the accumulation of intracellular zinc (15). Zrc1 and Cot1 are members of the cation diffusion facilitator family that also includes the mammalian zinc transporters ZnT-1 to ZnT-5 (22, 23). ZnT-1 is located at the plasma membrane and functions in zinc efflux (9). ZnT-2 and ZnT-3 are involved in the intracellular trafficking of zinc (12, 13, 24). ZnT-3 functions in zinc uptake into vesicles within neurones (13). ZnT-2 contributes to zinc efflux and the loading of vesicles in the intestine, kidney, and testis (12). Transfection with ZnT-2 confers a degree of zinc resistance, indicating that storage in intracellular compartments maintains a tolerable number of zinc atoms within the cytosol (12). ZnT-4 performs specialized functions in the brain, intestine, and mammary gland (25), whereas ZnT-5 transports zinc into secretory granules in pancreatic beta cells (23). During the writing of this paper, Clemens *et al.* (26) reported a cation diffusion facilitator transporter Zhf located in the endoplasmic reticulum and linked to

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[1] The abbreviations used are: MT, metallothionein; LZM, low zinc medium; MAPK, mitogen-activated protein kinase; PMPS, *p*-(hydroxymercuri)phenylsulphonate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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the accumulation of zinc within this compartment in *Schizosaccharomyces pombe*. We had coincidentally recovered the gene encoding the same protein from a screen designed to identify the major determinant(s) of zinc tolerance, confirming similar phenotypes and additionally observing impaired growth of *zhf*Δ on low zinc medium (LZM).

Here we examine the action of a gene designated *zym1*, encoding a 50-residue cysteine-rich polypeptide. By analogy to fungal metallothioneins, it might be anticipated that *zym1* transcripts would accumulate in response to copper, but we establish that zinc not copper enhances transcript abundance. Expression is substantially reduced in either *wis1*Δ or *pcr1*Δ cells, implicating the Sty1 MAPK pathway (27) in *zym1* regulation, although this pathway is not obligatory for zinc perception because zinc-dependent expression is retained albeit at a lower level. The deletion of *zym1* confers a "minimal" reduction in zinc tolerance in a wild-type background. Surprisingly, *zym1*Δ does show some reduction in cadmium tolerance, even though phytochelatins are the principal determinants of resistance in *S. pombe* (28, 29). We have exploited *zhf*$^-$ cells to demonstrate that *zym1* overexpression does substantially rescue zinc hypersensitivity of this genotype. The deletion of *zym1* also reduces zinc accumulation but does not alter the accumulation of cadmium. We report *in vitro* analyses of the zinc-binding properties of Zym1 and establish that 10–12 equivalents of *p*-(hydroxymercuri)phenylsulphonate (PMPS) displace four equivalents of zinc, confirming the coordination to most if not all 12 cysteine residues. As a zinc metallothionein from a fungal species, Zym1 provides the opportunity for functional and regulatory studies in a genetically tractable organism.

EXPERIMENTAL PROCEDURES

Strains and Culture—The routine culturing of *S. pombe* was performed in either rich medium (YE5S) or minimal medium (EMM) as described previously (30) using standard techniques. LZM was prepared as described previously (31). The strains used in this study were NT5, *h*$^-$ *ura4-D18*; IS537, *h*$^-$ *ura4-D18 zhf-537*; SW60, *h*$^-$ *ura4-D18 zhf*::*ura4*$^+$; SW62, *h*$^+$ *ura4-D18 zym1*::*ura4*$^+$; SW61, *h*$^-$; JX125, *h*^{90} *pcr1*::*ura4*$^+$; and GD1682, *h*$^-$ *wis1*::*ura4*$^+$. All of the strains are *ade6*$^-$ and *leu1*$^-$. The *zhf* open reading frame (SPAC23C11.14) was disrupted using a PCR-based approach as described previously (32). Oligonucleotides 5′-KO (5′-ATTTACCTTATTCCTTTTTCTCCCTGTGAAATTCAACCCTTACCATTTTTTTGAAATTGGTGACTTTTCGTTTTCAAACTGCTTAGCTACAAATCCCACT-3′) and KO-3′ (5-′-AATATAAAATTTTACCTCAAGAATAAATCAAAAGATGTGACACAATAGATTAACCACGTTAAATTTATAATGTTCGCAAATCTGACATAAAACGCCTAGG-3′) were used to amplify a 1.6-kilobases of *ura4*$^+$ containing fragment from pRep42. The amplified fragment was used to transform NT5 to Ura$^+$, thus creating SW60. Integration at the correct locus was confirmed by PCR analysis. A strain in which the *zym1*$^+$ open reading frame (SPAC22H10.13) was deleted was achieved using the same approach. The oligonucleotides used for amplification of the *ura4*$^+$ containing the disrupting fragment were 5′-KO (5′-TCTCACCTTTTTTACCACTTTAAGCGTGCTTGAGACAATCGACTTTCAACAACTCATATCACCCTAACAAACTTTCAACGGCTTAGCTACAAATCCCACT-3′) and KO-3′ (5′-ACAATAAGACGAGGCGGACATTGCTTTATGGATGAAATATTTCGTCAAGGCCAGAACTACAGTCCGTAAATCTCATATAGTCTGACATAAAACGCCTAGG-3′). Random mutagenesis was performed by plating exponentially growing colonies onto rich (YE5S) agar plates (~1×10^3 cells/plate) and subjecting them to UV irradiation using a Stratalinker at a dosage that resulted in ~70% killing. Plates were incubated in the dark at 30 °C for 4–5 days. The resulting colonies were replica-plated onto rich agar (YE5S) plates that were either supplemented or not supplemented with 2 mM $ZnSO_4$.

Plasmids—A plasmid (pRep1-Zym1) for the over-expression of *zym1* in *S. pombe* was created by using PCR products corresponding to *zym1*, generated using primers 5′-*zym1* (5′-GCGATCATATGGAACACACTACCCAATG-3′) and *zym1*-3′ (5′-CGTCTGGATCCGTCAAGGCCAGAACTACAGTC-3′). The resulting fragment was digested with *Nde*I and *Bam*HI followed by ligation to the *Nde*I and *Bam*HI sites of the pRep1 vector to create pRep1-Zym1. A plasmid for the overexpression of *zym1* in *E. coli* was produced by ligating the *zym1* PCR product into the *Nde*I and *Bam*HI sites of pGEM-T (Promega). The *zym1* DNA was then excised using *Nde*I and *Eco*RI and ligated to the prokaryotic expression vector pET29a to create pMHNR6.1.

RNA Analysis—RNA was extracted from 0.3×10^9 cells as described previously (33) with some modification. Cell pellets were washed in H_2O and resuspended in 200 μl of RNA buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 50 mM EDTA, pH 8.0, 0.25% (w/v) SDS) with 200 μl of phenol:chloroform in a 2-ml screw-capped Eppendorf tube. The cells were ruptured with 0.75 ml of 0.5-mm glass beads (Biospec) in a Ribolyser (Hybaid) using two 10-s bursts at full power. An additional 0.75 ml of RNA buffer was added followed by spinning in a microfuge for 5 min. The aqueous layer was subjected to further phenol:chloroform extractions before the RNA was precipitated with 0.1 volumes of sodium acetate, pH 5.2, 0.6 volumes of isopropyl alcohol. RNA pellets were washed in 70% (v/v) ethanol and resuspended in H_2O. A 10–15-μg sample of total RNA was denatured with glyoxal, separated on either a 1.2 or 1.4% (w/v) agarose gel prepared in 15 mM sodium phosphate, pH 6.5, and transferred to a GeneScreen Plus hybridization membrane (PerkinElmer Life Sciences). DNA probes were produced by PCR amplification from genomic DNA using the appropriate primers. All of the probes were labeled with [α-^{32}P]dCTP by use of a Prime-a-Gene labeling kit (Promega).

Analysis of Growth in Response to Metals—Exponentially growing cells (~2×10^4) were inoculated into 5 ml of medium supplemented with increasing concentrations of metal. A_{595} was measured either after an overnight incubation at 30 °C for rich medium (YE5S) or minimal medium (EMM) or after a 20-h incubation at 30 °C for LZM.

Quantification of Metal Contents—Cell pellets from aliquots (1 ml) of metal-supplemented cultures were washed in SSW (1 mM EDTA, 20 mM trisodium citrate, pH 4.2, 1 mM KH_2PO_4, 1 mM $CaCl_2$, 5 mM $MgSO_4$, 1 mM NaCl) and resuspended in 1 ml of 70% (v/v) HNO_3. Zinc contents were determined by atomic absorption spectrophotometry.

Production of Recombinant Zym1—pMHNR6.1 was used to transform BL21 (DE3) *E. coli* cells and protein expression induced in cells grown to 0.7 (A_{595}) by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. Zinc sulfate (0.5 mM) was added to the *E. coli* growth media, and the cells were allowed to grow for an additional 5 h before harvesting by centrifugation. The aliquots (2 ml) of cleared lysate were applied to a gel filtration column (Sephadex G-75, 2.5 × 60 cm, Amersham Biosciences) equilibrated with 25 mM Tris, pH 9.7, 1 mM dithiothreitol. Fractions containing Zym1 were pooled, concentrated (Vivaspin 20, 2.5 kDa of molecular mass cut off, Sartorius), and exchanged into 25 mM Tris, pH 9.7, by gel filtration (PD10, Amersham Biosciences). Zym1 was applied to an anion exchange column (1-ml HiTrapQ, Amersham Biosciences) equilibrated in 25 mM Tris, pH 9.7), and eluted with 40 ml of 0–500 mM linear NaCl gradient. The purity and identity of the recombinant protein were confirmed by polyacrylamide gel electrophoresis. SmtA was expressed and purified as described previously (34).

Analyses of Zinc-binding and Thiol Reactivities—The amount of Zn^{2+} bound to Zym1 (1 nmol) was determined using the metallochromic indicator, 4-(2)-(pyridylazo)resorcinol, and the cysteine-specific reagent, PMPS, as described previously (35). Thiol reactivity was assessed by incubating either recombinant SmtA (1 nmol) or Zym1 (0.75 nmol) with 37.5 μM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 50 mM KH_2PO_4, pH 7.6. Therefore, the concentration of protein thiols was equal in each sample. The formation of 5-thio-2-nitrobenzoate was monitored by the increase in absorbance at 405 nm, and the values calibrated by reacting known amounts of free cysteine with DTNB.

RESULTS

Identification of Zhf as a Major Determinant of Zinc Tolerance—To identify proteins responsible for either sequestering or storing surplus zinc ions, we undertook a genetic screen to isolate mutants with reduced zinc tolerance. *S. pombe* cells were subjected to random mutagenesis via UV irradiation, and colonies were identified that exhibited impaired growth on rich agar (YE5S) supplemented with 2 mM $ZnSO_4$. Approximately, 50,000 colonies were screened from which 12 strains with increased sensitivity to zinc were isolated. One strain subsequently designated *zhf-537* was non-viable on rich agar (YE5S) containing 0.4 mM $ZnSO_4$ (Fig. 1*A*). An isogenic wild type formed colonies on rich agar (YE5S) containing 3 mM $ZnSO_4$. Exponentially growing cells were inoculated into rich medium (YE5S) containing increasing concentrations of zinc and were incubated overnight. The growth of the wild type was not

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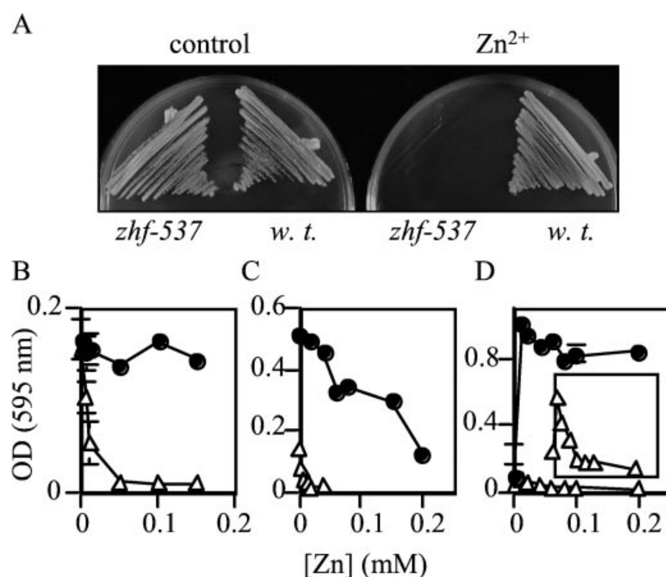

FIG. 1. **Zhf is required for growth on high and low zinc.** *A*, wild-type NT5 (*w. t.*) and IS537 (*zhf-537*) cells were grown on rich agar (YE5S) either supplemented with 0.4 mM $ZnSO_4$ ($+Zn^{2+}$) or not supplemented ($-Zn^{2+}$) for 3 days at 30 °C. *B*, approximately 2×10^4 exponentially growing *zhf-537* (*open triangles*) and wild-type NT5 (*closed circles*) cells were inoculated into 5 ml of rich medium (YE5S) supplemented with an increasing concentration of $ZnSO_4$. A_{595} was measured following overnight incubation at 30 °C. *C*, *zhf*Δ SW60 (*closed triangles*) and wild-type NT5 (*open circles*) with treatments described in *B*. *D*, approximately 2×10^4 exponentially growing *zhf*Δ SW60 (*open triangles*) and wild-type NT5 (*closed circles*) cells were inoculated into 5 ml of LZM supplemented with an increasing concentration of $ZnSO_4$. A_{595} was measured following a 20-h incubation at 30 °C. The *inset* represents *zhf*Δ SW60 (*open triangles*) alone on the exploded *y* axis, maximum A_{595} = 0.1. Data are the mean ± S.E. of triplicate determinations, and equivalent trends have been observed on two further occasions.

inhibited by up to 150 μM $ZnSO_4$, whereas *zhf-537* showed some inhibition at 5 μM $ZnSO_4$ and complete inhibition at 50 μM (Fig. 1*B*). The mutant had impaired growth on medium enriched with cobalt but enhanced growth in the presence of cadmium compared with control cells (data not shown).

As a first step to identify the mutation, we searched the *S. pombe* genomic data base for homologues of genes with known function in the cell biology of zinc. Open reading frame SPAC23C11.14 encodes a polypeptide with greater than 40% identity to Cot1 and Zrc1 of *S. cerevisiae* (19–21). Therefore, we recovered and sequenced SPAC23C11.14 from *zhf-537* and identified a nonsense cytosine to thymine substitution converting codon Gln-298 to stop and truncating the carboxyl terminus of Zhf by 89 residues. To confirm that zinc sensitivity resulted from loss of Zhf, we created a strain in which *zhf* was replaced with the *ura4*-selectable marker. It was noted that the introduction of *ura4* alone enhanced growth in rich medium without added zinc, although supplementation with $ZnSO_4$ in the range of 50–150 μM restored growth at rates more similar to strains deficient in *ura4* (Fig. 1, *B* and *C*, compare control strains). Most importantly, the *zhf*Δ strain was hypersensitive to zinc showing impaired growth on rich medium (YE5S) compared with the equivalent control strain and was unable to grow in medium supplemented with 20 μM $ZnSO_4$ (Fig. 1*C*). A genetic cross between *zhf-537* and *zhf*Δ yielded only zinc-hypersensitive progeny, confirming that the mutation of *zhf* is the major determinant of zinc resistance and responsible for the aberrant phenotype of *zhf-537*.

Zhf Is Required for Growth on Low Zinc—Intracellular sequestration by cation diffusion facilitator transporters in *S. cerevisiae* provides a supply of zinc for efficient use, especially when cells are transferred to zinc-depleted conditions (15). Does Zhf also contribute toward the efficient use of zinc, and does *zhf*Δ have impaired growth in zinc-limiting medium? The *ura4* containing control strain had poor growth on LZM unless supplemented with at least 20 μM $ZnSO_4$ (Fig. 1*D*). Significantly, *zhf*Δ failed to grow on LZM, although slight growth was restored in 20 μM $ZnSO_4$. This was not to control the levels (Fig. 1*D*, *inset*). In the absence of Zhf, *S. pombe* is sensitive to both high and low zinc such that in minimal medium with limited buffering capacity, we were unable to establish conditions supporting growth equivalent to the respective control strain.

Zinc but Not Copper Induce zym1 Metallothionein Transcripts—*S. pombe* synthesizes cadmium-binding phytochelatins termed class III metallothioneins (36–38), but there is also open reading frame SPAC22H10.13 within the *S. pombe* genome encoding a hypothetical protein with similarity to mammalian class I metallothioneins (Fig. 2). The predicted protein contains 12 cysteine residues, is shorter than mammalian metallothioneins (50 *versus* 61 amino acids), and the arrangement and spacing of the cysteine residues differ. In addition, the hypothetical *S. pombe* metallothionein also contains a single histidine residue. Is SPAC22H10.13, hereafter referred to as *zym1*, transcribed, and if so do the transcripts accumulate in response to elevated concentrations of copper in common with other fungal metallothioneins?

Zinc caused the accumulation of *zym1* transcripts with up to 10–20-fold increase 30 min after the addition of $ZnSO_4$ (Fig. 3, *A* and *C*). There was a direct correlation between *zym1* transcript abundance and zinc concentration. In contrast, equivalent exposures to $CuSO_4$ did not result in a detectable increase in the abundance of *zym1* transcripts (Fig. 3*B*). To detect any subtle change in *zym1* transcript abundance in response to copper, the blot was subjected to an extended exposure and this revealed low abundance and longer *zym1*-specific transcripts in control cells. The proportion of longer *zym1* transcripts increased in cells exposed to copper, although the overall *zym1* transcript abundance did not increase (Fig. 3*B*).

The Sty1 Pathway Positively Controls zym1 Expression but Is Not Obligatory for Induction by Zinc—The copper-binding transcriptional activator Ace1 modulates copper metallothionein expression in *S. cerevisiae*, but the induction of *zym1* by zinc (Fig. 3*A*) implies a different mode of control (10, 11). In *S. pombe*, the Sty1 MAPK pathway plays a central role in coordinating changes in gene expression in response to a wide range of environmental stresses (27). We reasoned that this pathway could control *zym1* and therefore examined transcript abundance in a strain lacking Wis1, a MAPK kinase critical for the activation of Sty1 (38–40). Both the basal and zinc-induced levels of *zym1* transcripts were severely reduced in wis1Δ (Fig. 3*C*). The Sty1 pathway clearly controls the expression of *zym1*, but this pathway is not obligatory for zinc perception because zinc induction was retained in Wis1 mutants albeit at reduced magnitude. The abundance of *zym1* transcripts was also reduced in cells lacking Pcr1, a bZIP transcription factor that in conjunction with Atf1 functions downstream of Sty1 (41, 42) (Fig. 3*C*).

Overexpression of zym1 Suppresses Zinc Hypersensitivity of zhf-537—Zinc-dependent *zym1* expression (Fig. 3*A*) suggests a role in the binding and storage of zinc *in vivo*. We investigated whether or not the overexpression of *zym1* suppressed zinc hypersensitivity associated with the loss of Zhf. The *zhf-537* strain was transformed with either pRep1-Zym1, which expresses *zym1* from the *nmt1* promoter (43), or an empty vector (pRep1), and the resulting strains assayed for growth on minimal agar (EMM) with or without, 0.4 mM $ZnSO_4$. The strain containing pRep1-Zym1 grew on zinc-enriched agar (EMM)

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FIG. 2. ***S. pombe* metallothionein Zym1.** Alignment of Zym1 (SPAC22H10.13) with mouse MT-I using ClustalW 1.7. The Zym1 cysteine residues are indicated with *asterisks*. The Zym1 histidine residue is *underlined*.

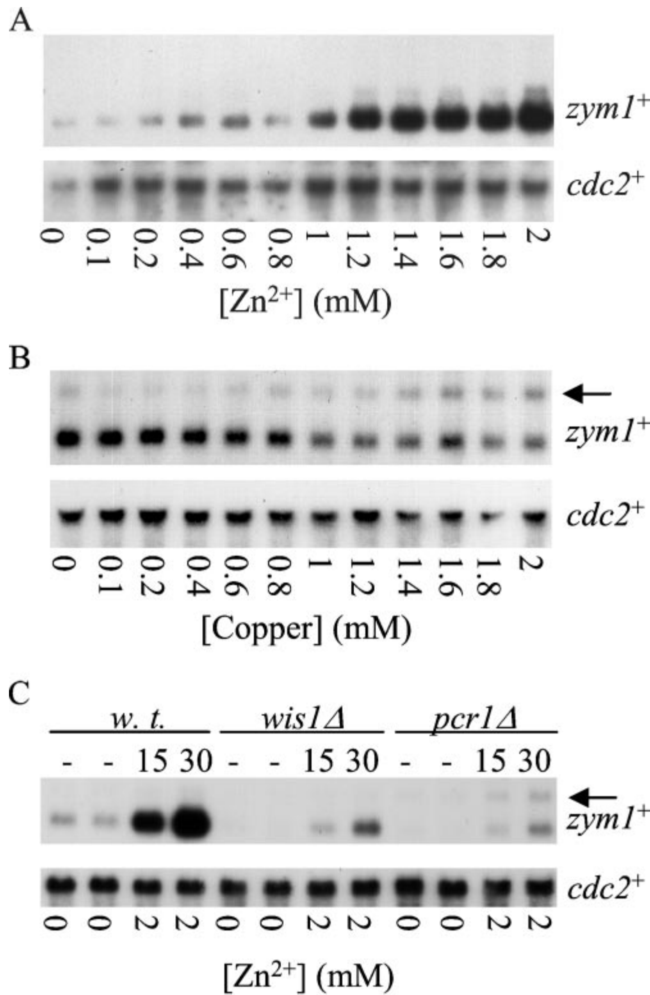

FIG. 3. **Expression of *zym1* is induced by zinc but not copper.** *A*, exponentially growing wild-type NT5 cells were treated with $ZnSO_4$ at the indicated concentration for 30 min. Cells were harvested, and total RNA was prepared and subjected to northern blotting using *zym1* and *cdc2* probes. *B*, *zym1* mRNA is not induced by copper. The *arrow* indicates the presence of a longer transcript. Details are as for *A* with the exception that $CuSO_4$ was used in place of $ZnSO_4$. *C*, exponentially growing wild-type NT5 (*w. t.*), *wis1*Δ (GD1682), and *pcr1*Δ (JX125) cells were treated with $ZnSO_4$ (2 mM) for either 15 or 30 min as indicated. Cells were harvested, and total RNA was prepared and subjected to northern blotting using *zym1* and *cdc2* probes. To detect background variability, two cell extracts (−) were prepared in each experiment.

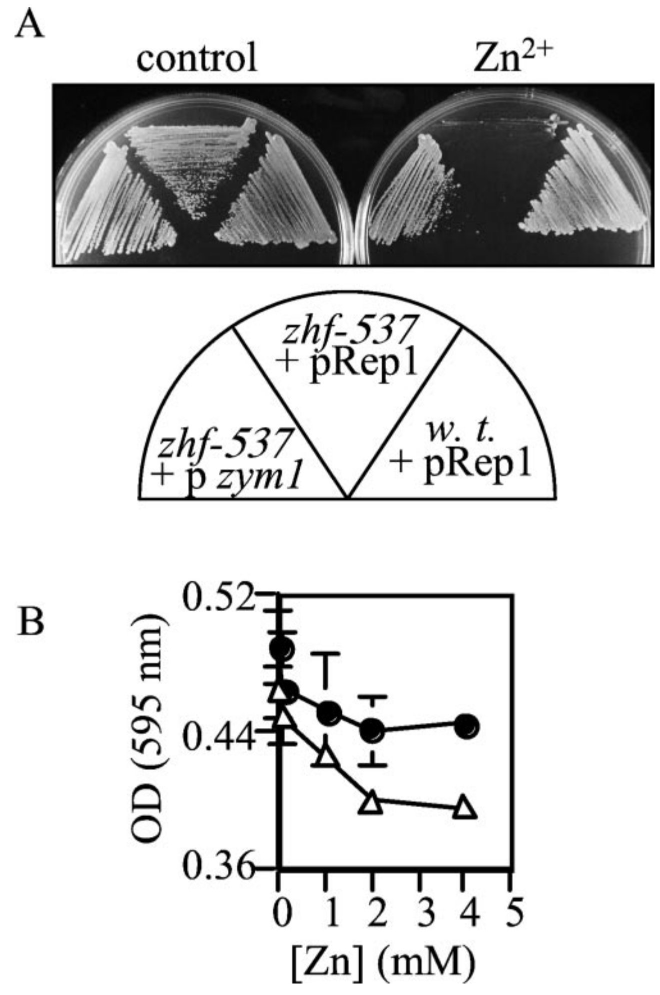

FIG. 4. **Overexpression of *zym1* partly rescues zinc hypersensitivity of *zhf-537*.** *A*, *zhf-537* (IS537) cells transformed with either empty vector (pRep1) or with a plasmid expressing *zym1* (p-*zym1*) were cultured on minimal agar (EMM) either supplemented with 0.4 mM $ZnSO_4$ as indicated or not supplemented. Wild-type (NT5) cells transformed with pRep1 (*w. t.* + pRep1) were included as a control. Plates were incubated for 4 days at 30 °C. *B*, *zhf-537* (IS537) cells carrying empty vector pRep1 (*open triangles*) or a plasmid expressing *zym1*, p-*zym1* (*closed circles*), were grown to early log phase (A_{595} = 0.2) in minimal medium (EMM) before adding $ZnSO_4$ to the appropriate concentration. A_{595} was measured following incubation at 30 °C for 6 h. Data are the means of triplicate determinations with S.E., and equivalent trends have been observed on two further occasions.

unlike the control strain (Fig. 4*A*). Suppression appeared incomplete, because wild-type cells grew better on 0.4 mM $ZnSO_4$ than *zhf-537* containing pRep1-Zym1. Exponentially growing liquid cultures of *zhf-537* containing pRep1-Zym1 showed enhanced cell division in the presence of zinc compared with equivalent cells containing pRep1 alone (Fig. 4*B*).

Deletion of zym1 Reduces Zinc Accumulation but Only Slightly Impairs Zinc Tolerance—A role in zinc homeostasis was further tested by creating a strain in which *zym1* was inactivated by insertion. The growth of the resulting *zym1*Δ showed only a small but reproducible impairment in rich medium (YE5S) supplemented with 10–100 μM zinc (Fig. 5*A*). The analyses of cellular metal content by atomic absorption spectroscopy revealed less atoms of zinc per *zym1*Δ cell compared with wild-type cells (Fig. 5*B*).

Zym1 Affords Some Protection against Cadmium—Phytochelatins are the major determinants of cadmium resistance in *S. pombe* (28, 29). We tested the growth of *zym1*Δ in cadmium and observed a decrease in tolerance (Fig. 6*A*). However, *zym1*Δ showed no difference in cadmium content as determined by atomic absorption spectroscopy (data not shown), and in this respect, the action of Zym1 is different in the cellular handling of cadmium and zinc. A loss of Zym1 has the greatest effect on resistance to cadmium (compare Fig. 5 with 6*A*) but only modulates the accumulation of zinc (Fig. 5*B*).

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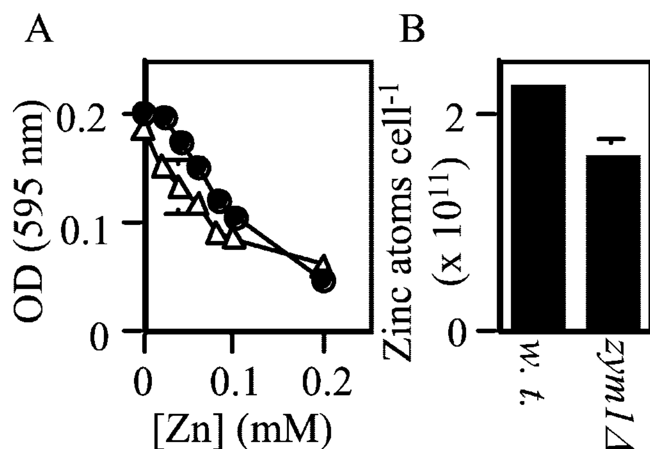

FIG. 5. **Zinc tolerance and zinc accumulation of *zym1*Δ cells.** *A*, approximately 2 × 10^4 exponentially growing *zym1*Δ SW61 (*open triangles*) and wild-type SW62 (*closed circles*) cells were inoculated into 5 ml of rich medium (YE5S) supplemented with increasing concentration of $ZnSO_4$ as indicated. A_{595} was measured following overnight incubation at 30 °C. *B*, intracellular zinc content of *zym1*Δ SW61, and wild-type cells SW62 (*w. t.*) growing in rich medium (YE5S) supplemented with 80 µM $ZnSO_4$ was determined using atomic absorption spectrophotometry. Data are the means ± S.E. of triplicate determinations, and equivalent trends have been observed on two further occasions.

Cadmium Promotes Accumulation of Longer zym1 Transcripts—Evidence that Zym1 confers some protection against cadmium indicates that the gene product is present in cadmium-challenged cells. Basal *zym1* expression in culture may be sufficient to mediate this level of resistance. Alternatively *zym1* may be induced by cadmium, and this theory was tested. Cadmium increased the proportion of longer *zym1* transcripts as observed previously following the exposure to copper (Fig. 6*B*). The longer transcript is unlikely to result from cadmium-mediated inhibition of splicing, the *zym1* gene containing no predicted introns. It is formally possible that cadmium selects an alternative *zym1* transcription start or termination site. There is a precedent for stress in the form of DNA-damaging agents, influencing the selection among three optional termination sites in the *rhp51* gene of *S. pombe* (44). Transcript abundance continues to increase up to 2 mM zinc (Fig 3*A*). Of course, cadmium is toxic at lower concentrations than zinc (Figs. 5*A* and 6*A*, compare *x* axes on showing inhibition by cadmium under these exposure conditions at approximately two orders of magnitude lower concentrations than zinc), precluding comparative analysis of viable cells at 2 mM. However, it is notable that transcript abundance (the sum of both transcripts) does not substantially increase at cadmium concentrations greater than 0.1 mM, indicating that optimal induction has been achieved (Fig. 6*B*). The fold increase in *zym1* transcript abundance in response to zinc (Fig. 3*A*) is greater than that observed in response to cadmium (Fig. 6). It is formally possible that a response to cadmium could indirectly result from the displacement of intracellular zinc.

Zym1 Binds Four Zinc Atoms in Vitro via up to 12 Cysteines—Several lines of evidence suggest that Zym1 can sequester zinc *in vivo*. To test the putative zinc-binding properties of Zym1, recombinant Zym1 was generated in *E. coli* (supplemented with 0.5 mM zinc) and purified from cell homogenates by gel filtration and anion exchange chromatography. Purity was tested by polyacrylamide gel electrophoresis, identity confirmed by amino-terminal sequence analysis, and association with zinc was confirmed by atomic absorption spectroscopy. To investigate stoichiometry and the nature of liganding, Zym1 was incubated with increasing concentrations of PMPS in the presence of the metallochromic indicator

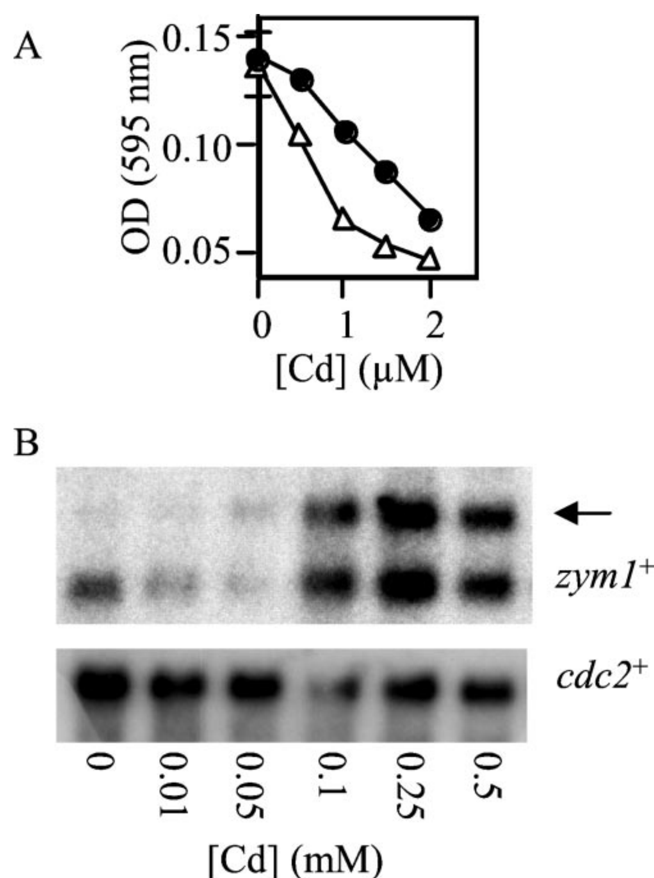

FIG. 6. **Loss of *zym1* decreases cadmium tolerance, and cadmium induces the expression of *zym1*.** *A*, approximately 2 × 10^4 exponentially growing *zym1*Δ SW61 (*open triangles*) and wild-type SW62 (*closed circles*) cells were inoculated into 5 ml of rich medium (YE5S) supplemented with increasing concentration of $CdSO_4$ as indicated. A_{595} was measured following overnight incubation at 30 °C. No difference in cadmium content was detected using atomic absorption spectrophotometry (data not shown). Data are the means ± S.E. of triplicate determinations, and equivalent trends have been observed on two further occasions. *B*, exponentially growing wild-type SW62, cells were treated with $CdSO_4$ for 30 min. Cells were harvested, and total RNA was prepared and subjected to northern blotting using *zym1* and *cdc2* probes. The *arrow* indicates the presence of a longer transcript.

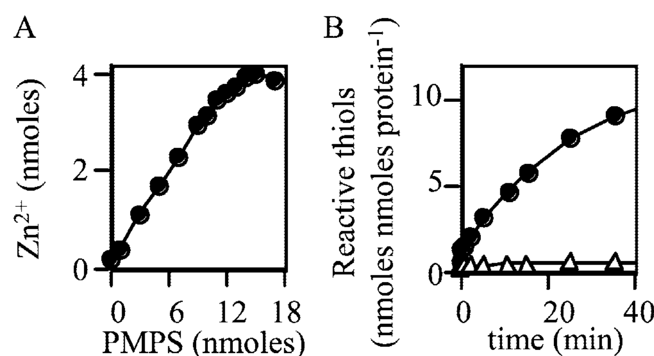

FIG. 7. **Zinc binding properties of Zym1 *in vitro*.** *A*, release of metal ions from Zym1 by titration with PMPS. An aliquot of protein (1 nmol) was titrated with PMPS, and metal ion release was quantified via the increase in A_{492} following reaction with 4-(2)-(pyridylazo)resorcinol. *B*, a comparison of thiol accessibility between Zym1 (*closed circles*) and the prokaryotic metallothionein SmtA (*open triangles*). Aliquots of Zym1 (0.75 nmol) and SmtA (1 nmol) were incubated with 37.5 µM DTNB, and the reaction of thiols was quantified via the increase in A_{402}.

4-(2)-(pyridylazo)resorcinol (35). Between 10 and 12 molar equivalents of PMPS displaced approximately four equivalents of zinc from Zym1, indicating that up to 12 cysteine

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residues are involved in binding four atoms of zinc (Fig. 7*A*). All of the 12 cysteine residues appear to contribute ligands. Bridging ligands are probable, because there are insufficient cysteines to provide four independent tetravalent sites.

The Thiols in Zym1 are 40-fold More Reactive with 5-Thio-2-nitrobenzoate Than Those of SmtA—Is Zym1-bound zinc likely to act as a labile zinc store? Thiol reactivities of metallothioneins correlate with the propensity of the cluster to engage in zinc transfer reactions (45). Recombinant Zym1 was incubated with an excess of DTNB, and the formation of 5-thio-2-nitrobenzoate was monitored by the increase in absorbance at 405 nm. Values were calibrated by reacting known amounts of free cysteine, and bacterial metallothionein SmtA was also used for comparison (35). The thiols react faster in Zym1 than SmtA (Fig. 7*B*). An analysis of the data as first order reactions gave values of $6.8 \times 10^{-4}\ s^{-1}$ for Zym1 and $0.2 \times 10^{-4}\ s^{-1}$ for SmtA. Zym1 is the more probable to exchange zinc *in vivo*, although both proteins are less reactive than mammalian metallothionein at $28 \times 10^{-4}\ s^{-1}$ (45).

DISCUSSION

Zym1 is a metallothionein revealed by its cysteine-sulfur metal coordination characteristics (Fig. 7), size, and sequence (Fig. 2) and further supported by an accumulation of *zym1* transcripts in response to metal (Fig. 3). Several lines of evidence (Figs. 3–5 and 7) implicate Zym1 in the cellular handling of zinc: zinc but not copper promotes a large accumulation of *zym1* transcripts (Fig. 3, *A* and *B*); the overexpression of *zym1* "substantively" restores zinc tolerance to *zhf* mutants (Fig. 4); and the deletion of *zym1* "slightly" reduces zinc tolerance and causes a decrease in the number of zinc atoms $cell^{-1}$ (Fig. 5). Surprisingly, *zym1*-deficient cells also show slight sensitivity to cadmium (Fig. 6*B*). A longer *zym1* transcript predominates in response to cadmium. The Sty1 pathway up-regulates *zym1* but is not essential for zinc signaling to *zym1*, because zinc-dependent expression is retained in *wis1*Δ (Fig. 3*C*). The endoplasmic reticulum zinc transporter Zhf is confirmed to be the major determinant of zinc resistance recovered in a phenotypic screen of *S. pombe* mutants, and we established that *zhf* is required for rapid growth under zinc deficiency (Fig. 1*B*). Both Zhf and Zym1 contribute toward the intracellular handling of zinc.

In *S. cerevisiae*, Zrc1 and Cot1 reduce the pool of toxic cytosolic zinc and hence confer the resistance to high zinc concentrations (15). Zhf from *S. pombe* has an extensive sequence similarity to Cot1 and Zrc1 and has been localized to the endoplasmic reticulum (26) rather than the vacuole. The detailed analyses of the intracellular distribution of zinc clearly demonstrate that Zhf contributes toward the compartmentalization of zinc within the endoplasmic reticulum and thereby detoxifies surplus (26). Fig. 1*D* confirms that Zhf also acts in the efficient cellular supply of zinc, because the growth of *zhf*Δ is severely impaired on LZM. Zym1 is fundamentally different from the metallothioneins of other fungal species that accumulate in response to and bind copper not zinc (10, 11). However, a loss of *zym1* resulted in only a small decrease in tolerance to elevated zinc (Fig. 5*A*), consistent with Zhf rather than Zym1 in representing the primary mechanism for detoxifying excess. This finding is similar to the situation in mammals in which cell lines or mutant mice that do not produce MT-I and MT-II exhibit only slight decreases in zinc tolerance with zinc transporters acting as the principal determinants of resistance (46). It is intriguing that *zhf*Δ and *zym1*Δ strains show inverse phenotypes with respect to excess cadmium, the *zhf*Δ strain being cadmium resistance and the *zym1*Δ strain being cadmium-sensitive. That fission yeast lacking *zym1* shows any reduction in cadmium tolerance was unexpected because *S. pombe* rapidly produces cadmium-binding phytochelatins, $(\gamma\text{-GluCys})_n$Gly, from glutathione in response to cadmium challenge (36, 37). Cadmium accumulation in contrast to zinc accumulation was unaffected by the loss of Zym1 (data not shown), indicating that this metallothionein does not influence cadmium sequestration by phytochelatins. The binding of cadmium by Zym1 might provide some supplemental buffering of cadmium in transit across the sensitive cytosol prior to vacuolar deposition as cadmium-phytochelatin complexes. Alternatively, Zym1 might scavenge cytosolic zinc displaced from metalloproteins by cadmium.

How is *zym1* regulated? In addition to direct metalloregulation, MT-I and MT-II are also expressed in response to agents that generate reactive oxygen species (6), and metallothioneins protect against oxidative damage (47, 48). Preliminary data (not shown) indicate that *zym1* transcripts accumulate in response to H_2O_2. Cadmium (Fig. 6*B*) and copper stress (Fig. 3*B*) cause some production of longer transcripts (Fig. 3*C*). Critically, the Sty1 pathway substantially enhances the overall level of expression of *zym1* in both the absence and presence of zinc. "Preemptive" up-regulation of *zym1* by stresses that promote zinc liberation from metalloproteins may involve general stress signal pathways such as Sty1, elevating *zym1* expression under culture conditions. However, the Sty1 kinase Wis1 is not obligatory for the perception of zinc (Fig. 3*C*). Some independent zinc-sensing factor also acts on *zym1*. The *S. pombe* genome does not encode proteins with a high homology or similar number of zinc fingers to the *S. cerevisiae* low zinc sensor Zap1 (49), and it is unclear how *S. cerevisiae* genes are activated in response to zinc surplus. In mammals, the zinc finger factor MTF-I regulates MT-I and MT-II in response to elevated zinc (6). MTF-I homologues have not been found in lower eukaryotes, but there are several uncharacterized open reading frames in *S. pombe* that have some similarity to the zinc finger regions of MTF-I and are therefore candidates for the control of *zym1*.

Compartmentalization by Zhf is required for optimal growth in zinc limitation and excess. Zinc released from metalloproteins as a result of normal turnover or by oxidants and metals of greater affinity is likely to form gratuitous partnerships with other cytosolic ligands and may not be accessible for transport by Zhf. One attractive hypothesis is that Zym1 scavenges zinc associated with adventitious sites to inhibit deleterious interactions, aiding cellular retention and recycling.

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Surplus Zinc Is Handled by Zym1 Metallothionein and Zhf Endoplasmic Reticulum Transporter in *Schizosaccharomyces pombe*

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