A Transporter in the Endoplasmic Reticulum of Schizosaccharomyces pombe Cells Mediates Zinc Storage and Differentially Affects Transition Metal Tolerance*

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The cation diffusion facilitator (CDF) family represents a class of ubiquitous metal transporters. Inactivation of a CDF in Schizosaccharomyces pombe, Zhf, causes drastically different effects on the tolerance toward various metals. A deletion mutant is Zn²⁺/Co²⁺-hypersensitive yet displays significantly enhanced Cd²⁺ and Ni²⁺ tolerance. Accumulation of zinc, cobalt, and cadmium is reduced in mutant cells. Non-vacuolar zinc content, as measured by analytical electron microscopy, is lower in zhf⁻ cells compared with wild-type cells in the presence of elevated Zn²⁺ concentrations. The protective effect against cadmium toxicity is independent of the phytochelatin detoxification pathway. Phytochelatin synthase-deficient cells show extremely enhanced (about 200-fold) cadmium tolerance when zhf is disrupted. Immunogold labeling indicates endoplasmic reticulum (ER) localization of Zhf. Electron spectroscopic imaging shows that accumulation of zinc coincides with Zhf localization, demonstrating a major role of the ER for metal storage and the involvement of Zhf in cellular zinc homeostasis. Also, these observations indicate that Cd²⁺ ions exert their toxic effects on cellular metabolism in the ER rather than in the cytosol.

Ions of heavy metals such as iron, copper, zinc, cobalt, or nickel are essential micronutrients, required for function of a large number of proteins. At supraoptimal concentrations, however, these metal ions can be detrimental. Furthermore, living organisms can be exposed to the highly toxic ions of cadmium, lead, mercury, and other metals that are generally considered non-essential. Consequently, a complex network of transport, chelation, and sequestration processes has evolved that functions to maintain the concentrations of essential metal ions in different cellular compartments within the narrow physiological range and to minimize the damage caused by the entry of non-essential metal ions into the cytosol (1, 2). The exquisitely tight control of free ion concentrations has been demonstrated for copper (3). Most recent evidence suggests a similar degree of control also for zinc (4). The intracellular mechanisms of storage and cellular distribution, however, are largely unknown.

Proteins belonging to the cation diffusion facilitator family (CDF) (5, 6) could potentially play a major role in metal homeostasis. They have been shown in bacteria and budding yeast to confer tolerance of Zn²⁺, Co²⁺, or Cd²⁺ ions (7–10). ZRC1 and COT1 in Saccharomyces cerevisiae localize to the vacuolar membrane and are hypothesized to contribute to the storage of Zn²⁺ and Co²⁺ ions, respectively (11, 12). Mamalian members of the CDF family appear to be involved mainly in the removal of Zn²⁺ ions from the cytosol either through the plasma membrane (Zn-T1) (13) or into endosomal vesicles (Zn-T2) (14).

We are interested in the physiological role of CDFs and other putative metal transporters for cellular metal homeostasis, tolerance, and accumulation in organisms that express phytochelatin synthases, using fission yeast as the most suitable model system. The synthesis of phytochelatins (PCs), small metal-binding peptides derived from glutathione (15, 16), represents one of the main metal chelation and detoxification mechanisms in plants, fungi, marine diatoms, and also certain animals (17–19). Here we report on the functional characterization of a Schizosaccharomyces pombe CDF (named Zhf, for zinc homeostasis factor), whose function affects tolerance to a range of metal ions in drastically different fashion; disruption of the gene renders S. pombe cells Zn²⁺- and Co²⁺-hypersensitive yet significantly enhances tolerance toward Cd²⁺ and Ni²⁺. Electron microscopic protein and zinc localization indicate Zhf-dependent zinc accumulation in the ER. Our findings represent novel evidence for the role of the respective compartment in metal homeostasis and identify a major pathway of zinc storage in the ER. Furthermore, the data provide new insights into the still poorly understood cellular mechanisms of cadmium toxicity.

EXPERIMENTAL PROCEDURES

S. pombe Strains and Media — The S. pombe strains employed in this study were derived from FY261 (14) ade6-M216 leu1-32 ura4Δ18 can1-I, kindly provided by Susan Forsburg, The Salk Institute, La Jolla, CA and the previously described phytochelatin synthase-deficient Δpcs strain Sp27 (20). Cells were cultivated in Edinburgh's minimal medium (EMM) supplemented appropriately. Transformation of S. pombe was performed according to the protocol of Bähler et al. (21). The zhf gene was disrupted in FY261 in two ways: insertion of leu2 marker into an internal HindIII site and insertion of EcoRI/HindIII-digested ura4 marker between the respective sites in the zhf coding sequence. Deletions were confirmed by Southern analysis.

Construction of Epitope-tagged Protein — The S. pombe zhf and Arabidopsis ZAT coding sequences were amplified with Pfu polymerase

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1 The abbreviations used are: CDF, cation diffusion facilitator; PC, phytochelatin; Zhf, zinc homeostasis factor; ER, endoplasmic reticulum; EMM, Edinburgh’s minimal medium; MES, 4-morpholinethanesulfonic acid; EDX, energy-dispersible x-ray analysis; ESI, electron spectroscopic imaging; HA, hemagglutinin.
using primers carrying a BglII and a SacI site. The resulting fragments were ligated into pSGP73 (kindly provided by Dr. Susan Forssburg, Salk Institute) to add a triple hemagglutinin tag to the N terminus of the protein. The zhf and ZAT sequences were confirmed by automatic sequencing.

**Metal Toxicity Tests and Metal Accumulation Assays**—To test metal sensitivity, cells grown to log phase were diluted to an A600 of 0.1 and incubated in the presence of different metal concentrations. After 24 h, cell density was measured. For metal accumulation assays, cells were grown to mid-log phase, harvested, washed, and resuspended in uptake buffer (10 mM MES, 20 g/liter glucose, pH 6.1). Accumulation was started by adding 20 µl of either 100 mM CdCl2 (37 MBq/µg; Amersham Biosciences), 50 µM ZnCl2 (185 GBq/µg; DuPont), or 50 µM CoCl2 (315 MBq/µg; Amersham) to a final concentration of 100 µM. At different time points 450-µl aliquots were harvested on filters (0.45 mm, Schleicher and Schuell, Dassel, Germany) and washed three times with washing buffer (1 mM NaCl, 10 mM MgCl2, 2 mM CaCl2, 1 mM KH2PO4, 20 mM trisodium citrate, 1 mM EDTA, pH 4.2). The radioactivity that remained on the membrane filter was determined with a scintillation counter (LS6500, Beckman, Munich, Germany). The dry weight was determined via the optical density and an equilibration curve.

**Phytocelatin and Glutathione Assay**—Phytocelatins and glutathione were analyzed essentially as described by Sneller et al. (22). S. pombe cells were lyophilized and extracted with 0.1% trifluoroacetic acid. Following centrifugation the supernatant was derivatized with monobromobimane and analyzed essentially as described by Sneller et al. (22). The radioactivity that remained on the membrane filter was determined with a scintillation counter.

**Western Blotting**—Total cell extracts were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblots were performed essentially as described by Sneller et al. (22). An anti-HA antibody (1:1000 dilution) was used to detect HA-tagged proteins. 

**Elemental Mapping**—For elemental mapping by electron spectroscopic imaging (ESI) the specimens were analyzed with an energy-dispersible x-ray analysis (EDX) system (Link eXIII, Oxford Analytical Instruments, Bensheim, Germany), and embedded in ERL (Plano, Wetzlar, Germany). Sections (50 nm, Leica, Bensheim, Germany), and freeze-substituted in acetone (CSauto, Balzers, Liechtenstein), were cut with a ultramicrotome (EM912 OMEGA, LEO). Images were recorded with a SIT-TV camera (EM912 OMEGA, LEO). The spot size at 80 keV was 20 eV at 80 keV. The background was subtracted by computers with the two-window method (Analysis 3.0, Soft Imaging System, Münster, Germany), and the colored elemental images were overlaid with the zero loss image.

**Immunolocalization—zhf knockout cells**—Immunolocalization was performed with an N-terminally HA-tagged Zhf which is best suited for visualizing Zhf. The HA-tagged Zhf was DNA-based and the fusion was detected by indirect immunofluorescence. The antibody used was specific for the HA tag and was prepared with an HA antibody (Roche, Mannheim, Germany). Immunolocalization was performed essentially as described by Sneller et al. (22).

**Miscellaneous Procedures**—Standard methods for the manipulation of DNA and for PCR as well as for SDS-PAGE and Western analysis were used (23). Total RNA was extracted following the Trizol reagent protocol (Invitrogen). First-strand cDNA synthesis starting from 1 µg of total RNA was performed using Moloney murine leukemia virus reverse transcriptase. A zhf fragment was amplified with primers 5'-gatagttg-gaaagatgtc-3' and 5'-ttatatcaggaaggtgca-3' (25 cycles). A fragment of the actin gene act1 (DBB/EMBL/GenBank™ accession no. Y100447) was amplified with primers 5'-gtattttggagaggctg-3' and 5'-ggctgcttcagcct-3' (25 cycles). HA-tagged Zhf protein was detected with HA monoclonal antibody. Homologous DNA and protein sequences were identified by searching within the DDBJ/EMBL/GenBank™ data base using BLAST (24). Amino acid sequences were analyzed with TMAPred (25) for the presence of putative transmembrane spans.

**RESULTS**

**Deletion of zhf from the S. pombe Genome Caused Zinc and Cobalt Sensitivity but Enhanced Tolerance to Nickel and Cadmium**—The first CDF family member sequenced in the process of the Sanger Center S. pombe program was submitted to DDBJ/EMBL/GenBank under accession no. Z98559. The derived protein (DBB/EMBL/GenBank™ accession no. D89236), designated Zhf, displayed 43% identity to ZRC1 from S. cerevisiae and 21% identity to ZAT from Arabidopsis. Typical features such as the CDF signature sequence (6), six putative transmembrane domains, and a His-rich cytoplasmic loop between transmembrane helices 4 and 5 were revealed by sequence analysis. The corresponding gene was amplified from S. pombe genomic DNA and cloned. Weak stringency Southern hybridization indicated it as a single copy gene in S. pombe (data not shown). The zhf gene was disrupted in the haploid wild-type strain FY261 by inserting the leu2 marker. Cells of the resulting Δzhf strain were viable and showed only a minor reduction in growth rate under control conditions without any added heavy metal salts. In the presence of elevated Zn2+ levels, however, they were severely growth-inhibited (Fig. 1, A and B). Whereas wild-type cells grew normally in EMM containing up to the maximum soluble Zn2+ concentration of 5 mM, half-maximal inhibition of growth at about 150 µM Zn2+ was extrapolated from the data for the Δzhf strain.

Similarly, the absence of a functional Zhf protein rendered the S. pombe cells Co2+-hypersensitive (Fig. 1C). At 1 mM Co2+ in EMM, wild-type cells showed 94% (±11%) of the optical density of untreated control cells, whereas Δzhf cells reached only 36% (±3%). In contrast to that, the zhf disruption was found to significantly protect cells from toxicity of Cd2+ ions, the third known substrate of CDF proteins. At the IC50 concentration for wild-type cells of 100 µM, Δzhf cells were inhibited only by 4% (±3%) (Fig. 1D). In the presence of Cd2+ concentrations that completely inhibited growth of wild-type cells, Zhf-deficient cells still reached 70% of their growth rate under control conditions. When the responses to other metal ions were assayed, a pronounced protective effect of the Zhf inactivation could be detected also for Ni2+. The IC50 value was shifted from about 180 µM for wild-type cells to 1000 µM for Δzhf (Fig. 1E). Cu2+ effects on growth were only slightly less severe for Δzhf cells compared with wild-type (data not shown).

**Disruption of zhf in PC-deficient Cells**—The protection of the Zhf inactivation against Cd2+ toxicity could potentially be explained by indirect effects on the Cd2+ detoxification mechanisms. To determine whether the main pathway for Cd2+ detoxification in S. pombe, the formation of phytocelatins, is required for the Δzhf-dependent protection, the zhf gene was disrupted in the Δpcs strain Sp27 (20). Toxicity assays showed that the protective effect was even more pronounced in this genetic background. The IC50 value of the Sp27 strain for Cd2+ was shifted from 0.5 µM back to the wild-type level of 100 µM as a consequence of the zhf disruption (data not shown). This equaled a complete reversion of the severe Cd2+ hypersensitivity phenotype caused by PC deficiency in S. pombe. Furthermore, the levels of glutathione, which may also contribute to Cd2+ detoxification, were not altered in Zhf-deficient cells (data not shown). Thus, an enhanced complexation of the cation in the cytoplasm via activation of the main Cd2+ detoxification system apparently was not the cause for the enhanced Cd2+ tolerance of Δzhf cells. Zn2+, Ni2+, and Co2+ tolerance phenotypes were the same in zhf− and zhf−pcs− cells.

**Metal Accumulation by Δzhf Cells**—Given the activities of characterized CDF members, the extreme Zn2+ sensitivity of the knockout strain was likely to be related to a defect in removal of Zn2+ from Zn2+-sensitive sites. To discriminate...
between Zn$^{2+}$/H$_{11001}$ efflux across the plasma membrane and the intracellular sequestration of Zn$^{2+}$/H$_{11001}$, Zn$^{2+}$/H$_{11001}$ accumulation studies were performed. Metal ion accumulation by whole cells of the $\Delta$zhf mutant strain was always lower than accumulation by wild-type cells (Fig. 2). Compared with control cells, half as much cadmium was accumulated by the mutant (Fig. 2B), although initial uptake rates were the same for both strains. The differences were striking when zinc accumulation was considered; wild-type cells accumulated within 2 h nearly 20 mol of zinc/g, dry weight, but the mutant cells accumulated only about 10% of this value (Fig. 2A). Cobalt accumulation in the mutant cells was also diminished (Fig. 2C).

Analytical electron microscopy was used to more specifically assess the effects of Zhf inactivation on the cellular zinc content. Wild-type and $\Delta$zhf cells were compared by EDX, a technique that allows the measurement of zinc outside the vacuoles. No significant differences were found in the number of zinc atoms/reference volume for cells grown in normal medium.

**FIG. 1.** Disruption of $zhf$ affects tolerance of *S. pombe* cells to a range of transition metals differentially. *S. pombe* wild-type (○) and $\Delta$zhf cells (●) were grown on EMM in the absence and presence (250 μM) of added Zn$^{2+}$ (A) in liquid EMM in the presence of different Zn$^{2+}$ (B), Co$^{2+}$ (C), Cd$^{2+}$ (D), or Ni$^{2+}$ (E) concentrations. Cultures were diluted to an $A_{600}$ of 0.1, and cell density was measured after 24 h. Error bars represent mean ± S.D., $n = 5$.

**FIG. 2.** $\Delta$zhf cells show a significant reduction in accumulation rates for zinc, cadmium, and cobalt. *S. pombe* wild-type (○) and $\Delta$zhf cells (●) were assayed for accumulation of $^{65}$ZnCl$_2$ (A), $^{109}$CdCl$_2$ (B), and $^{57}$CoCl$_2$ (C). The concentration was 100 μM. Aliquots were taken at different time points, and radioactivity was measured. Error bars represent mean ± S.D., $n = 3$. d.w., dry weight.
for 20 h (Fig. 3). Growth in EMM supplemented with 100 μM Zn$^{2+}$ for 20 h, however, resulted in an increase in non-vacular zinc content for wild-type cells by a factor of 5, whereas the zhf$^-$ knock-out cells showed only a slight increase.

Cellular cadmium content is too low to be measured reliably by analytical electron microscopy. However, the rate of phytochelatin synthesis can be used as a marker for cytosolic cadmium content because the phytochelatin synthase is activated by binding of its substrate GSH-cadmium (26). Thus, the rate of PC formation is correlated with availability of cadmium. PC accumulation was measured over a range of Cd$^{2+}$ concentrations below and above the toxicity threshold for wild-type cells. Δzhf$^-$ cells consistently showed a reduced accumulation of PC2 and PC3 (Fig. 4). To reach the amount of PCs accumulating in wild-type cells exposed to 5 μM Cd$^{2+}$, Δzhf$^-$ cells had to be treated with 50 μM Cd$^{2+}$. This was interpreted as an indication for a reduced level of available cytosolic cadmium in Δzhf$^-$ cells, which matched the diminished amount of whole cell-bound cadmium of the mutant cells.

Localization of Zhf—The zhf$^-$ gene is expressed constitutively. No increase in message level was observed upon increasing the zinc concentration of the medium to 1 mM (Fig. 5). From this, it was concluded that Zhf is involved in the intracellular storage of zinc. Western analysis showed a single band migrating at a molecular mass of about 49 kDa. No signal was detected in extracts from control cells carrying the empty pSGP73 plasmid. The complemented cells were used for the immunolocalization with a monoclonal anti-HA antibody. Sections of control cells did not show any significant staining following immunogold labeling (Fig. 6C). In Zhf-HA samples, signals appeared in structures inside the cell and underneath the plasma membrane, which are typical for the endoplasmic reticulum. Also, staining of the nuclear envelope was found (Fig. 6, A and B). No staining was detectable at the plasma membrane, around the vacuoles, or associated with any other organelle. We concluded that Zhf is located in the ER and in the nuclear envelope of S. pombe cells.

Electron Spectroscopic Imaging of Zinc—To determine how the proposed localization of Zhf affected cellular zinc distribution, zinc was visualized by ESI of wild-type and Δzhf$^-$ cells grown in Zn$^{2+}$-supplemented medium. ESI analysis of wild-type cells showed accumulation of zinc in structures again inside the cell and at the cell periphery underneath the plasma membrane (Fig. 7A). No such accumulation could be detected in Δzhf$^-$ cells (Fig. 7B). EDX analysis of cell wall and cellular material near the plasma membrane showed that no zinc is detectable in the cell wall (Fig. 7, C and D). Hence, the observed staining was not due to cell wall binding of zinc. Furthermore, when ESI analysis was carried out on samples used for immunolocalization, zinc accumulation was detected in structures staining for Zhf-HA (Fig. 8). Thus, zinc accumulation in wild-type and Zhf-complemented cells coincided with localization of Zhf.

Complementation of Δzhf$^-$ Cells—Complementation of the Zhf deficiency with the endogenous gene was compared with that by ZAT, the first characterized CDF from Arabidopsis thaliana (27). ZAT displays specificity for Zn$^{2+}$ ions and does not transport Cd$^{2+}$ or Cd$^{3+}$ ions as indicated by the results of reconstitution experiments in proteoliposomes (37). ZAT and zhf$^-$ were subcloned into an S. pombe expression vector carrying a leu2 marker. These constructs were used to transform an S. pombe strain with a ura4 insertion in the zhf$^-$ gene. Plasmid-encoded Zhf restored the Zn$^{2+}$- and Cd$^{2+}$-related phenotypes. Cells were able to grow in the presence of elevated Zn$^{2+}$ and became as Cd$^{2+}$-sensitive as wild-type cells (Fig. 9). ZAT, however, restored only the Zn$^{2+}$ hypersensitivity of the Zhf-deficient cells. The increased Cd$^{2+}$ tolerance as compared with wild-type was partially maintained. ZAT-expressing cells still grew to an absorbance of 53.5% (±12.2%) of controls in the presence of 200
DISCUSSION

Transport of metal ions across intracellular membranes is a key aspect of metal homeostasis. It provides a driving force for the uptake of metal ions across the plasma membrane and contributes to the trafficking and storage of essential metal ions as well as to the detoxification of non-essential metal ions. Members of the CDF family have been implicated in these still poorly understood processes in a diverse range of model systems. Few of them, however, have been assigned a physiological role. Examples include the mammalian Zn\(^{2+}\) transporter ZnT-3, required for the accumulation of zinc in vesicles in nerve cells (28, 29), and CzcD of Bacillus metalloidiurans, which is involved in the regulation of the metal-pumping Czc system by mediating the export of inducing cations (10).

Metal concentrations in the various compartments of a cell are the function of transport and chelation activities. Thus, it is important to understand the contribution and interaction of such different processes. Is there, for instance, in cells that synthesize phytochelatins, a contribution of transporters independent of the PC pathway to Cd\(^{2+}\) sequestration? In this context a study on CDFs in S. pombe, the most suitable phytochelatin synthase-expressing model system, was initiated. Zhf is one of at least three members of this family in S. pombe (DDBJ/EMBL/GenBank\(^{TM}\) accession numbers CAC05733 and T43145). A mutant strain was generated and characterized with respect to metal tolerance and accumulation. Effects on tolerance toward a range of metal ions were found to be different to an unprecedented degree (Fig. 1).

Sequestration capacity and degree of tolerance are very often positively correlated. Chelation and sequestration activities that confer tolerance also drive the uptake of metal ions across the plasma membrane. Synthesis of phytochelatins in S. cerevisiae cells leads to increased tolerance and accumulation of cadmium (20). A number of CDFs, e.g. mammalian ZnT-2 and ZAT from Arabidopsis, have been shown to mediate Zn\(^{2+}\) tolerance as well as increased accumulation of zinc (14, 27). Correspondingly, the Zhf-deficient cells were Zn\(^{2+}\)-hypersensitive and accumulated less zinc than wild-type cells upon Zn\(^{2+}\) feeding (Figs. 3 and 4). Also, initial Zn\(^{2+}\) uptake rates were only slightly reduced in zhf\(^{−}\) cells. Thus, a role in zinc uptake was ruled out, and Zhf was hypothesized to mediate storage of zinc inside the fission yeast cell.

The vacuole is generally considered the main storage site for metals in yeast and plant cells. The EDX data (Fig. 3), however, already demonstrated zinc accumulation in zinc-treated wild-type cells outside the vacuoles. This accumulation was not detectable in Zhf-deficient cells. Furthermore, our immunogold labeling data (Fig. 6) indicated localization of the protein in the endoplasmic reticulum/nuclear envelope. The tagged protein was functional as shown by complementation, and no specific signal for the vacuolar membrane, any other cellular compartment, or the plasma membrane was detectable. This gives the results a high degree of confidence despite the expression from a plasmid under control of the constitutive nmt1 promoter. No signal was detectable in sections of cells grown in thiamine-containing medium repressing the promoter. However, localization was further supported by the ESI data (Figs. 7 and 8) that indicated that the sites of reduced zinc accumulation in Δzhf\(^{−}\) cells likely correspond to the ER structures stained by immunogold labeling.

These data, as well as another recent study, demonstrate a major role in zinc homeostasis for the endoplasmic reticulum. The reported localization of MSC2, a CDF family member from S. cerevisiae, in the ER/nucleus and the higher nuclear zinc content of msz2\(^{−}\) cells suggested a requirement for transporter-facilitated exchange of zinc between cytosol and nucleus at

\[\mu\text{M Cd}^{2+}\], whereas Zhf-expressing cells reached only 20.4% (±6.7%), and cells carrying the empty vector reached 80.3% (±2.4%).
least under certain stress conditions (30). Zhf is hypothesized to mediate the transport of zinc into the ER and the nuclear envelope in fission yeast. Zhf would thereby contribute to zinc storage and to supplying zinc to zinc-requiring proteins processed in the endoplasmic reticulum. The extreme Zn^{2+} sensitivity of \textit{zhf} cells demonstrates that Zhf-dependent transport represents a major pathway of zinc storage in \textit{S. pombe} cells. The fact that Zhf deficiency is not lethal, however, suggests the existence of additional transport systems providing at least the minimum required zinc influx into the ER. This influx may well be too low to result in any accumulation detectable by ESI.

Overall accumulation and cytosolic concentrations of available cadmium were found to be also reduced in \textit{zhf}-deficient cells. The apparent decrease in sequestrating capacity, however, was not accompanied by an increase in sensitivity, as was observed for instance for the vacuolar membrane-localized transporters ZRC1 in \textit{S. cerevisiae} (7) or Hmt1 in \textit{S. pombe} (31). In these cases, a transporter residing in an endomembrane detoxifies cadmium and drives the uptake of cadmium. Zhf, however, appears to drive cadmium uptake in a similar fashion yet does not contribute to detoxification. On the contrary, \textit{zhf} disruption resulted in a dramatic elevation of Cd^{2+} tolerance. Different hypotheses to explain this surprising observation were assessed. The Cd^{2+} tolerance phenotype of \textit{zhf} cells might be an indirect result of the effects on zinc content and distribution. However, no evidence for a dependence of this phenotype on the major known cadmium detoxification pathways could be found. Cd^{2+} ions compete with Zn^{2+} ions for binding sites in proteins. Higher Zn^{2+} levels due to the inactivation of a zinc storage mechanism therefore could suppress...
Cd\textsuperscript{2+} toxicity. Cytosolic Zn\textsuperscript{2+} content under control conditions, however, does not appear to be increased in the zhf knock-out strain as indicated by the results of EDX analysis. Is the Cd\textsuperscript{2+} tolerance phenotype then a mere consequence of reduced uptake of Cd\textsuperscript{2+} into the cells? Zn\textsuperscript{2+} transporters represent one of the entry pathways for Cd\textsuperscript{2+} (32, 33). However, no effect of Zhf inactivation on short-term uptake rates of Cd\textsuperscript{2+} could be detected (Fig. 2B). Together with the fact that zhf disruption caused a dramatic protective effect in pcs, cells which are impaired in cytosolic cadmium binding, this led us to hypothesize that Zhf mediates cadmium transport to a particularly sensitive site inside the cell, which based on the protein and zinc localization data would be the endoplasmic reticulum. To find additional evidence as to whether the Cd\textsuperscript{2+} phenotype is an indirect effect or dependent on a cadmium transport activity of Zhf, we compared complementation of Δzhf cells by the endogenous transporter with complementation by a CDF whose substrate specificity had been studied. Although a number of CDFs studied to date seem to transport Zn\textsuperscript{2+}, Co\textsuperscript{2+}, Cd\textsuperscript{2+}, and also Ni\textsuperscript{2+} ions, as most recently shown for Thlaspi transporters (34), Arabidopsis ZAT apparently transports Zn\textsuperscript{2+} but not Cd\textsuperscript{2+} (37). When cells were transformed with the respective genes it was found that both are functionally expressed and complement the Zn\textsuperscript{2+} hypersensitivity of Δzhf cells. In contrast to plasmid-encoded Zhf, ZAT only partially restored the Cd\textsuperscript{2+} sensitivity. This uncoupling of the zinc- and cadmium-related phenotypes suggests that interference with zinc homeostasis is not the cause of the protection against Cd\textsuperscript{2+}. Rather, these data indicate an important insight into the mechanisms of Cd\textsuperscript{2+} toxicity that to date are poorly understood. Displacement of Zn\textsuperscript{2+} in proteins is one current hypothesis to explain the harmful effects of Cd\textsuperscript{2+} exposure (35). Zhf appears to directly mediate Cd\textsuperscript{2+} toxicity by transporting Cd\textsuperscript{2+} ions into the ER and the nuclear envelope, a cellular compartment that apparently is particularly cadmium-sensitive. Cd\textsuperscript{2+} ions may interfere with the folding of proteins in the ER or more specifically the loading of zinc-requiring proteins with Zn\textsuperscript{2+} ions. Also, transport of Cd\textsuperscript{2+} ions into the ER lumen may lead to disturbances in Ca\textsuperscript{2+} homeostasis as recent evidence suggests a role of Ca\textsuperscript{2+} ions as a signaling molecule in the ER (36).

The transport dependence of Cd\textsuperscript{2+} toxicity would also have implications for the efficiency of cytosolic chelation of cadmium by phytochelatins, the main cadmium buffer in many different organisms. The PC detoxification pathway is not sequestering cadmium quantitatively. A fraction is available for transport into the endoplasmic reticulum or other organelles. Hence, Δzhf cells accumulate slightly less cadmium than wild-type cells. In pcs cells that are impaired in the cytosolic binding of cadmium, this fraction is much larger, which would explain why the zhf disruption results in such an extreme protection against Cd\textsuperscript{2+} in PC-deficient cells.

In conclusion, our data suggest that the transporter Zhf is localized in the ER/nuclear envelope and plays an important role in cellular zinc homeostasis by mediating the transport of zinc into the endoplasmic reticulum. Zhf activity provides a driving force for the accumulation of Zn\textsuperscript{2+}, Co\textsuperscript{2+}, and Cd\textsuperscript{2+} ions. Zhf-dependent removal from the cytosol results in a protection against Zn\textsuperscript{2+} and Co\textsuperscript{2+} toxicity. In contrast, Cd\textsuperscript{2+} toxicity is enhanced because the transport of cadmium into a particularly cadmium-sensitive organelle is mediated by Zhf.

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