DROSOPHILA ABC TRANSPORTER, DmHMT-1, CON芙ERS TOLERANCE TO CADMIUM. DmHMT-1 AND ITS YEAST HOMOLOG, SpHMT-1, ARE NOT ESSENTIAL FOR VACUOLAR PHYTOCHELATIN SEQUESTRATION.*

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Running head: HMT-1 from Drosophila confers tolerance to cadmium

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Half molecule ATP-binding cassette transporters of the HMT-1 (heavy metal tolerance factor 1) subfamily are required for Cd²⁺ tolerance in Schizosaccharomyces pombe, Caenorhabditis elegans and Chlamydomonas reinhardtii. Based on studies of S. pombe, it has been proposed that SpHMT-1 transports heavy metal-phytochelatin (PC) complexes into the vacuolysosomal compartment. PCs are glutathione derivatives synthesized by PC synthases (PCS) in plants, fungi and C. elegans in response to heavy metals. Our previous studies in C. elegans, however, suggested that HMT-1 and PCS-1 do not necessarily act in concert in metal detoxification. To further explore this inconsistency, we have gone on to test whether DmHMT-1, an HMT-1 from a new source, Drosophila, whose genome lacks PCS homologs, functions in heavy metal detoxification. In so doing, we show that heterologously-expressed DmHMT-1 suppresses the Cd²⁺ hypersensitivity of S. pombe hmt-1 mutants and localizes to the vacuolar membrane, but does not transport Cd·PC complexes. Crucially, similar analyses of S. pombe hmt-1 mutants extend this finding to show that SpHMT-1 itself either does not transport Cd·PC complexes, or is not the principal Cd·PC/apoPC transporter. Consistent with this discovery and with our previous suggestion that HMT-1 and PCS-1 do not operate in a simple linear metal detoxification pathway, we demonstrate that unlike PCS-deficient cells, which are hypersensitive to several heavy metals, SpHMT-1-deficient cells are hypersensitive to Cd²⁺, but not to Hg²⁺ or As³⁺. These findings significantly change our current understanding of the function of HMT-1 proteins and invoke a PC-independent role for these transporters in Cd²⁺ detoxification.

The adverse health effects of heavy metals such as cadmium (Cd³⁺), mercury (Hg²⁺) and lead (Pb²⁺) from food and air are well established (1-4). Despite this knowledge, exposure to heavy metals continues, and has even increased in some areas, due to their sustained production and emission into the environment. At the cellular level, the toxicity of heavy metals results from the displacement of endogenous co-factors from their cellular binding sites, the oxidation of essential enzymes and other proteins, and promotion of the formation of reactive oxygen species (ROS) (3, 4). The variety of ways by which heavy metals exert their effects places demands on a wide range of distinct cellular detoxification mechanisms in which ATP-binding cassette (ABC) transporters are clearly implicated (5-9).

The ABC transporter family is one of the largest families of membrane proteins. Although 60 ABC transporter family members are known in Caenorhabditis elegans, 49 in humans, 57 in Drosophila, 103 in Arabidopsis, 30 in Saccharomyces cerevisiae, and 11 in Schizosaccharomyces pombe (10-13), the exact role played by the many that are implicated in heavy metal detoxification remains to be determined. What is known is that ABC transporters mediate the Mg·ATP-energized transmembrane transport of a wide range of substrates, reside on different cellular membranes
and, although functionally diverse, share a common architecture.

Canonical, “full-molecule” ABC transporters consist of four domains: two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) that contain the Walker A and B boxes and the ABC signature motif (14). “Half-molecule” ABC transporters contain a single TMD and NBD. Some members of either the full- or half-molecule subfamilies of ABC proteins possess a hydrophobic N-terminal extension (NTE). The NTE encompasses five to six transmembrane spans (the TMD0 domain) and a cytosolic linker sequence (L0) contiguous with the TMD or NBD (5, 13). Among ABC transporters, the structure of HMT-1 proteins is unique: they are the only half molecule ABC proteins with an NTE. This domain organization of HMT-1 proteins is conserved across species and distinguishes the HMT-1 protein subfamily from other members of ABC transporter superfamily (5, 7, 9).

The first HMT-1-like protein identified, SpHMT-1, was isolated from S. pombe in mutant screens for genes involved in phytochelatin (PC)-mediated heavy metal tolerance (7). PCs are small, cysteine-rich peptides with the general structure (γ-(EC)nXaa, where n = 2-11. PCs are synthesized in the presence of heavy metals from glutathione (GSH) and related thiols by PC synthases (PCS), bind heavy metals with high affinity, and facilitate heavy metal sequestration into the vacuole, a lysosome-like compartment of plant and fungal cells (15, 16). It has been suggested that in plants, metal-PC complexes are transported into vacuoles by unidentified ABC transporter(s) (17). Based on in vitro transport assays, it has been proposed that in S. pombe, SpHMT-1 is a vacuolar membrane Cd·PC and/or apoPC transporter that functions downstream of PC formation in the PCS-dependent pathway (18). However, it remains to be determined directly if the transport of PCs is the mechanism by which SpHMT-1 alleviates Cd2+ toxicity in vivo. Indeed, several considerations indicate that the function of HMT-1 in metal detoxification is more complex than previously thought. First, studies of the HMT-1-like protein from C. elegans, CeHMT-1, yielded findings that suggest an alternate and/or auxiliary role for HMT-1 in heavy metal detoxification, which is not obligatorily dependent on the upstream synthesis of PCs (5). Second, the Chlamydomonas reinhardti SpHMT-1 homolog, CrCDS1, confers heavy metal tolerance, yet localizes to the mitochondrion, an organelle that does not directly participate in intracellular Cd·PC sequestration (9). Third, genes encoding HMT-1 homologs have not been detected in the genomes of vascular plants, which utilize the PC-dependent pathway (12, 13). Fourth, ABC transporters with an HMT-1-type domain organization have been identified in the genomes of organisms that do not have PCS genes. Examples are the fly, Drosophila melanogaster, HMT-1 (DmHMT-1 alias CG4225) and its counterparts in mammals, including human MTABC3 and mouse ABCB6 (5). Evidently, these HMT-1 proteins do not ordinarily transport metal-PC complexes and/or apoPCs because these substances they would never encounter in vivo.

To further our understanding of the role of HMT-1-type transporters, we have sought to determine whether the HMT-1 from Drosophila is involved in metal detoxification. If it is, this would imply that HMT-1 proteins from different species share a conserved role in heavy metal detoxification, but one that does not depend on the synthesis of PCs. The results presented here establish the need for revision of the role that HMT-1 proteins have been considered to play in the detoxification of heavy metals, invoke a specific requirement for this transporter in the detoxification of Cd2+, but not other heavy metals while at the same time explain why some of the organisms that engage in PC-dependent metal detoxification lack strict HMT-1 homologs.

Experimental Procedures

Yeast strains and growth conditions- The S. pombe strains used in these studies were the wild type strain YF016 (h+ leu 1-32, ura 4-C190T ade7::ura4) and its isogenic hmt-1Δ mutant (h+ leu 1-32, ura 4-C190T ade7::ura4; hmt-1::URA4) (19). The wild type strain Sp286 (h+/h+ ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32) and its isogenic pcs-1Δ mutant (h+/h+ ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 pcs-1::KanMX4). Cells were grown at 30°C in Edinburgh Minimal Medium (EMM) which in addition to leucine, adenine (225 mg/L each), 2% (w/v) dextrose and in the case of YF016
and Sp286 cells, uracil (225 mg/L), contained: 14.7 mM potassium hydrogen phthalate, 15.5 mM Na3HPO4, 93.5 mM NH4Cl, 0.26 M MgCl2·6H2O, 4.99 mM CaCl2·2H2O, 0.67 M KCl, 14.1 mM Na2SO4, 80.9 mM boric acid, 23.7 mM MnSO4, 13.9 mM ZnSO4·7H2O, 7.4 mM FeCl2·6H2O, 2.47 mM molybdate, 6.02 mM KI, 1.60 mM CuSO4·5H2O, 47.6 mM citric acid, 4.20 mM pantothenic acid, 81.2 mM nicotinic acid, 55.5 mM inositol and 40.8 µM biotin. S. pombe transformants were selected for leucine prototrophy in EMM. For the assessment of Cd2+ tolerance, the EMM growth media were supplemented with CdCl2 at the concentrations indicated.

Isolation and heterologous expression of dm-hmt-1- The cDNA corresponding to dm-hmt-1 was obtained from the Drosophila Genomics Resource Center (DGRC), Indiana University, Bloomington, IN. Primers for amplification of the ORF for dm-hmt-1 were designed to generate XhoI and NotI restriction sites at the 5’- and 3’-termini, respectively, of the dm-hmt-1 amplification product. The sequences of the two primers yielding the 2.6-kb dm-hmt-1 amplification product were 5’-CCGGTCGAGATGCTGTACTGCCCGC CCAACG-3’ and 5’-ATAGTTTAGCGGCCGCC TAGCGTGCTCCCCCA-3’. The resulting cDNA (GenBank accession number ACE60575) was subcloned into the XhoI and NotI restriction sites of the S. pombe-Escherichia coli shuttle vector, pTN197 (19) to place dm-hmt-1 under the control of a thiamine-repressible promoter of the nmt1 gene. The resulting pTN197-dm-hmt-1 construct, or pTN197 vector lacking the dm-hmt-1 insert were expressed in S. pombe hmt-1Δ cells. To permit direct comparisons with isogenic wild type YF016 cells grown under identical conditions, the pTN197 vector was expressed in YF016 cells.

Transformation of S. pombe- S. pombe cells were transformed using a standard lithium acetate procedure (20). Transformed cells were selected for leucine prototrophy in EMM medium as described above.

Isolation of intact vacuoles- For the isolation of intact vacuoles, YF016/pTN197, hmt-1Δ/pTN197, or hmt-1Δ/DmHMT-1 cells were subjected to cell wall digestion, disruption, and fractionation by differential centrifugation. Two hundred-milliliter volumes of stationary phase cultures were diluted into 1.5 L of EMM medium containing supplements and grown for 4-6 hours at 30°C to an OD600nm of approximately 0.6 after which time CdCl2 (500 µM) was added to the cultures to activate PC production. Thereafter the cells were cultured in the presence of CdCl2 for an additional 18 h at 30°C to an OD600 of approximately 1.2, collected by centrifugation, and used for the isolation of intact vacuoles by a modification of the procedure described in ref. 18. Briefly, the sedimented cells were washed in water and harvested by centrifugation at 3,000 x g for 5 min. After resuspension in 20 mM 2-mercaptoethanol and 100 mM Tris-HCl (pH 9.4), the cells were incubated for 20 min at 30°C with gentle shaking. The cells were then pelleted, resuspended in 100 ml of digestion medium (DM) containing 1.2 M sorbitol, 10 mM 2-mercaptoethanol, 20 mM potassium phosphate, pH 7.5, and converted to spheroplasts by the addition of 50 mg of Zymolyase 20T (ICN) and 100 mg of lysing enzymes from Trichoderma harzianum (Sigma-Aldrich, St. Louis, MO). The suspension was incubated for 2 h at 30°C with gentle shaking, and pelleted by centrifugation at 3,000 x g for 5 min. The spheroplasts were washed free of DM by resuspension in 50 ml of ice-cold homogenization medium (HM) consisting of 1.6 M sorbitol, 10 mM MES-Tris (pH 6.9), 0.5 mM MgCl2, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each of leupeptin, aprotenin, and pepstatin. The pelleted spheroplasts were lysed in the same medium (20 ml) by homogenization in a 50-ml glass Dounce homogenizer. The crude lysate was cleared of cell debris and unbroken cells by centrifugation at 4,000 x g at 4°C for 10 min. The pellet was resuspended in another 20 ml of homogenization medium, homogenized again, and recentrifuged. Partially-purified vacuoles (the P13,000 fraction) were collected by centrifugation of the supernatant at 13,000 x g at 4°C for 30 min, resuspended in HM, layered onto a Percoll step gradient (18%/30% v/v) prepared in HM) and pelleted at 68,320 x g at 4°C for 1 h. The resulting vacuolar pellet was resuspended in HM and layered on a cushion of 50% (v/v) of Percoll, prepared in HM and re-pelleted at 68,320 x g for 1 h. The pellet containing purified vacuoles was washed free of Percoll by three rounds of resuspension in suspension medium (SM) containing 1.6 M sorbitol, 100 mM KCl, 10 mM MES-Tris, pH 6.9, 5 mM MgCl2 and protease.
inhibitors, and centrifuged at 4°C at 13,000 rpm for 10 min in an Eppendorf microcentrifuge. The final vacuolar preparation was used immediately or stored at -80°C.

**Assessment of integrity of vacuole preparations** - The integrity of the vacuoles prepared in this way was assessed by testing their ability to retain the fluorescent glutathione S-conjugate of monochlorobimane (MCB), bimane-GS (6, 21). MCB is a membrane-permeant, nonfluorescent compound that is specifically conjugated with GSH by cytosolic glutathione S-transferases (GSTs) to generate the intensely fluorescent, membrane-impermeant product bimane-GS that is actively transported into and sequestered within the vacuole of intact cells.

Seventy-five milliliter volumes of stationary phase *S. pombe* cell cultures were diluted into 500 ml of EMM medium containing MCB (150 µM) and grown for 20 h at 30°C, after which time the cells were harvested and converted to spheroplasts for the purification of intact vacuoles as described above. Cells, spheroplasts and vacuoles from this source were examined without fixation by fluorescent microscopy.

**Enzyme assays** - The purity of the vacuolar fractions was evaluated by marker enzyme assays. α-mannosidase activity, a vacuolar membrane marker, was employed to enumerate enrichment of the partially purified (P13,000) and final vacuolar fractions. Cytochrome c oxidase and glucose-6-phosphate dehydrogenase activity were employed to assess contamination of these fractions with mitochondria and cytosolic components, respectively. α-mannosidase was determined using p-nitrophenyl-α-D-mannopyranoside as substrate (22). Glucose-6-phosphate dehydrogenase was assayed by measuring the rate of glucose-6-phosphate-dependent NADPH formation (22). The activity of cytochrome c oxidase activity was assayed using a colorimetric assay based on the decrease in absorbance of ferrocyanochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase (23).

**Measurement of PC content** - The PC contents of the isolated intact vacuole preparations were estimated by a combination of reverse-phase (RP)-HPLC and thiol quantitation after reaction with Ellman’s reagent (24). Aliquots of vacuoles (10-20 µg of protein) were made 5% (w/v) with 5-sulfosalicylic acid, protein was pelleted by centrifugation, and aliquots of the supernatant (50 µl) were loaded onto an Econosphere C18, 150 x 4.6-mm RP-HPLC column (Alltech). The column was developed with a linear gradient of water/0.05% (v/v) phosphoric acid, 17% (v/v) acetonitrile/0.05% (v/v) phosphoric acid at a flow rate of 1 ml/min. For the quantitation of PCs, thiols were estimated spectrophotometrically at 412 nm by reacting aliquots (500 µl) of the column fractions with 0.8 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (500 µl) dissolved in 250 mM phosphate buffer, pH 7.6 (25). Calibration was with GSH. Individual PC fractions were identified on the basis of their co-migration with PC standards synthesized in vitro by purified AtPCS1-FLAG (26) and by mass spectrometry as described below.

**Electrospray ionization mass spectrometry (ESI-MS) and tandem liquid chromatography matrix-assisted laser desorption ionization mass spectrometry (LC-MALDI-MS)** - Intact vacuoles were subjected to LC-MALDI-MS and ESI-MS analyses for the identification of PCs. The MALDI analysis utilized an LC Packings UltiMate nano-LC system. Mobile phase A consisted of 0.1% trifluoroacetic acid (TFA) in water and mobile phase B consisted of 0.1% (w/v) TFA in 80% acetonitrile (v/v). Injectins (6.4 µL) of the PC samples dissolved in 0.1% TFA were loaded for 5 minutes onto a trapping column (C18 PepMap100, 300 µm ID x 5 mm, 5 µm particle size, 100 Å pore size) in 20 µL/min mobile phase A. Thereafter, a linear 40 min gradient of 0-55% B was directed through the trap column onto an analytical column (C18 PepMap 100, 75 µm ID x 15 cm, 3 µm particle size) at a flow rate of 250 nL/min. The column effluent was directed to an LC Packings Proben fraction collector where it was mixed at a constant 705 nL/min flow rate with 7.5 mg/mL α-cyano-4-hydroxycinnamic acid and 20 fmol/µL [Glu]1-fibrinopeptide B dissolved in 2% (w/w) ammonium citrate. This mixture was spotted onto MALDI plates at 20 second collections/fraction. The MALDI plates were analyzed in an Applied Biosystems/MDX Sciex 4700 MALDI TOF/TOF Proteomics Analyzer operated in positive ion mode. An m/z range from 400 to 4000 was scanned for each fraction with internal calibration at an m/z of 1570.677 corresponding to the GluFib added to the matrix solution. PCs were detected by integrating all fractions for representative m/z values.
values to produce ion current chromatograms across each plate. PC2 in the vacuolar extracts was identified as an \( m/z \) 540.2 species (theoretical mean isotopic mass \([\text{M+H}]^+\) of PC2 (\(\gamma\)-Gly-Cys)\(_2\)Gly is 540.14).

The ESI analyses were performed using a Perkin Elmer 200 micro LC system. Mobile phase A consisted of 0.1% (v/v) formic acid in water and mobile phase B consisted of 0.1% formic acid in 90% (w/v) acetonitrile. After injection of a 10 µL sample, the column (Vydac C\(_18\) MassSpec, 1 mm ID x 150 mm, 5 µm particle size, 300 Å pore size) was developed with 100% A (0 to 2 min); a linear gradient to 60% B (2 to 30 min); and 60% B (30-35 min) at a flow rate of 100 µL/min. The column effluent was directed to an Applied Biosystems/MDX Sciex API 150 single quadrupole mass spectrometer with a turbo ion spray source. The instrument was operated in positive ion mode with the settings optimized for GSH by scanning in the \( m/z \) range 300-1600. PCs were detected by extracting the ion currents for their representative \( m/z \) values from the total ion current for each separation. The presence of PC3 and PC4 was inferred from \([\text{M+H}]^+\) \( m/z \) ratios of 772.2 and 1004.2 respectively (theoretical mean isotopic mass \([\text{M+H}]^+\) of PC3 (\(\gamma\)-Gly-Cys)\(_3\)Gly is 772.19, of PC4 (\(\gamma\)-Gly-Cys)\(_4\)Gly is 1004.2).

Analyses of cadmium content of isolated intact vacuoles by inductively-coupled plasma atomic emission spectroscopy (ICP-AES)- Aliquots of the intact vacuole fractions (70 µl) were placed in 20.0 ml quartz tubes and digested with 0.25 ml of a 50/50 mixture of concentrated nitric acid and perchloric acid at 120°C until dry before a second 0.25 ml of a 50/50 mixture of concentrated nitric acid and perchloric acid was added and heated at 220°C until dry. The ash was dissolved in 15.0 ml of 2% nitric acid and analyzed on an axially viewed ICP trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, Waltham Ma). To minimize matrix effects, short depth of field optics were employed (US Patent No. 6,122050).

Protein estimations- Protein was estimated by the dye-binding method (27).

Chemicals- All of the general reagents were obtained from Fisher, Research Organics, Inc., Invitrogen or Sigma-Aldrich.

RESULTS AND DISCUSSION

Identification and cloning of dm-hmt-1, dm-hmt-1 (Drosophila melanogaster heavy metal tolerance factor 1 (GenBank accession number ACE60575) encoding a 97.9 kDa polypeptide, DmHMT-1, was identified by systematic domain comparisons among the half-molecule ABC transporters in sequence databases by scanning for proteins with an HMT-1-specific organization: the presence of a single TMD, containing six transmembrane spans and an NBF, containing Walker A and B boxes (sequences GPSGAGKS and IVLLD respectively), separated by an ABC signature motif (sequence LSGGEKQRVAIARTL), and an NTE consisting of an approximately 200 residue TMD0 domain containing five hydrophilicity minima and an approximately 50 residue L0 domain. DmHMT-1, which shares 55% sequence similarity (37% sequence identity) to SpHMT-1 and 57% sequence similarity (44% sequence identity) to CeHMT-1, possesses a 244-amino-acid residue NTE, consisting of a 192-amino-acid TMD0 encompassing five hydrophilicity minima and a 54-amino-acid residue L0 domain, oriented in tandem with the TMD and NBF domains (Fig. 1).

The presence of the NTE distinguishes DmHMT-1 from its closest homologs, the ATMs (ABC transporters of the mitochondrion), which possess a mitochondrial-targeting signal peptide instead of an NTE domain, are implicated in iron homeostasis, and localize to the inner-mitochondrial membrane (28). Phylogenetic analysis of the sequences of representative HMT-1 and ATM subfamily members from yeast, Arabidopsis, C. elegans, Drosophila and mammals demonstrated that the HMTs form a common subcluster, distinct from that of the ATMs (Fig. 2). As is evident from Fig. 2, DmHMT-1, CeHMT-1 and MTABC3 group together within the HMT-1 subcluster, but are distinct from SpHMT-1, which might imply evolutionary and possibly functional divergence of the former three HMTs from SpHMT1.

The cDNA corresponding to the predicted ORF of dm-hmt-1 was isolated by PCR from a cDNA clone obtained from the Drosophila Genomics Resource Center (DGRC). After confirming the fidelity of the 2.6 kb amplification
product by sequencing, it was used for the experiments described below.

**Heterologously expressed DmHMT-1 partially suppresses the Cd\(^{2+}\)-hypersensitivity of S. pombe hmt-1Δ mutants.** All HMT-1-like proteins characterized to date have been isolated from organisms possessing PC synthase genes and have been shown to contribute to the alleviation of Cd\(^{2+}\) toxicity. Based on studies in *S. pombe*, they have been implicated in the vacuolysosomal sequestration of Cd-PC complexes. Since the *Drosophila* genome does not possess PC synthase homologs, the question of whether DmHMT-1 confers heavy metal tolerance was intriguing. If *dm-hmt-1* encodes a protein that is functionally equivalent to or has significant functional overlap with SpHMT-1, its heterologous expression in a Cd\(^{2+}\) hypersensitive *S. pombe hmt-1* mutant strain (hmt-1Δ) should alleviate the hypersensitive phenotype. To probe its functional capabilities, *dm-hmt-1* cDNA was subcloned into the *S. pombe-E. coli* shuttle vector pTN197 under control of the thiamine-repressible promoter of the *nmt1* gene, transformed into SpHMT-1-deficient (hmt-1Δ) *S. pombe* cells, and tested for its ability to suppress the Cd\(^{2+}\)-hypersensitive phenotype resulting from disruption of *hmt-1*.

In this way we established that heterologous expression of DmHMT-1 in *hmt-1Δ* cells (hmt-1Δ/DmHMT-1 cells) partially suppressed Cd\(^{2+}\) sensitivity regardless of whether growth was monitored as colony formation by serially diluted cell inocula after 8 days of growth on solid media (Fig. 3A), or as cell density after 24 h of growth in liquid media containing Cd\(^{2+}\) (Fig. 3B). As determined from the concentrations of Cd\(^{2+}\) required to inhibit growth by 50% in liquid media, heterologous expression of *dm-hmt-1* increased the Cd\(^{2+}\) tolerance of *hmt-1Δ* cells by at least 10-fold (Fig. 3B).

We then tested whether the increased Cd\(^{2+}\) tolerance of *hmt-1Δ/DmHMT-1* cells was specifically attributable to the heterologous expression of DrHMT-1. Since *dr-hmt-1* cDNA was placed under the control of the thiamine-repressible promoter of the pTN197 vector (see *Experimental Procedures*), this question could be readily examined by adding thiamine to the growth medium (29). It was thereby determined that the repression of *dm-hmt-1* expression by the addition of thiamine to the growth medium abolished the Cd\(^{2+}\) tolerance that was otherwise conferred by the pTN197-dm-hmt-1 construct (Fig. 3C). Evidently, the observed Cd\(^{2+}\) tolerance of *hmt-1Δ/DmHMT-1* cells is specifically attributable to the expression of *dm-hmt-1*.

**Heterologously expressed DmHMT-1 localizes to the vacuolar membrane of S. pombe.** SpHMT-1 resides on the vacuolar membrane of *S. pombe* (18). To test whether heterologously-expressed DmHMT-1 is also targeted to vacuolysosomal membranes in this system, the full-length coding sequence of *dm-hmt-1* was fused in-frame with the coding sequence for the red-shifted variant of GFP, EGFP, and cloned behind the *nmt1* promoter of the pTN197 vector. The resulting construct was transformed into *hmt-1Δ* cells. Fluorescence microscopy of *hmt-1Δ/DmHMT-1-EGFP* cells revealed that the intense green fluorescence associated with this fusion localized to the periphery of vesicular, vacuole-like structures (Fig. 4A). These vesicular structures also accumulated LysoTracker, a lipophilic, weakly basic red fluorescent dye that selectively accumulates in cellular compartments with low internal pH such as vacuoles and lysosomes (Fig. 4B). On the basis of superimposition of the fluorescence from the green fluor of DmHMT-1-EGFP and the red fluor of LysoTracker it was concluded that heterologously expressed DmHMT-1 is incorporated into the vacuolar membrane of *S. pombe*.

Vacuoles from Cd\(^{2+}\)-treated *hmt-1Δ* cells heterologously expressing DmHMT-1 accumulate less Cd-PCs in comparison with vacuoles from wild type cells. Given that heterologously expressed DmHMT-1 localizes to the vacuolar membrane as does SpHMT-1, the simplest explanation for its ability to suppress the Cd\(^{2+}\) hypersensitivity of *hmt-1Δ* cells is that, although DmHMT-1 is derived from an organism that does not produce PCs *in vivo*, it is be capable of transporting Cd-PC complexes in the heterologous system. To test this hypothesis we assayed the PC and Cd\(^{2+}\) contents of intact vacuoles isolated from Cd\(^{2+}\)-grown wild type YF016 cells transformed with the empty pTN197 vector (YF016/pTN197), *hmt-1Δ* cells expressing DmHMT-1 (*hmt-1Δ/DmHMT-1*), and *hmt-1Δ* transformed with the empty pTN197 vector (*hmt-1Δ/pTN197*). If the
hypothesis is correct and DmHMT-1 suppresses the Cd²⁺ hypersensitivity of \textit{hmt-1Δ} cells by sequestering Cd·PC complexes into vacuoles and if, as suggested previously, SpHMT-1 is the sole Cd·PC transporter on the vacuolar membrane of \textit{S. pombe}, then it would be expected that: 1) vacuoles from Cd²⁺-treated wild type \textit{YF016/pTN197} cells would contain Cd²⁺ and PCs; 2) vacuoles from \textit{hmt-1Δ/pTN197} cells would lack detectable PCs and since PCs are involved in Cd²⁺ sequestration, would accumulate less Cd²⁺; 3) heterologously-expressed DmHMT-1 would restore the accumulation of PCs (and Cd²⁺) in vacuoles of \textit{hmt-1Δ} cells.

Vacuoles were prepared from \textit{S. pombe} spheroplasts as described in the “Experimental Procedures”. To assess the integrity of these vacuole preparations, cells of \textit{S. pombe} were cultured in the presence of MCB, which resulted in vacuolar accumulation of its fluorescent derivative, bimane-GS (Fig. 5A). As would be expected if a sizeable fraction of the isolated vacuoles maintained their integrity throughout the purification procedure, the bimane-GS-associated fluorescence was retained by the vacuoles from this source (Fig. 5B). Since bimane-GS, a relatively low molecular weight substituted tripeptide, was retained by the vacuole fraction, it was reasoned that PCs would be also substantially retained in vacuoles isolated in this way.

The purity of the vacuole preparations was assessed by assaying for enrichment of the activity of the vacuolar membrane marker enzyme, α-mannosidase, and decreases in the activities of the mitochondrial membrane and cytosolic marker enzymes, cytochrome c oxidase and glucose-6 phosphate dehydrogenase, respectively (Table 1). An at least a 2.5-fold enrichment in the activity of α-mannosidase in purified vacuoles in comparison with partially purified preparations (P13,000 fraction, see “Experimental Procedures”) was determined, while the activities of the mitochondrial and cytosolic markers were substantially decreased. On the basis of these results and the retention of bimane-GS, it was concluded that this purification protocol yielded intact vacuoles without significant contamination with other cellular components.

Reverse-phase (RP)-HPLC analysis of the non-protein thiol content of intact vacuoles isolated from Cd²⁺-treated \textit{YF016/pTN197} cells not only revealed a prominent peak corresponding to the 2-mercaptoethanol that was carried over from the vacuolar preparation medium, but also several other peaks that eluted later (Fig. 6A). The chromatographic properties of the peaks that eluted later were indistinguishable from those of PC₂, PC₃, and PC₄ that had been synthesized \textit{in vitro} by purified AtPCS1-FLAG (26). The aggregate thiol content of these PC-related thiol peptides was 396 ± 35.0 nmol/mg protein (Table 2). The corresponding fractions from vacuoles of cells grown in media lacking Cd²⁺ were devoid of PC-like nonprotein thiols (Fig. 6D), which was expected since exposure to heavy metal is an essential prerequisite for net PC synthesis from GSH (15, 24).

Parallel inductively coupled plasma atomic emission spectroscopy (ICP-AES) analyses demonstrated that vacuoles from Cd²⁺-cultured \textit{YF016/pTN197} cells accumulate 1050.5 ± 120 nmol Cd²⁺/mg of proteins (Table 2). It is notable that the Cd²⁺ content of the vacuoles from \textit{YF016/pTN197} cells exceeds their PC content by about four-fold. The reason for this is not known, but it is possible that other carriers (e.g., Cd²⁺/H⁺ antiport and/or the full-molecule ABC transporter, SpYCF1, a homolog of \textit{S. cerevisiae} YCF1 [yeast cadmium factor 1] (6, 18)) contribute to PC-independent vacuolar Cd²⁺ accumulation, albeit to an extent insufficient to override the hypersensitivity of \textit{hmt-1Δ} cells (Fig. 3, 7).

Vacuoles from Cd²⁺-treated \textit{hmt-1Δ/DmHMT-1} cells also accumulated both Cd²⁺ and PCs (Table 2 and Fig. 6B), although the amounts of PCs and Cd²⁺ estimated were 1.6- and 1.8-fold lower by comparison to the equivalent vacuolar fractions purified from \textit{YF016/pTN197} cells.

To determine if the accumulation of PCs in vacuoles from Cd²⁺-treated \textit{hmt-1Δ/DmHMT-1} cells is due to the activity of DmHMT-1, the PC contents of vacuoles from Cd²⁺-treated \textit{hmt-1Δ/pTN197} cells were determined. It was at this point that an unprecedented finding was made.

\textit{SpHMT-1 is not a bona fide Cd-PC transporter}. If SpHMT-1 is the sole Cd-PC and/or apoPC transporter, vacuoles from CdCl₂-grown \textit{hmt-1Δ/pTN197} cells should lack detectable PCs and accumulate less Cd²⁺. However, RP-HPLC analysis of the non-protein thiol compounds in vacuoles from \textit{hmt-1Δ/pTN197} cells after growth in media containing CdCl₂ disclosed peaks eluting at...
the same positions as in vitro synthesized PC₂, PC₃ and PC₄ standards (Fig. 6C). Consistent with the Cd²⁺-dependent formation of PCs from GSH, the corresponding fractions from vacuoles of hmt-1Δ/pTN197 cells cultured in media lacking Cd²⁺ did not contain non-protein thiols other than those associated with the 2-mercaptoethanol peak (data not shown). ESI-MS and LC-MALDI analysis confirmed the identity of the putative PC peaks in vacuoles from Cd²⁺-grown hmt-1Δ/pTN197 cells as PC₂, PC₃ and PC₄ (Fig. 6E, F, G).

Since finding PCs in vacuoles from hmt-1Δ/pTN197 cells was unexpected, these experiments were repeated using another hmt-1Δ S. pombe strain, LK100 (18). Regardless of which S. pombe hmt-1-mutant strain was used for the analyses of vacuolar PC content, LK100 or hmt-1Δ/pTN197, PCs were found in Cd²⁺-treated hmt-1Δ deficient cells (data for LK100 not shown).

As an additional control, the PC contents of vacuoles from Cd²⁺-treated pcs-1Δ S. pombe cells were examined. These cells lack a functional PC synthase gene and are deficient in PC synthesis (30). These experiments were performed because it has been suggested that in S. cerevisiae PCs are produced in vacuoles by carboxypeptidase C, in a PC synthase-independent manner. As would be expected if vacuolar PC accumulation depends on the sequestration from the cytosol, where their synthesis is mediated by S. pombe PC synthase (SpPCS-1, (30)), thiol-containing peaks in vacuoles of Cd²⁺-grown pcs-1Δ cells at the positions corresponding to PC₂, PC₃ and PC₄ standards were all below the limit of detection (not shown). In contrast, vacuoles from Cd²⁺-grown isogenic wild type Sp286 cells, contained PCs at the aggregate level of 454 ± 54 nmol/mg of protein. These data indicate that the PCs in vacuoles of hmt-1Δ cells accumulate in a PCS-dependent manner and must be transported into this compartment by an unidentified transporter.

The finding that PCs accumulate in vacuoles of hmt-1Δ mutants of S. pombe implied that SpHMT-1 is not the primary vacuolar Cd·PC and/or apoPC transporter. A different and/or additional Cd·PC transport activity, whose identity has yet to be determined, must be present on the vacuolar membrane of S. pombe.

SpHMT-1, but not DmHMT-1, might contribute to the transport of short-chain, Cd·PC₂ and/or apoPC₂, complexes. Against this background, it should nevertheless be noted that the chromatographic profiles of the PCs of vacuoles from hmt-1Δ/pTN197 and YF016/pTN197 cells are readily distinguishable (Figs. 6A, C). Although vacuoles of Cd²⁺-treated hmt-1Δ/pTN197 cells accumulated the short-chain phytochelatin, PC₂, they did so at a level of only 72.8 ± 14.1 nmol/mg protein, which was 3-fold lower than that achieved by the equivalent organelle fraction from wild-type cells (Table 2). By contrast, the aggregate vacuolar content of the longer-chain PCs, PC₃ and PC₄, was similar in the two cell lines (Table 2). In some cases, an increase in PC₃ accumulation in CdCl₂-treated hmt-1Δ/pTN197 cells in comparison with wild type was observed (Table 2), but these differences were not statistically significant.

The apparent decrease in the PC₂ levels of vacuoles from hmt-1Δ/pTN197 cells was not attributable to thiol oxidation. Regardless of whether the vacuolar extracts were subjected to reduction with sodium borohydride (NaBH₄) or not, the PC₂ contents of those from Cd²⁺-treated hmt-1Δ/pTN197 cells were lower than those from Cd²⁺-treated wild type cells (data not shown). These findings imply that although another Cd·PC₂-specific transport activity is present on the vacuolar membrane of S. pombe, and is responsible for accumulation of this PC in the vacuoles of hmt-1Δ/pTN197 cells, SpHMT-1 might still contribute to the vacuolar sequestration of PC₂, despite its inability to mediate the accumulation of longer-chain PCs.

Consistent with the contention that PCs facilitate the vacuolar sequestration of Cd²⁺ (15, 31), the decrease in PC₂ accumulation in vacuoles from Cd²⁺-treated hmt-1Δ/pTN197 cells is accompanied with a 2.1-fold decrease in the accumulation of Cd²⁺ (Table 2).

Heterologous expression of DmHMT-1 in hmt-1Δ cells does not restore vacuolar PC₂ or Cd²⁺ accumulation (Fig 6A, B; Table 2). On this basis it was concluded that DmHMT-1 does not transport Cd²⁺ and/or Cd·PC₂ and apoPC₂ complexes. Instead, it must alleviate the Cd²⁺ hypersensitivity of hmt-1Δ cells by another mechanism. In this regard, the content of PC₂ and of total PCs are lower in vacuoles of both hmt-1Δ/pTN197 and hmt-1Δ/DmHMT-1 cells (Table 2). However, hmt-
ΔDmHMT-1 cells tolerate Cd^2+ in culture medium, whereas, hmt-1Δ/pTN197 cells are acutely Cd^2+ hypersensitive (Fig. 3). This observation implies that the acute Cd^2+ hypersensitivity of hmt-1Δ/pTN197 cells does not result from a decrease in their ability to vacuolarly sequester Cd^2+ and PCs. Apparently, an alternative and/or auxiliary PC-independent mechanism is deployed when HMT-1 confers Cd^2+ tolerance.

HMT-1 and PCS-1 confer tolerance to different heavy metals. PC synthase has been reported to confer tolerance to a wide range of heavy metals and metalloids, including Hg^2+ and As^3+ as well as Cd^2+ (26, 30, 32, 33). If the HMT-1 and PCS-1 pathways overlap, it would be expected that HMT-1 confers tolerance to the same range of heavy metals. However, this is not what is seen. Analysis of the growth of serially diluted hmt-1Δ/pTN197; hmt-1Δ/pTN197-DmHMT-1; PC synthase-deficient (pcs-1Δ), and isogenic wild type Sp286 S. pombe cell-inocula on solid media with or without Cd^2+, Hg^2+ or As^3+ revealed that, as expected, pcs-1Δ, cells were exquisitely sensitive to all three metals. In striking contrast, however, while hmt-1Δ cells were sensitive to Cd^2+, they were not sensitive to Hg^2+ or As^3+ (Fig. 7). These findings complement the biochemical studies described above and re-enforce the notion that PC synthases and HMT-1 proteins do not operate in a simple linear metal detoxification pathway. These observations are consistent with our previous genetic studies of hmt-1 and pcs-1 in C. elegans, suggesting that these genes do not act in the same pathway for heavy metal detoxification (5). The finding that of the metals and metalloids tested, hmt-1Δ cells are sensitive only to Cd^2+, but not to Hg^2+ or As^3+, indicates that Cd^2+ exerts its effects in ways that can only be remedied by HMT-1.

**CONCLUDING REMARKS**

The results presented necessitate revision of our understanding of the roles played by HMT-1 proteins in metal detoxification. First, we have demonstrated for the first time that an HMT-1 from an animal that lacks the machinery for PC synthesis contributes to Cd^2+ detoxification. Because of the high degree of functional conservation among HMT-1 proteins tested thus far, it is likely that this newly-identified pathway mediates Cd^2+ detoxification in other animals, possibly humans. Second, despite what was previously thought about their function, at least HMT-1 proteins of S. pombe and Drosophila are not the primary heavy metal-PC and/or apoPC transporters. This would explain why HMT-1-homology-based searches for Cd·PC transporters in plants that utilize the PC-dependent pathway for heavy metal detoxification, have consistently failed. Third, the discovery that of the heavy metals and metalloids tested, S. pombe hmt-1Δ cells are hypersensitive only to Cd^2+, but not to the other heavy metals screened (Hg^2+ and As^3+), indicates a specific role for HMT-1 proteins in the detoxification of Cd^2+ and/or the products of its action.

**REFERENCES**


**FOOTNOTES**

*We thank Dr. Takegawa, Kagawa University, Japan for providing *S. pombe* mutant strains and *S. pombe* expression vectors, Dr. Julian Schroeder, University of California, San Diego for providing the *S. pombe* *pcs-1* mutant strain, Dr. Daniel Buckley, Cornell University, for use of his Zeiss Axioscope 2 Plus Microscope. We thank Drs. Elizabeth Bucher Emerson and Beth Ahner for reading and advising on the manuscript. This work has been funded by start-up funds from Cornell’s College of Agriculture and Life Sciences Start-up funds awarded to O.K.V. O.K.V. initiated this project when she was in Dr. Rea’s laboratory and was partially supported by United States Department of Energy (Energy Biosciences) Grant DE-FG02- 91ER20055 awarded to P.A.R.

†These authors contributed equally to this work

The abbreviations used are: ABC transporters, ATP-binding cassette transporters; HMT-1, heavy metal tolerance factor 1; DmHMT-1, *Drosophila melanogaster* heavy metal tolerance factor 1; CeHMT-1, *Caenorhabditis elegans* heavy metal tolerance factor 1, SpHMT-1, *Schizosaccharomyces pombe* heavy metal tolerance factor 1; PC, phytochelatin; GSH, glutathione; SpPCS-1, *Schizosaccharomyces pombe* phytochelatin synthase 1; TMD, transmembrane domain; NBD, nucleotide-binding domain; NTE,
hydrophobic N-terminal extension; 2-mercaptoethanol, 2-ME; ESI-MS, electrospray ionization mass spectrometry; LC-MALDI-MS, tandem liquid chromatography matrix-assisted laser desorption ionization mass spectrometry; ICP-AES, inductively coupled plasma-atomic emission spectrometry; monochlorobimane, MCB; bimane-S-glutathione, bimane-GS.

FIGURE LEGENDS

**Fig. 1.** Domain organization (A) and hydropathy plot (B) of DmHMT-1 polypeptide. The different domains are color-coded as follows: black, transmembrane domains (TMD0 of the N-terminal extension [NTE] and TMD1), white, nucleotide-binding domain (NBD1); light gray, linker domain (L0) of the NTE. Hydropathy was computed and putative transmembrane spans were predicted according to Kyte and Doolittle (35) over a running window of 19 amino acid residues. The hydrophobic N-terminal extension in the hydropathy plot is shown in light gray.

**Fig. 2.** Phylogenetic analysis of the HMT-1 and ATM subfamily sequences from *S. pombe*, *C. reinhardtii*, *Arabidopsis*, *C. elegans*, and mammals. The full amino acid sequences were aligned using ClustalX (version 1.83) and subjected to phylogenetic analysis by the distance with neighbor-joining method using the phylogenetic analysis program PAUP (version 4.0). Gaps were treated as missing and ZK484.2 (AAK39394), a half molecule ABC transporter from *C. elegans* that is not involved in Cd$^{2+}$ detoxification ((5)), was used as an outgroup. The bootstrap percentages for 1000 replicates are shown at each branch point. Branch lengths are proportional to phylogenetic distance. The accession numbers for the HMT-1 and ATM family members used for this analysis (accession numbers in parenthesis) are as follows: DmHMT-1 (ACE60575), *S. pombe*, SpHMT-1 (Q02592), *C. elegans*, CeHMT-1 (AAM33381), *C. reinhardtii*, CrCDS1 (AAQ19847), *Homo sapiens*, HsMTABC3 (AB039371), *S. cerevisiae*, ScATM1 (X82612); *S. pombe*, SpATM1 (NP_594288); *C. elegans*, ABTM-1 alias Y74C10AR.3 (ABA00166); *Drosophila*, CG7955-PA (AAF47525); *Arabidopsis*, AtATM1 (At4g28630), AtATM2 (At4g28620), AtATM3 (At5g58270); *Homo sapiens*, HsABC7 (AF133659).

**Fig. 3.** Suppression of Cd$^{2+}$-hypersensitivity of *hmt-1*Δ cells of *S. pombe* by heterologously expressed DmHMT-1. **A.** Wild-type YF016 *S. pombe* cells transformed with empty pTN197 vector (*YF016/pTN197*), *hmt-1*Δ mutant cells transformed with empty vector (*hmt-1*Δ/pTN197), and *hmt-1*Δ cells transformed with pTN197-DmHMT-1 (*hmt-1*Δ/DmHMT-1) were grown overnight to an OD$_{600}$ of 1.7. Aliquots of the cell suspensions were then serially diluted by 0-, 2-, 5-, 10-, or 20-fold and spotted onto solid EMM supplemented with glucose, adenine, and the indicated concentrations of CdCl$_2$. Colonies were visualized after incubating the plates for 8 days at 30°C. **B, C.** *S. pombe* wild-type (*YF016/pTN197, ○*) and *hmt-1*Δ cells transformed with empty pTN197 vector (*hmt-1*Δ/pTN197, •), and *hmt-1*Δ cells transformed with pTN197 containing the *dm-hmt-1* insert (*hmt-1*Δ/DmHMT-1, □) were grown in liquid EMM. Aliquots (200 µl) from standard overnight cultures were inoculated into 2 ml of the same medium, with or without CdCl$_2$ at the concentrations indicated. To control expression of *dm-hmt-1* from the thiamine-repressible *nmt1* promoter of the pTN197 vector, thiamine (5 µg/ml) was either omitted (B) or added (C) to the medium. OD$_{600}$ was measured after growth at 30°C for 24 h.

**Fig. 4.** Subcellular localization of heterologously expressed *dm-hmt-1::egfp* in *S. pombe*. **A.** Nomarski (DIC), fluorescence (GFP) or superimposed (Merge) photomicrographs of *S. pombe* *hmt-1*Δ cells transformed with pTN197-*dm-hmt-1::egfp*. **B.** Nomarski (DIC), fluorescence (GFP) and superimposed (Merge) photomicrographs of *S. pombe* *hmt-1*Δ cells transformed with pTN197-*dm-hmt-1::egfp* after staining the vacuoles with LysoTracker (*Lyso*). Cells were examined at a magnification of 100 x. The Nomarski images, GFP and LysoTracker fluorescence images were captured using a Zeiss Axioskope 2 Plus, equipped with GFP- and rhodamine-specific filter sets.
Fig. 5. Photomicrographs of wild type *S. pombe* cells (A) and intact vacuoles (B) isolated from *S. pombe* cells after incubation with monochlorobimane (MCB). Cells and intact vacuoles were examined in bright field (Bf) and fluorescence (bimane-GS) modes. Log-phase cells and intact vacuoles were examined at a magnification of 100 x using a Zeiss Axioskop 2 microscope equipped with the appropriate filter sets.

Fig. 6. Reverse-phase (RP)-HPLC (A-D) and mass spectrometry (E-G) analyses of the nonprotein thiols in vacuoles from *S. pombe*. A. RP-HPLC profiles of PCs from vacuoles isolated from wild-type YF016 cells expressing the empty pTN197 vector (YF017/pTN197) after growth for 18 h in media supplemented with 500 µM CdCl₂. The peaks designated PC₂, PC₃ and PC₄ were identified on the basis of their co-migration with PC₂, PC₃ and PC₄ standards synthesized in vitro using purified AtPCS1-FLAG (26). The 2-mercaptoethanol (2ME) was carried over from the media used to fractionate the vacuoles. B. RP-HPLC profiles of PCs from vacuoles isolated from hmt-1Δ cells transformed with pTN197-DmHMT-1 after growth for 18 h in media supplemented with 500 µM CdCl₂. C. RP-HPLC profiles of PCs from vacuoles of hmt-1Δ cells transformed with empty TN197 vector after growth for 18 h in media supplemented with 500 µM CdCl₂. D. RP-HPLC analysis of vacuoles from YF016/pTN197 cells, after growth in media lacking CdCl₂. E-G. Mass spectra of PCs in vacuoles from Cd²⁺-grown hmt-1Δ/pTN197 cells. The peaks designated PC₂, PC₃ and PC₄ were identified on the basis of their mass/charge (m/z) ratios (see Experimental Procedures).

Fig. 7. hmt-1Δ and pcs-1Δ cells of *S. pombe* confer tolerance to different heavy metals. Wild-type YF016 *S. pombe* cells transformed with the empty pTN197 vector (YF016/pTN197), hmt-1Δ mutant cells transformed with empty vector (hmt-1Δ/pTN197), hmt-1Δ cells transformed with pTN197-DmHMT-1 (hmt-1Δ/DmHMT-1), and PC synthase-deficient (pcs-1Δ), and isogenic wild type Sp286 (PCS-1) *S. pombe* cells were grown overnight to an OD₆₀₀ nm of 1.7. Aliquots of the cell suspensions were then serially diluted by 0, 2-, 5-, 10-, or 20-fold and spotted onto solid EMM supplemented with glucose, adenine, and the indicated concentrations of CdCl₂, HgCl₂ or As₂O₃. Colonies were visualized after incubating the plates for 4 days at 30°C.
Table 1. Comparison of the specific activities of marker enzymes in partially purified and purified intact vacuoles from *S. pombe*. Intact vacuoles were isolated by differential centrifugation in Percoll gradients as described in Experimental Procedures. The purity of the vacuolar preparations was assessed by assaying for enrichment of the vacuolar membrane marker enzyme, α-mannosidase, and for decreases in the activities of mitochondrial membrane marker enzyme, cytochrome c oxidase, and the cytosolic marker enzyme glucose-6 phosphate dehydrogenase. Partially purified vacuoles correspond to the pellet after centrifugation at 13,000 x g; purified vacuoles correspond to the fraction obtained at the end of the purification procedure.

<table>
<thead>
<tr>
<th>Marker enzyme</th>
<th>Partially-purified vacuoles</th>
<th>Purified vacuoles</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c-oxidase</td>
<td>156.1 ± 38.3</td>
<td>16.3 ± 5.6</td>
<td>0.1</td>
</tr>
<tr>
<td>α-mannosidase</td>
<td>278.6 ± 60.7</td>
<td>651.6 ± 108.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>102.1 ± 18.7</td>
<td>1.5 ± 0.1</td>
<td>0.01</td>
</tr>
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</table>
Table 2. The aggregate content of PCs of different chain lengths, total PCs and Cd\(^{2+}\) in vacuoles from CdCl\(_2\)-grown, wild-type YF016 S. pombe cells transformed with empty pTN197 vector (YF016/pTN197), hmt-1\(\Delta\) mutant cells transformed with empty vector (hmt-1\(\Delta\)/pTN197), and hmt-1\(\Delta\) cells transformed with pTN197-DmHMT-1 (hmt-1\(\Delta\)/DmHMT-1). Vacuoles were prepared from cells cultured for 18h in growth medium supplemented with 500 µM CdCl\(_2\). PC\(_2\), PC\(_3\) and PC\(_4\) were separated and quantitated in aliquots (20 µg) of the vacuole preparations by RP-HPLC. The cadmium content of the vacuoles was estimated by ICP-AES.

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Individual PCs (nmol thiols equivalents/mg proteins)</th>
<th>Total PCs (nmol/mg protein)</th>
<th>Cadmium (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YF016/pTN197</td>
<td>hmt-1(\Delta)/pTN197</td>
<td>hmt-1(\Delta)/DmHMT-1</td>
</tr>
<tr>
<td>PC(_2)</td>
<td>241.7 ± 38.2</td>
<td>72.8 ± 14.1</td>
<td>93.1 ± 21.1</td>
</tr>
<tr>
<td>PC(_3)</td>
<td>127.8 ± 7.1</td>
<td>140.7 ± 35.3</td>
<td>132.3 ± 37.1</td>
</tr>
<tr>
<td>PC(_4)</td>
<td>45.5 ± 27.4</td>
<td>32.2 ± 9.1</td>
<td>33.3 ± 10.4</td>
</tr>
<tr>
<td>Total PCs</td>
<td>396.9 ± 35.1</td>
<td>232.8 ± 50.9</td>
<td>242.1 ± 66.6</td>
</tr>
<tr>
<td>Cadmium</td>
<td>1050.5 ± 120</td>
<td>495.7 ± 32.4</td>
<td>581.3 ± 122.9</td>
</tr>
</tbody>
</table>
Figure 1

A

\[ \text{NTE} \]

NH\textsubscript{2} \quad \text{TMD0} \quad \text{L0} \quad \text{TMD1} \quad \text{NBD1} \quad \text{COOH} 

B

![Graph showing amino acid residue](http://www.jbc.org/)

**Amino acid residue**

0 100 200 300 400 500 600 700 800 900
Figure 2
Figure 3

A

B

C

0 µM CdCl₂
25 µM CdCl₂
50 µM CdCl₂
100 µM CdCl₂

[YF016/pTN197]
[hmt-1Δ/pTN197]
[hmt-1Δ/DmHMT-1]

OD₆₀₀nm vs. [CdCl₂] (µM)

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4

0
50
100
150
200

[ CdCl₂ ] (µM)

100 µM CdCl₂

[YF016/pTN197]
[hmt-1Δ/DmHMT-1]
[hmt-1Δ/pTN197]
Figure 4
Figure 6
Figure 7

- YF016/pTN197
- hmt-1Δ/pTN197
- hmt-1Δ/DmHMT-1
- PCS-1
- pcs-1Δ

Control
CdCl₂ (10 µM)

HgCl₂ (12.5 µM)
As₂O₃ (50 µM)
Drosophila abc transporter, DmHMT-1, confers tolerance to cadmium. DmHMT-1 and its yeast homolog, SpHMT-1, are not essential for vacuolar phytochelatin sequestration

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