A new pathway for heavy metal detoxification in animals: phytochelatin synthase is required for cadmium tolerance in Caenorhabditis elegans

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Abbreviations: AtPCS1, Arabidopsis thaliana phytochelatin synthase 1; CePCS1, Caenorhabditis elegans phytochelatin synthase 1; GSH, glutathione; MT, metallothionein; NGM, nematode growth medium; PCn, phytochelatin containing n γ-Glu-Cys repeats; PCS, phytochelatin synthase; HPLC, high pressure liquid chromatography
ABSTRACT
Increasing emissions of heavy metals such as cadmium, mercury and arsenic into the environment pose an acute problem for all organisms. Considerations of the biochemical basis of heavy metal detoxification in animals have focused exclusively on two classes of peptides: the thiol tripeptide glutathione (GSH, γ-Glu-Cys-Gly), and a diverse family of cysteine-rich low molecular weight proteins the metallothioneins (MTs). Plants and some fungi, however, not only deploy GSH and MTs for metal detoxification but also synthesize another class of heavy metal-binding peptides termed phytochelatins (PCs) from GSH. Here we show that PC-mediated heavy metal detoxification is not restricted to plants and some fungi but extends to animals by demonstrating that the *ce-pcs-1* gene of the nematode worm *Caenorhabditis elegans* encodes a functional PC synthase whose activity is critical for heavy metal tolerance in the intact organism.
INTRODUCTION

Plants and some fungi posttranslationally synthesize novel peptides termed phytochelatins (PCs) when exposed to heavy metals. Fabricated from the ubiquitous thiol tripeptide glutathione (GSH) and related thiols in a novel transpeptidation reaction catalyzed by PC synthases (γ-glutamylcysteine dipeptidyl transpeptidases; EC 2.3.2.15), PCs have the general structure \((\gamma\text{-Glu-Cys})_n\text{-Xaa}\), contain 2-11 \(\gamma\text{-Glu-Cys}\) repeats, chelate heavy metals at high affinity, and facilitate the vacuolar sequestration of heavy metals, most notably \(\text{Cd}^{2+}\) (1-3). Although it is more than a decade since the first report of the partial purification of a heavy metal-, primarily \(\text{Cd}^{2+}\)-, activated PC synthase from plant extracts (1), it is only recently that the small family of genes encoding these enzymes has been identified in plants and the fission yeast \(\text{Schizosaccharomyces pombe}\) (4-6). As exemplified by the clone from \(\text{Arabidopsis thaliana}\) (AtPCS1), these genes encode 45-55 kDa proteins that are sufficient for heavy metal-activated PC synthesis from GSH both \(\textit{in vivo}\) and \(\textit{in vitro}\) (6,7).

An unexpected outcome of the cloning of AtPCS1 and its equivalents from other plants and \(\text{S. pombe}\) was the identification of a single-copy gene homolog (accession number Z66513) in the nematode worm \(\text{Caenorhabditis elegans}\) (4-6). Designated \textit{ce-pcs-1}, this gene encodes a hypothetical 40.8 kDa protein (CePCS1) bearing 32% identity (45% similarity) to AtPCS1 in an overlap of 367 amino acid residues (6). Disclosure of a PCS1 homolog in the genome of \(\text{C. elegans}\) was surprising in that it raised for the first time the possibility that not only GSH and metallothioneins (8) but also PCs might participate in metal homeostasis in at least some animals.

In the report that follows we demonstrate unequivocally that \textit{ce-pcs-1} encodes a bona fide PC synthase whose activity is necessary for the detoxification of heavy metals in the intact
organism. Discovery of the PC synthase-dependent pathway in the model organism C. elegans establishes a firm basis for determining the ubiquity of this pathway in other animals and for elucidation of the identity and organization of the cellular machinery likely involved in the eventual elimination, sequestration and/or metabolism of heavy metal-PC complexes in animals.

MATERIALS AND METHODS

Isolation and heterologous expression of ce-pcs-1. ce-pcs-1 was isolated from C. elegans N2 total RNA by RT-PCR(9) and the resulting cDNA (accession numbers AF299332, AF299333) was subcloned into yeast-Escherichia coli shuttle vector pYES3 (10) to place ce-pcs-1 under control of the constitutive yeast PGK gene promoter. For immunodetection of the translation product, vector pYES3-CePCS1::FLAG, containing the ce-pcs-1 insert engineered to code CePCS1 C-terminally fused with a FLAG (DYDDDDK) epitope tag, was constructed. Both constructs or empty vector pYES3 were used to transform (11) S. cerevisiae ycf1Δ strain DTY167 (MATα ura3-52 leu2-3, -112 his-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 ycf1::hisG), a Cd²⁺-hypersensitive mutant deficient in the YCF1-mediated vacuolar sequestration of Cd.glutathione complexes (12,13). The pYES3-CePCS1, pYES3-CePCS1::FLAG and empty vector transformants were selected for uracil prototrophy by plating on AHC medium supplemented with glucose (2% w/v) and tryptophan (50 µg/ml). pYES3-AtPCS1::FLAG-transformed DTY167 cells were generated as described (6).

Preparation of S. cerevisiae cell extracts. For investigations of their PC content and PC synthase activities, aliquots of stationary phase cultures of CePCS1::FLAG-, AtPCS1::FLAG- or empty vector-transformed DTY167 cells were inoculated into 40 ml volumes of AHC medium with or without added CdCl₂ (25 µM), grown for 16-18 h at 30°C to an OD₆₀₀ of 0.8-1.0 and harvested by centrifugation. After resuspension and disruption of the cells by the glass bead
method (14) in homogenization buffer (10 mM Tris-HCl, 10% (w/v) glycerol, 10 mM 2-mercaptoethanol, pH 7.6) containing 1 mM phenylmethyl-sulfonylfluoride and 1 µg/ml each of leupeptin, pepstatin and aprotinin, the homogenates were cleared by centrifugation at 10,000 g for 20 min and assayed immediately for PCs and PC synthase activity.

Measurement of PCs and PC synthase activity. The cellular PC content and PC synthase activities of the cell-free extracts from yeast CePCS1::FLAG-, AtPCS1::FLAG- and empty vector transformants were estimated as described (7) by a combination of reverse-phase HPLC and thiol quantitation with Ellman’s reagent. Individual PC fractions were identified by estimating their Glu/Gly ratios (ratio = n = number of Glu-Cys repeats per Gly) after acid hydrolysis and amino acid analysis of the appropriate HPLC fractions and/or on the basis of their comigration with PC standards synthesized \textit{in vitro} by purified AtPCS1-FLAG (7).

SDS-PAGE and immunoblot analyses. Protein samples were prepared for and subjected to SDS-PAGE for immunoblot analysis against anti-FLAG M2 antibody (Sigma) as described (6).

dsRNA synthesis. For \textit{in vitro} ce-pcs-1 RNA synthesis, the cDNA insert of pYES3-CePCS1 was subcloned into pBluescript and RNA was transcribed from the T3 and T7 promoters using an \textit{in vitro} RNA transcription kit (Stratagene). After DNase digestion of the template, the sense and antisense preparations were gel-purified, mixed in an equimolar ratio in 1 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, heated for 1 min in a boiling water and annealed at room temperature (15). The single electrophoretic species corresponding in size to that expected of dsce-pcs-1 RNA was purified by phenol/chloroform extraction, dissolved in water, diluted to a concentration of 500 ng/µl with injection buffer (16) and stored at –80°C.

dsRNA microinjection and analysis of phenotypes. The \textit{ce-pcs-1} RNA interference experiments were performed on wild type (N2) worms. The body cavities of adult
hermaphrodites were injected as described (16). Injected worms were cultured for 12-18 h on standard NGM plates seeded with *E. coli* OP50 before transfer onto plates supplemented with CdCl$_2$. Two treatment regimes were used to examine the effects of Cd$^{2+}$. In the first regime, individual worms were transferred sequentially onto NGM plates supplemented with 0, 25, 50 and 100 µM CdCl$_2$. After transfer onto each Cd$^{2+}$ concentration, egg laying was allowed to proceed for 6 h, after which time the worms were transferred to the next higher Cd$^{2+}$ concentration for another round of egg laying. In this way, it was determined that 25 µM CdCl$_2$ was sufficient to cause severe morphological and developmental changes in the CePCS1-deficient worms but not the controls. In the second regime, individual injected worms were transferred to NGM plates supplemented with 0, 5, 10, 25, 50 or 100 µM CdCl$_2$, allowed to lay eggs for 6 h, after which time they were transferred for another 6 h onto plates containing 25 µM CdCl$_2$. Only the progeny of worms that exhibited the CePCS1-deficient phenotype upon transfer to plates containing 25 µM Cd$^{2+}$ (65-75% of the progeny of the total worms injected) were scored at the other concentrations. Control worms were treated in an identical manner except that they were injected with injection buffer alone. The phenotypes of the worms were examined 4-6 d after hatching. Features scored included gross morphology, developmental stage and fertility. The specificity of the effects exerted by the injection of dsce-pcs-1 RNA and the efficacy of the injection protocol employed were assessed by the injection of wild type N2 worms and strain EE86 worms (genotype upIs1 [mup-4::gfp(CeMup-4.99.1617); rol-6(pRF4)]) with ds gfp RNA by the same procedure. In both cases the worms were screened for Cd$^{2+}$ tolerance; in the latter case the worms were also screened for diminished hypodermal cell MUP-4::GFP expression (17). GFP levels were assessed under a Leica stereomicroscope equipped with UV illumination.
RESULTS

CePCS1 catalyzes PC synthesis. The functional equivalence of CePCS1 with its plant and *S. pombe* homologs was determined by heterologous expression in *Saccharomyces cerevisiae*, which otherwise lacks PC synthase (4-6). For this purpose and to render its translation product immunodetectable, *ce-pcs-1::FLAG* fusions were cloned into expression vector pYES3 and these (pYES3-CePCS1::FLAG), the untagged construct (pYES3-CePCS1) and empty vector were transformed into Cd²⁺-hypersensitive *S. cerevisiae* strain DTY167 (Materials and Methods).

The involvement of CePCS1 in heavy metal tolerance was examined at three levels: by determining its capacity for alleviating Cd²⁺ hypersensitivity; by defining its facility for promoting the Cd²⁺-dependent intracellular accumulation of PCs; by determining its ability for *de novo* Cd²⁺-activated synthesis of PCs from GSH *in vitro*.

Regardless of the level at which it was examined, CePCS1 had the properties expected of a PC synthase. Plasmid-borne *ce-pcs-1::FLAG* suppressed the Cd²⁺-hypersensitivity of yeast strain DTY167, as did untagged *ce-pcs1*, at efficacies comparable to those of *AtPCS1::FLAG* (and *AtPCS1*, data not shown). CePCS1::FLAG and CePCS1 increased the concentration of CdCl₂ in the growth medium required for 50% inhibition of growth by 12.0- and 12.4-fold and 1.5- and 1.4-fold versus empty vector-transformed controls or *AtPCS1::FLAG*-transformed cells (Fig. 1). As shown previously for *AtPCS1* (6), the tolerance conferred by CePCS1::FLAG (and CePCS1) was not restricted to Cd²⁺ but extended to other soft metals and metalloids, including mercury (as HgCl₂) and arsenic (as AsO₄³⁻ or AsO₂⁻) (data not shown).

Whenever enhanced Cd³⁺ tolerance was observed, it was associated with Cd²⁺-dependent intracellular PC accumulation. Reverse-phase HPLC analysis of nonprotein thiols in the soluble fraction from *ce-pcs-1*-transformed DTY167 cells after growth in media containing CdCl₂.
revealed prominent peaks eluting after the GSH/2-mercaptoethanol injection peak whose chromatographic properties were indistinguishable from those of PCs (PC₂, PC₃, PC₄, PC₅) synthesized in vitro by purified AtPCS1::FLAG (7) (Fig. 2A). The aggregate thiol content of these nonprotein thiol peptides was 178 nmol/mg soluble protein. The corresponding fractions from CdCl₂-grown empty vector control cells and from ce-pcs-1-transformed cells after growth in medium lacking Cd²⁺ were devoid of PC-like nonprotein thiols (Fig. 2A), indicating that both CePCS1 and exposure to heavy metal were essential prerequisites for net PC synthesis in vivo.

The CePCS1- and Cd²⁺-dependent synthesis of PCs measured in vivo was precisely replicated in vitro. Analyses of the capacity of CePCS1::FLAG yeast cell-free extracts for the Cd²⁺-dependent incorporation of GSH into PCs demonstrated net synthesis of PC₂ and PC₃ at an aggregate rate of 28.5 ± 4.3 nmol/mg/min (Fig. 2B). No peaks other than those corresponding to GSH were detected when the same extracts were incubated in the media lacking CdCl₂. The corresponding fraction from DTY167 cells transformed with AtPCS1::FLAG catalyzed PC₂ and PC₃ synthesis at an aggregate rate of 107.0 ± 36.0 nmol/mg/min (Fig. 2b). By contrast, net PC synthesis by the cell-free extracts from vector controls was undetectable (< 0.01 nmol/mg/min) (data not shown). Since approximate proportionality between net PC synthetic activity in vitro and the amount of recoverable fusion protein in the extracts was demonstrable immunologically (Fig. 2, Inset), CePCS1::FLAG and AtPCS1::FLAG were inferred to have similar intrinsic catalytic capacities.

CePCS1 is required for cadmium tolerance in the intact organism. The discovery of ce-pcs-1 in C. elegans and the demonstrated facility of its translation product for Cd²⁺-dependent PC synthesis was completely unexpected. PCs had never before been considered to be involved in metal homeostasis in animals (8). Hence, to determine if PC synthase-mediated PC synthesis
might indeed contribute to metal detoxification in *C. elegans*, the *in vivo* function of *ce-pcs-1* was examined at the level of the whole organism. Toward this end, the double-stranded RNA interference (RNAi) technique (18) was employed. This technique for the targeted suppression of specific genes, was thought to be particularly applicable to the study of *ce-pcs-1*, a single-copy gene. Wild type (N2) or a green fluorescent protein- (GFP-) expressing line (EE86) of *C. elegans* were injected with injection buffer alone, injection buffer containing ds*gfp* RNA or with injection buffer containing ds*ce-pcs-1* RNA, and cultured on standard nematode growth medium (NGM) or on NGM containing different concentrations of CdCl₂. Four days later, the progeny of these worms were scored for gross morphology, development and fertility.

The progeny of control worms injected with either injection buffer or ds*gfp* RNA were indistinguishable from wild type (Figs. 3, 4A), although all of the progeny of the ds*gfp* RNA-injected GS86 worms exhibited markedly diminished or no GFP expression as determined by fluorescence microscopy. At low (5, 10 and 25 µM) concentrations of Cd^{2+}, the wild type control progeny developed into normal-sized, gravid adults, comparable to those grown in the media devoid of heavy metal (Figs. 3, 4B). At higher concentrations (50 and 100 µM), the controls grew more slowly, but nevertheless reached adulthood and laid eggs after about 6 days (data not shown). In contrast, marked differences were evident in the progeny of worms injected with ds*ce-pcs-1* RNA, even at lowest concentrations of Cd^{2+}. When raised on 5 and 10 µM Cd^{2+}, the CePCS1-deficient animals reached adulthood (Fig. 3); however, at 5 µM most individuals retained their eggs, and at 10 µM showed severe necrosis (Fig. 4B), producing fewer eggs. A fraction (21%) of the worms reached adulthood at 25 µM CdCl₂ (Fig. 3) but they were small, necrotic, sterile and eventually died. At 50 and 100 µM Cd^{2+}, CePCS1-deficient worms arrested at the L2-L4 larval stage, were extensively necrotic and had died by day 6 (Figs. 3 and 4C).
differential effects seen were conditional on exposure to Cd$^{2+}$ since CePCS1-deficient worms were identical to wild type when grown on standard NGM plates (Figs. 3 and 4a). As would be expected if the effects of Cd$^{2+}$ on the CePCS1-deficient worms were irreversible, transfer of arrested larvae to standard NGM plates did not restore growth (data not shown).

**CONCLUSIONS**

The findings reported here represent the first demonstration of the PC synthase-mediated detoxification of Cd$^{2+}$ and probably other heavy metals in an animal. Moreover, while other gene products have been inferred to contribute to Cd$^{2+}$ tolerance in *C. elegans*, CePCS1 is the first for which a firm biochemical basis has been established for the effects seen at the whole organism level. Elegant studies of *C. elegans* mutants for an MRP (multidrug resistance-associated protein) subclass ATP-binding cassette transporter gene (19) and for a MAP kinase kinase (MEK1) gene (20) have revealed an increase in sensitivity to Cd$^{2+}$ versus wild type but the effects seen were less acute and/or less specific than those reported here for CePCS1-deficient worms and in neither case could be attributed to a specific biochemical process or event.

The ubiquity of the PC synthase-dependent pathway in other animals and in groups of organisms other than plants remains to be delineated but the fact that many nematode species are pathogenic and that the EST databases for the parasitic protists *Eimeria*, *Leishmania* and *Plasmodium* contain cDNAs whose predicted proteins are also PC synthase homologs markedly extends the significance of the findings reported here. On the one hand, its discovery in *C. elegans*, in conjunction with the prominence of heavy metals as environmental toxins (21) implicated in many disease states, including cancers in humans (22), may mean that PC synthase-dependent metal detoxification in animals will prove to be of environmental toxicological significance. On the other hand, the likely operation of equivalent metal
detoxification pathways in pathogenic nematodes and protists – organisms responsible for untold human suffering and agronomic losses (23,24) – may, in view of the conditional lethality of the ce-pcs-1 RNAi phenotype, spawn new chemotherapeutic and agrochemical approaches for combating the many infections caused by these pathogens.

REFERENCES


Acknowledgments

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LEGENDS TO FIGURES

**Fig. 1.** Suppression of Cd\textsuperscript{2+}-hypersensitivity of *S. cerevisiae ycf1Δ* mutant strain DTY167 by plasmid-borne CePCS1. Yeast *ycf1Δ* strain DTY167 was transformed with pYES3-CePCS1 (○), pYES3-CePCS1::FLAG (●), pYES3-AtPCS1::FLAG (□) or empty pYES3 vector (■). Cells were grown at 30°C to an OD\textsubscript{600} nm of approximately 1.8 in AHC medium supplemented with glucose and tryptophan before inoculating aliquots into 2 ml volumes of the same medium containing the indicated concentrations of CdCl\textsubscript{2}. OD\textsubscript{600} nm was measured after growth for 36 h.

**Fig. 2.** Cadmium-activated CePCS1-FLAG-dependent PC synthesis *in vivo* and *in vitro*. **A.** Reverse-phase HPLC analysis of nonprotein thiols in the soluble fractions extracted from CePCS1::FLAG-transformed DTY167 cells after growth in liquid medium containing CdCl\textsubscript{2} (25 µM). **B.** Reverse-phase HPLC analysis of the nonprotein thiols formed after incubation of whole cell extracts prepared from CePCS1::FLAG-transformed DTY167 cells (180 µg protein/ml) with GSH (3.3 mM) and CdCl\textsubscript{2} (25 µM). The peaks designated “PC\textsubscript{2}” and “PC\textsubscript{3}” were identified on the basis of their Glu/Gly ratios (2.1 ± 0.1 and 3.0 ± 0.2, respectively) and comigration with PC\textsubscript{2} and PC\textsubscript{3} standards synthesized *in vitro* using purified AtPCS1-FLAG (7). **Inset:** Immunoblot analysis of CePCS1-FLAG and AtPCS1-FLAG in whole cell extracts from CePCS1::FLAG-, AtPCS1::FLAG- and empty vector-transformed DTY167 cells. The M\textsubscript{r} 46,000 and 58,000 species were the only anti-FLAG antibody-reactive polypeptides detected in the extracts.

**Fig. 3.** Increased sensitivity of CePCS1-deficient worms to Cd\textsuperscript{2+}. Shown are the percentages of the progeny of dsce-pcs-1 RNA-injected (●), dsgfp RNA-injected (numbers) and injection buffer-injected worms (○) that had reached adulthood 4 d after hatching on NGM plates supplemented with the indicated concentrations of CdCl\textsubscript{2}. Since the two treatment regimes
described in Materials and Methods yielded identical results, the data presented incorporate both sets of results. The number of worms injected and the number of progeny scored at each concentration of Cd\(^{2+}\) were as follows. Injection buffer control: 0 µM Cd\(^{2+}\) - 8 injections, 268 progeny; 5 µM Cd\(^{2+}\) - 6 injections, 144 progeny; 10 µM Cd\(^{2+}\) - 5 injections, 191 progeny; 25 µM Cd\(^{2+}\) - 10 injections, 206 progeny; 50 µM Cd\(^{2+}\) - 10 injections, 295 progeny; 100 µM Cd\(^{2+}\) - 9 injections, 178 progeny. ds\(gfp\) RNA injection control: 0 µM Cd\(^{2+}\) - 4 injections, 74 progeny; 5 µM Cd\(^{2+}\) - 4 injections, 76 progeny; 10 µM Cd\(^{2+}\) - 3 injections, 140 progeny; 25 µM Cd\(^{2+}\) - 3 injections, 62 progeny; 50 µM Cd\(^{2+}\) - 3 injections, 65 progeny; 100 µM Cd\(^{2+}\) - 3 injections, 156 progeny. ds\(ce-pcs-1\) RNA injection: 0 µM Cd\(^{2+}\) - 17 injections, 248 progeny; 5 µM Cd\(^{2+}\) - 12 injections, 278 progeny; 10 µM Cd\(^{2+}\) - 10 injections, 234 progeny; 25 µM Cd\(^{2+}\) - 19 injections, 300 progeny; 50 µM Cd\(^{2+}\) - 16 injections, 264 progeny; 100 µM Cd\(^{2+}\) - 18 injections, 397 progeny.

**Fig. 4.** Morphology of control worms (*Control*) and CePCS1-deficient worms (*CePCS1*) after growth on NGM plates containing (A) 0 µM CdCl\(_2\), (B) 10 µM CdCl\(_2\), or (C) 50 µM CdCl\(_2\). Five day-old worms were immobilized in buffer containing 5 mM sodium azide and photographed within 5 min. Nomarski images were collected using a Leica DAS microscope equipped with a DMR camera.
Figure 1
Figure 2
Figure 3
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