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A pseudo-phytochelatin synthase in the ciliated protozoan *Tetrahymena thermophila*

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ABSTRACT

Phytochelatins (PCs) and metallothioneins (MTs) are the two major heavy metal chelating peptides in eukaryotes. We report here on the identification of a biosynthetically inactive pseudo-phytochelatin synthase enzyme (TtψPCS) in the ciliate *Tetrahymena thermophila*, the first of this kind (pseudo-PCS) to be described in eukaryotes. TtψPCS which resembles a true PCS at the N-terminal region, while it is most divergent in its Cys-poor C-terminal region, was found to be up-regulated under cadmium stress conditions. However, only glutathione (GSH) hydrolysis products, but not PCs, could be detected in extracts from Cd-treated cells. The latter feature is reminiscent of pseudo-PCS enzymes recently identified in cyanobacteria, which are also biosynthetically inactive, but capable to hydrolyze GSH.

1. Introduction

Phytochelatins (PCs), glutathione (GSH) and metallothioneins (MTs) are cysteine-bearing (poly)peptides involved in heavy metal detoxification (Cobbett, 2000). PCs are synthesized enzymatically from GSH by phytochelatin synthase (PCS) (γ-glutamylcysteinyltranspeptidase; EC 2.3.2.15). PCs, which have the general structure (γ-Glu-Cys)$_n$-Gly (n = 2–11), chelate heavy metals, especially Cd^{2+}, with high affinity and facilitate the vacuolar sequestration of these inorganic pollutants (Cobbett, 2000). The PCS enzyme catalyzes the transpeptidation of the γ-Glu-Cys moiety of GSH onto a second GSH molecule forming PC_2 or onto a preformed PC_n molecule to form the corresponding PC_{n+1} (Grill et al., 1989).

First discovered in the fission yeast *Schizosaccharomyces pombe* (Kondo et al., 1984), PCs were subsequently found in all plants examined to date (Grill et al., 1988) and in some microalgae (Grill et al., 1989). Likewise, genes encoding PCSs have been cloned from plants (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999), fungi (Clemens et al., 1999) and nematodes (Clemens et al., 2001; Vatamaniuk et al., 2001). Sequence alignment of different PCS polypeptides revealed a high degree of sequence similarity within the N-terminal, but not the C-terminal region (Cobbett and Goldsbrough, 2002). The N-terminal domain was indeed shown to be sufficient for catalysis, whereas the C-terminal domain was found to enhance PCS activity as well as protein stability and heavy metal responsiveness (Ruotolo et al., 2004). A number of putative PCS sequences have been retrieved recently from EST and genome sequence databases of organisms belonging to all kingdoms (Cobbett and Goldsbrough, 2002). These include a group of bacterial PCS-like proteins (Harada et al., 2004; Tsuji et al., 2004), whose main activity is the conversion of GSH into γ-glutamylcysteine rather than PC biosynthesis (Harada et al., 2004). Interestingly, a similar activity has been described recently for the *Arabidopsis* AtPCS1 enzyme, which has been shown to be involved in glutathione-conjugate degradation, besides heavy metal detoxification (Blum et al., 2007).

In ciliates, the main molecules involved in the response to heavy metal stress are MTs (Boldrin et al., 2002; Díaz et al., 2007; Amaro et al., 2008), which belong to two different subfamilies (CdMTs and CuMTs) as it has been documented recently in *Tetrahymena thermophila* (Díaz et al., 2007; Amaro et al., 2008). However, the potential involvement of other biochelators, particularly PCs, has not been investigated so far.

We report here the identification and characterization of a cDNA (*TtψPCS*) encoding a PCS homologous sequence from the ciliated protozoan *T. thermophila*, the first to be described in ciliates. A quantitative real-time PCR (qRT-PCR) expression analysis of *TtψPCS* has been carried out under different heavy metal stress conditions. Several experimental evidences suggest that this enzyme is biosynthetically inactive in PC formation, while it is likely endowed with a γ-glutamyltransferase (γGT) activity, which makes it the first pseudo-PCS to be described in eukaryotes.

2. Materials and methods

2.1. Ciliate culture conditions and heavy metal treatment

T. thermophila, strain SB1969, was grown axenically in PP210 medium (2% w/v aqueous solution of proteose peptone (Difco) supplemented

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with 10 μM $FeCl_3$ and 250 μg/mL of each antibiotic (streptomycin sulphate and penicillin to prevent bacterial growth) and maintained at 30 °C. Exponential growth phase cultures of this ciliate were exposed (1 or 24 h) to different salts of heavy metals; 44.5 μM Cd^{2+} ($CdCl_2$), 315 μM Cu^{2+} ($CuSO_4 \times 5H_2O$), 965 μM Pb^{2+} ($Pb(NO_3)_2$) and 100 μM As^{5+} ($Na_2HAsO_4 \times 7H_2O$), all of them were supplied by Sigma. The metal concentrations used were lower than the corresponding LC_{50} value for each heavy metal and resulted in negligible cell mortality.

2.2. Total DNA/RNA isolations, standard PCR, cDNA synthesis and cloning

Exponential cultures (100 mL) of *T. thermophila* were harvested by centrifugation at 500 g for 3 min. Total DNA was isolated according to the protocol described in Hamilton and Orias (2000). PCR reactions (50 μL) contained: 1× PCR buffer (GeneAmp Gold buffer), 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM each primer (PCSN1/PCSN3) (Table 1), 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and about 100 ng of DNA. Amplification was: 7 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 50 °C and 2 min at 72 °C, and 72 °C for 5 min. Amplified PCR products were cloned with the TOPO TA Cloning kit (Invitrogen) and sequenced.

Total RNA was isolated from control and metal-treated cultures using the RNAqueos™-4PCR kit (Ambion). All samples were treated with RNase-free DNase-I (Ambion), according to the protocol supplied by the manufacturer. The cDNA synthesis was carried out using 5 μg RNA, oligo d(T) primer (1.6 μg), AMV reverse transcriptase (Roche) (20 U) with RNase inhibitor (50 U) (Roche) and 5 mM dNTPs, in a total volume of 20 μL.

The *TtψPCS* cDNA was amplified by RT-PCR from Cd-treated *T. thermophila* cDNA using 1.25 U AmpliTaq Gold DNA polymerase (PE Applied Biosystems), 5 μL of cDNA (0.125 μg), 1× PCR buffer (GeneAmp Gold buffer), 2.5 mM $MgCl_2$, 0.2 mM each dNTP and 0.2 μM each primer (PCS1/PCS2) (Table 1). Amplification protocol, cloning and sequencing were as above indicated.

2.3. Quantitative real time RT-PCR

cDNA samples from control and heavy metal treated cells were amplified in duplicate in 96 microtiter plates (Applied Biosystems). Primers for qRT-PCR (PCSQA/PCSQB) (Table 1) for *TtψPCS* gene and (ATUB1/ATUB2) (Table 1) for *α-tubulin* gene (Acc. No. AAA21350) were designed with the Primer Express v2.0 software (Applied Biosystems), the *α-tubulin* gene was utilized as a housekeeping reference (Amaro et al., 2008; Díaz et al., 2007). Each PCR reaction (20 μL total volume) contained: 10 μL of SYBR Green PCR Master Mix (Applied Biosystems), 1 μL of each primer (at 300 nM final concentration), 3 μL of H_2O and 5 μL of cDNA. The size and sequence of each product (54 bp for *α-tubulin* and 96 bp for *TtψPCS*) was confirmed by gel electrophoresis and DNA sequencing. RT-PCR reactions were carried out in an ABI PRISM 7700 real time PCR apparatus, and the thermal cycling protocol was as follows: 10 min at 95 °C, followed by 40 cycles (15 s at 95 °C and 1 min at 50 °C). All controls (no template control and RT minus control) were negative. The specificity of each primer pair was tested by qPCR; a unique PCR product was obtained for all primer pairs as determined by melting curve analysis. To calculate the normalized relative gene expression levels we used a relative gene expression software tool (REST-MCS β version 2) (Pfaffl, 2001). The slopes of the resulting trend lines were −3.43 (*α-tubulin*) and −3.33 (*TtψPCS*), the correlation coefficients (R^2) were 100% for both genes, and the amplification efficiency (E) values were 1.96 and 2.00, respectively. Statistical analysis was carried out using the Pair Wise Fixed Reallocation Randomisation Test (REST-MCS β version 2 Software) (Pfaffl et al., 2002).

Table 1
PCR primers.

Primer	Sequence (5′ to 3′)
PCSN1	ATGTCTCGTTCACTATTCAA
PCSN3	GAAAAAGCCTAAAACAAATA
PCS1	ATGTCTCGTTCACTATTCAAGA
PCS2	TCAACTTTAAAAAAGATTAT
PCSQA	TATTAGAGGAAAAGAGTGAAGTAGGA
PCSQB	AGCGATCAATGGAGCATA
ATUB1	TGTCGTCCCCAAGGAT
ATUB2	GTTCTCTTGGTCTTGATGGT

2.4. DNA sequencing and phylogenetic analysis

DNA sequences were determined on both strands using an ABI PRISM™ 377 DNA automatic sequencer (PE Applied Biosystems), with the dideoxy technique, using appropriate primers and synthetic oligonucleotides (Big-dye™ terminator cycle sequencing ready reaction kit from AP Biosystems). Homology searches were performed using BLAST program at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). The PCS phylogenetic tree was derived from the alignment using the T-Coffee method (Tree-based Consistency Objective Function For alignmEnt Evaluation) (Notredame et al., 2000) of the N-terminal region of PCS amino acid sequences representative of the main PCS-containing lineages, and the PhyML 3.0 program (http://www.atgc-montpellier.fr/phyml/), a maximum likelihood-based phylogenetic inference method (Guindon and Gascuel, 2003; Guindon et al., 2005).

2.5. MS-HPLC analysis

Ciliate cells were harvested by centrifugation (500 g at 4 °C), washed once with 0.01 M Tris–HCl pH 6.8, 4 mM EDTA, lyophilized and stored at −80 °C. For HPLC analysis, ca. 100 mg of cell powder was extracted with 5% sulfosalycilic acid, 6.3 mM diethylenetriaminepentaacetic acid (DTPA) (De Knecht et al., 1994). HPLC analysis followed by post-column derivatization with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was carried out as described previously (Ruotolo et al., 2004). For mass spectrometry (MS)-HPLC analysis, extracts were fractionated on a reverse-phase C18 column (XTerra/Phenomenex, 150 mm × 2.1 mm) operated with a Waters Alliance 2690 HPLC apparatus (0–95% acetonitrile concentration gradient containing 0.1% formic acid; run time: 30 min; flow-rate: 0.2 mL/min). Peak fractions were directly subjected to MS analysis (Waters Micromass Quattro micro AP triple–quadrupole mass spectrometer). Analytical conditions were optimized using authentic GSH (Sigma), PC_2 (Applichem), and a $PC_{(2-6)}$ mixture generated *in vitro* using recombinant AtPCS1, as standards (Courbot et al., 2004). PC-producing yeast cells transformed with the *AtPCS1* gene (Ruotolo et al., 2004) were used as extraction controls.

2.6. Protein extraction

Lyophilized *T. thermophila* cells (2 × 100 mL cultures) washed with 0.01 M Tris–HCl pH 7.5 were resuspended in ice-cold TMD buffer (100 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol) and lysed by sonication at 4 °C. Protein was determined with the Bio-Rad protein assay. Reactions were carried out at 35 °C for 1 h in the presence of 1 μg total protein extract; reaction mixtures were extracted and analyzed for the presence of PCs by HPLC as described (Ruotolo et al., 2004).

2.7. Sequence accession numbers

The *TtψPCS* cDNA sequence from the ciliate *T. thermophila* has been deposited in the GenBank Database (Acc. No. DQ082725). GenBank accession numbers of other PCS polypeptides utilized in this article are: AtPCS1 (*Arabidopsis thaliana*, AAD41794), AtPCS2 (*A. thaliana*, Q9ZWB7), BjPCS (*Brassica juncea*, CAC37692), TaPCS (*Triticum aestivum*,