Toxicology Letters 180 (2008) 21–27

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

The role of the yeast ATP-binding cassette Ycf1p in glutathione and cadmium ion homeostasis during respiratory metabolism

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ARTICLE INFO

Article history:
Received 12 March 2008
Received in revised form 21 May 2008
Accepted 21 May 2008
Available online 29 May 2008

Keywords:
Ycf1p
GSH
Respiratory metabolism
Cadmium
Saccharomyces cerevisiae

ABSTRACT

Cadmium (Cd^{2+}) is a toxic environmental contaminant for biological systems, which can form complexes with reduced glutathione (GSH), and thus alter the intracellular redox state. In *Saccharomyces* (*S.*) *cerevisiae*, bis(glutathionato)cadmium (Cd–$[GS]_2$) complexes can be removed from the cytosol and transported into the vacuole by a glutathione-conjugated pump, Ycf1p. In this study, we investigated the role of Ycf1p in Cd^{2+} detoxification during respiratory metabolism of *S. cerevisiae*, and the correlation of Ycf1p with GSH intracellular homeostasis. The results showed that in respiratory condition the mutant *ycf1*Δ is more tolerant to Cd^{2+} and to the oxidants *t*-BOOH and H_2O_2 than wild-type strain. This tolerance is probably related to the high content of GSH present in *ycf1*Δ mutant. The expression of *YCF1* promoter in the wild-type strain is naturally down-regulated after the transition from fermentative to respiratory metabolism (diauxic shift), and its induction in response to Cd^{2+} is dependent on GSH availability. Our data suggest that Ycf1p is involved in the maintenance of intracellular GSH homeostasis and it can interfere with the oxidative tolerance of yeast. Moreover, the detoxification of Cd^{2+} is dependent on GSH availability and on cellular metabolic status.

1. Introduction

Cadmium (Cd^{2+}) is an environmental contaminant that shown a long biological half life (15–20 years) as a consequence of its low excretion rate in the organism (Nordberg, 1984; Jin et al., 1998). Moreover, Cd^{2+} is classified as a carcinogen by the International Agency for Research on Cancer [http://www.iarc.fr/index.html], due to its correlation with the development of lung and prostate cancer (Huff et al., 2007). The deleterious effects of Cd^{2+} are not fully understood, but its toxicity seem be related to two distinct mechanisms. One assertion is that Cd^{2+} strongly inhibits the mismatch-repair pathway (Jin et al., 2003). Another assertion is that free intracellular Cd^{2+} causes depletion of reduced glutathione (GSH; γ-Glu-Cys-Gly), a tripeptide ubiquitous in eukaryotic cells. GSH depletion can be threatening, as it is required for many biological processes, such as protein and DNA synthesis, cell cycle regulation, intracellular buffering, heat shock resistance, detoxification of xenobiotics, regulation of thiolredox status and antioxidant defense (DeLeve and Kaplowitz, 1990; Meister, 1984; Wang and Ballaroti, 1998).

Living organisms use several mechanisms to deal with the toxic effects of Cd^{2+}. In bacteria, the main system of resistance is the energy-dependent efflux of metal ions (Silver and Phung, 2005). In eukaryotes, Cd^{2+} can be chelated by GSH or other thiol-containing proteins like metallothioneins or phytochelatins, which are synthesized from GSH. These thiol-containing polypeptides are found in plants, worms, and the fission yeast, *Schizosaccharomyces pombe* (Gehig et al., 2000; Duncan et al., 2006; Li et al., 1997; Vido et al., 1999). In *S. cerevisiae* the best characterized pathway that protects against Cd^{2+} toxicity involves its binding with GSH, and the formation of bis(glutathionato)cadmium (Cd–$[GS]_2$) complexes. These complexes are removed from the cytosol and transported into the vacuole by specific transmembrane proteins such as the transporter pump Ycf1p (Li et al., 1997).

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doi:10.1016/j.toxlet.2008.05.010

Coded by the gene *YCF1*, Ycf1p is an ATP-binding cassette (ABC) protein that belongs to the multidrug resistance-associated protein subfamily (MRP/ABCC). The Ycf1p protein forms a vacuolar glutathione-conjugated pump that shares sequence homology to human MRP1, and to the human cystic fibrosis transmembrane conductance regulator (hCFTR) (Li et al., 1996; Szczypka et al., 1994). Ycf1p catalyzes the transport of a broad range of substrates, including *S*-(2,4-dinitrophenyl)glutathione, glutathione disulfide (GSSG), free GSH, and Cd–$[GS]_2$ complex. Another substrate transported by Ycfp1 is the endogenous compound phosphoribosylaminoimidazole-glutathione, a red pigment that accumulates in the vacuoles of yeast *ade1* or *ade2* mutants when grown under adenine limitation conditions (Li et al., 1996, 1997; Rebbeor et al., 1998; Sharma et al., 2002; Chaudhuri et al., 1997).

Most drugs are transported by MRPs as conjugates of GSH, glucoronide, or sulfate (Cole and Deeley, 2006). Alternatively, some drugs are co-transported with free GSH, because free GSH is itself a direct substrate for Ycf1p (Cole and Deeley, 2006; Ballatori et al., 2005). The Ycf1p transport system accounts for about 70% of the GSH transport activity in vacuoles. The remaining activity is due to the V-ATPase-coupled system (Mehdi et al., 2001). Interestingly, the transport of GSH is not seen in vacuolar membrane vesicles isolated from the yeast strains lacking a functional Ycf1p (Rebbeor et al., 1998; Sharma et al., 2002).

As damaging reactive oxygen species (ROS) are generated by mitochondrial electron transport chain activity, the maintenance of GSH homeostasis is particularly important for protection against oxidative stress during respiratory metabolism (Adam-Vizi and Chinopoulus, 2006). In *S. cerevisiae*, the transition from fermentative to respiratory metabolism in response to glucose exhaustion (diauxic shift) is accomplished by an increase in the expression of antioxidant defense genes—including the gene *GSH1*, which codes for the first enzyme of GSH biosynthesis (Maris et al., 2001). Since the main pathway for Cd^{2+} detoxification in yeast involves GSH sequestration by Ycf1p, and Cd^{2+} toxicity is at least partially associated with GSH depletion and oxidative stress, it is relevant to analyze the disruption caused by this heavy metal in the face of respiratory activity.

In this work, we use the eukaryotic model *S. cerevisiae* to investigate the role of Ycf1p in Cd^{2+} detoxification during respiratory metabolism. Remarkably, in the last years the use of *S. cerevisiae* has allowed the gain of insights about the mechanisms related to cadmium toxicity and detoxification (Gomes et al., 2002, 2008; Adamis et al., 2007; Jin et al., 2003). We performing cytotoxic and gene reporter assays in strains proficient and deficient in Ycf1p. Then, we compared these results with measurements of total intracellular GSH and Cd^{2+} to further understand the relationship of oxidative stress to Cd^{2+} detoxification.

2. Materials and methods

2.1. Yeast strains and plasmid

The strains of *S. cerevisiae* used in this study were WT BY4741 (Wild type; *MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and Yfc1p-deficient *ycf1Δ* (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *ycf1::kanMX4*), both obtained from Euroscarf (European *S. cerevisiae* Archive for Functional Analysis, Frankfurt, Germany). The *YCF1* reporter plasmid (pJAW736) was described in (Wemmie et al., 1994) and was provided by Dr. Scott Moye-Rowley (University of Iowa).

2.2. Cd^{2+} sensitivity assays

The strains were grown in rich medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C until stationary phase, then harvested by centrifugation (1 min/15,000 × *g*) and washed twice with distilled water. The cell number was determined by counting in a Neubauer chamber. The cellular assays were carried out in Synthetic Hartwell's complete medium (SC) (Burke et al., 2000) containing 5% glucose (for yeast fermenting cells) or 3% glycerol (for yeast respiring cells) as carbon sources. For the survival assay, 5×10^6 cells/mL were incubated for 24 h (180 rpm) at 30 °C in SC (with 5% glucose or 3% glycerol), supplemented or not with $CdCl_2$ (12, 48, 192 and 384 μM). After the treatments, cells were washed twice with distilled water and diluted to 1×10^3 cells/mL. Aliquots of 100 μL were plated in SC solid medium (2% glucose) and incubated at 30 °C for 2–3 days for the determination of survival colonies. For the drop test, cells were washed twice and diluted between 10^8 and 10^3 cells/mL, and 10 μL of each dilution were plated in SC solid medium containing 3% glycerol with either Cd^{2+} and *N*-acetylcysteine (NAC, a GSH precursor compound) or 1-chloro-2,4-dinitrobenzene (CDNB, a GSH depleting compound). The plates were incubated for 5–6 days at 30 °C. Drop tests were also performed in the presence of H_2O_2 and *tert*-butyl hydroperoxide (*t*-BOOH), instead of Cd^{+2}.

2.3. Quantification of total glutathione

Total glutathione (GSH + GSSG) was measured by photometric determination of 5-thio-2-nitrobenzoate (TNB), which was produced from DTNB in a kinetic assay, according to Akerboom and Sies (1981). For this procedure the strains were grown, washed and treated as described in Section 2.2. After 24 h, 2×10^8 cells were collected by centrifugation, washed twice with distilled water, resuspended in 1 mL of cold buffer (100 mM sodium phosphate, 4 mM EDTA; pH 7.0) and disrupted by cycles of agitation with glass beads and ice bath (8 cycles × 40 s). The suspension was treated by the addition of an equal volume of 2 M $HClO_4$ and centrifuged for 10 min/15,000 × *g*. The supernatant was neutralized to pH 7.0–7.2 with 2 M KOH/0.3 3-[*N*-morpholino] propanesulfonic acid, and the insoluble residues were removed by centrifugation (10 min/10,500 × *g*). The enzymatic determination of total GSH was performed using 250 μL of neutralized supernatant. Glutathione concentration was calculated from the standard curve of GSSG and is expressed as GSH equivalent/2×10^8 cells.

2.4. β-Galactosidase reporter assays

To access the pattern of *YCF1* expression during the growth of WT BY4741 strain in the presence of Cd^{2+}, the cells were transformed with reporter plasmid pJAW736. This plasmid contains a non-coding *YCF1* region (−736 to −1 bp) with a *lacZ* reporter gene (Wemmie et al., 1994). The yeast transformation was performed by the lithium acetate method (Gietz and Woods, 2002). Cells were grown in SC with 2% glucose lacking uracil (SC ura^-) at late exponential phase, then reinoculated in the same fresh medium (5×10^6 cells/mL) supplemented or not with 48 μM of $CdCl_2$. This $CdCl_2$ concentration was chosen because it is comparable to the levels found in the environment as well as levels that can accumulate in the human body (Jin et al., 2003; Satarug and Moore, 2004). Over 24 h, aliquots were withdrawn at intervals of 2–4 h, for cell number determination and *YCF1* expression measurement. The β-galactosidase activity was measured as in Ausubel et al. (1995) and activity was expressed as units per optical density at 600 nm (OD600) unit of cells.

2.5. Semiquantitative RT-PCR

The WT BY4741 strain was treated as described in section 2.4. After 6 h, cells were harvested for total RNA extraction using RNeasy® Mini Kit (Quiagen, USA) according to manufacturer's instructions. Equal amounts of total RNA (1.0 μg) previously treated with DNAse I amplification grade (Promega, USA) were subjected to first strand cDNA synthesis using the poly-T antisense primer, and the M-MLV reverse transcriptase (Promega, USA). PCR was carried out with *Taq* DNA polymerase using the first strand cDNA and specific primers for *YCF1* (5′-AAGACATTAGTTGGCGAGAAAGGG-3′ and 5′-TTTGGACCCAACACATGTTCGATCA-3′) or *ACT1* (actin, constitutive control) (5′-ATGGAAGATGGAGCCAAAGC-3′ and 5′-TCTGCCGGTATTGACCAAAC-3′). Both fragments were amplified with the same PCR program (one cycle of 94 °C for 1 min, followed by 28 cycles of 94 °C for 30 s/53 °C for 30 s/72 °C for 30 s, and a last cycle of 72 °C for 5 min). The size of fragments generated was 170 bp (with *YCF1* primers) and 164 bp (with *ACT1*primers). The semiquantitative RT-PCR was performed two times with RNA samples extract in independently days.

2.6. Atomic absorption

The yeast strains were prepared as they were for β-Galactosidase reporter assays (Section 2.4). To account for the ATP-dependent activity of Ycf1p, the procedure was carried in SC rather than in PBS buffer, which does not contain an energy source. To measure the residual Cd^{2+} concentration present in the media, 5-mL aliquots were centrifuged, and the supernatant solution was collected and subjected to atomic absorption (Atomic Absorption Spectrometer, PerkinElmer 3100). Cd^{2+} content was estimated by determining the difference in metal concentration between control medium without biomass and test medium containing biomass. The results were normalized with respect to sample cell density at each timepoint, and are expressed in percentage of Cd^{2+} absorbed by 10^7 cells.