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Lap4, a vacuolar aminopeptidase I, is involved in cadmium-glutathione metabolism

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Abstract In *Saccharomyces cerevisiae*, accumulation of cadmium-glutathione complex in cytoplasm inhibits cadmium absorption, glutathione transferase 2 is required for the formation of the complex and the vacuolar gamma-glutamyl transferase participates of the first step of glutathione degradation. Here, we proposed that Lap4, a vacuolar amino peptidase, is involved in glutathione catabolism under cadmium stress. *Saccharomyces cerevisiae* cells deficient in Lap4 absorbed almost 3-fold as much cadmium as the wild-type strain (wt), probably due to the lower rate of cadmium-glutathione complex synthesis in the cytoplasm. In wt, but not in *lap4* strain, the oxidized/reduced GSH ratio and the Gtt activity increased in response to cadmium, confirming that the mutant is deficient in the synthesis of the complex probably because the degradation of vacuolar glutathione is impaired. Thus, under cadmium stress, Lap4 and gamma-glutamyl transferase seem to work together to assure an efficient glutathione turnover stored in the vacuole.

Keywords Lap4 · Cadmium · Glutathione · Mutagenesis · *Saccharomyces cerevisiae*

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Introduction

Cadmium (Cd) is a heavy metal; roughly 13,000 tons are produced worldwide each year for nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings and alloys. The concern arises because Cd accumulates in particular food species, with potential consequences for human health (Satarug and Moore 2004). This metal is a well known carcinogen, even at low concentrations, and a strong mutagen that acts by inhibiting DNA mismatch repair (Jin et al. 2003; Martelli et al. 2006).

Last but not least, Cd causes an oxidative stress: when attacking the membranes, Cd leads to lipid peroxidation; it displaces Zn^{2+} and Fe^{2+} in proteins, resulting in their inactivation and in the iron release, which generates the highly reactive hydroxyl radical; it has a great affinity for thiols, specially glutathione (GSH), the most important antioxidant in aerobic organisms (Adamis et al. 2003; Fauchon et al. 2002; Valko et al. 2005). Oxidative stress increases ROS (reactive oxygen species) production, which has been implicated in cancer, aging, apoptosis and mutagenic effects (Jin et al. 2003; Valko et al. 2005).

The mechanism of Cd detoxification is very complex and not well understood. The structural and functional similarities of genes in lower eukaryotes, like *Saccharomyces cerevisiae*, and mammals suggest that elucidation of the molecular mechanisms by which yeast cells protect themselves from toxic metal ions will help to direct investigations of mechanisms in human cells.

A general defense mechanism against Cd in *S. cerevisiae* and mammals is the chelation of the metal by a ligand and, in some cases, its subsequent compartmentation as a ligand–metal complex. The main molecules responsible for the cytoplasmic sequestration of Cd include GSH and metallothioneins, small cysteine-rich proteins. In mammals, GSH complex is pumped out of the cell by the multi-drug resistance-associated protein, Mrp1, while in *S. cerevisiae* GSH–Cd complex is transported to vacuole through Ycf1, homologous to Mrp1 (Li et al. 1997). According to our results, vacuolar transport of Cd–GSH complex is necessary to allow Cd tolerance, probably, because once inside the vacuole, the complex can be decomposed, restoring the amino acids in the cytoplasm, which can be used to the synthesis de novo of GSH (Adamis et al. 2007). Thus, GSH would be recycled for protection against metals, xenobiotics and oxidative stress. The mechanism of degradation of GSH conjugated with Cd into the vacuole is not understood yet. Recently, we showed that gamma-glutamyl transferase (gamma-GT) is involved in GSH degradation under Cd stress, generating glutamate and cysteinylglycine (CysGly) for a new GSH synthesis (Adamis et al. 2007).

Hydrolysis of CysGly and its S-conjugates represent the second step in the degradation of GSH and glutathione-S-conjugates. A cysteinylglycine dipeptidase activity was found to be responsible for the hydrolysis of CysGly. Additionally, some peptidases have been described to catalyze the hydrolysis of CysGly-S-conjugates (Jösch et al. 2003). Several L-Cys-Gly-hydrolysing peptidases are produced by microorganisms and mammals, such as the vacuolar aminopeptidase I of *S. cerevisiae*, coded by *LAP4* (or *APE1*), a soluble metallo-exopeptidase (Mehdi et al. 2001). The activity of aminopeptidase increases as the cells approach the stationary growth phase, a phenomenon thought to reflect a release from carbon catabolite repression (Cueva et al. 1989). Furthermore, *LAP4* gene is induced more than 9-fold when *S. cerevisiae* was grown in the presence of Cd (Fauchon et al. 2002), suggesting the importance of Lap4 in the protection against this heavy metal.

The aim of this work was to evaluate the oxidative damages, cellular response and the role of protein Lap4 on Cd detoxification pathway using the yeast *S. cerevisiae* as experimental model.

Material and methods

Yeast strains and growth conditions

Wild-type strain BY4741 (*MATa*; *his3*; *leu2*; *met15*; *ura3*) and its isogenic mutant *lap4Δ*, harboring the gene *LAP4* interrupted by the gene *KanMX4*, were purchased from Euroscarf, Frankfurt, Germany. Cells were grown up to stationary phase (about 7×10^7 cells/ml) in liquid YPD medium (1% yeast extract, 2% glucose and 2% peptone), using an orbital shaker at 28°C and 160 rpm, with the ratio of flask volume/medium of 5/1.

Cadmium stress

Cells were harvested by centrifugation and washed twice with 50 mM sodium phosphate buffer, pH 6.0. Thereafter, they were resuspended in the same buffer containing or not 48 μM of $CdSO_4$ and maintained at 28°C/160 rpm. The final concentration of cell was about 7×10^7 cells/ml. After 24 h of treatment, aliquots were withdrawn to analyze residual metal in the supernatant and to perform some investigations with the pellet (Adamis et al. 2003, 2004, 2007).

Determination of cadmium absorption

Analyses of the capacity of *S. cerevisiae* cells to absorb Cd were determined by using atomic absorption spectrophotometry, as previously described (Adamis et al. 2007).

Determination of glutathione

Reduced (GSH) and oxidized glutathione (GSSG) concentrations were determined in neutralized trichloroacetic acid (10% TCA) extracts, before and after 24 h of metal treatment, according to Bernt and Bergmeyer 1974. The results were expressed as GSSG/GSH ratios.

Enzyme and protein assays

Extracts for enzymatic determinations were obtained by disruption of cells with glass beads in 0.1 M Tris–HCl buffer, pH 8.0 (Adamis et al. 2007). Glutathione reductase activity (GR) was assayed spectrophotometrically on the basis of NADPH consumption

(Gunasekaran et al. 1995). One unit (U) of GR activity is defined as consumption of 1 μmol of NADPH for 1 min. Glutathione transferase activity (GTT) was assayed by following GSH conjugation with 1-chloro-2,4-dinitrobenzene (CDNB) at 25°C (Choi et al. 1998). In a 1-ml cuvette, 0.8 ml of 0.1 M potassium phosphate buffer (pH 6.5), 0.05 ml of 20 mM GSH and 0.05 ml of 20 mM CDNB (ethanol solution) were added. The reaction mixture was preincubated at 25°C for 5 min, and the reaction was initiated by addition of 0.1 ml of cell lysate. The increase in absorbance at 340 nm was monitored for 3 min and the activity was calculated from the difference in the reaction mixtures with and without the cell lysate. One unit of glutathione transferase activity is defined as consumption of 1 μmol of CDNB for 1 min. Gamma-glutamyl transferase activity was estimated by spectrophotometric determination at 405 nm of the amount of *p*-nitroaniline released from a reaction medium containing L-gamma-glutamyl-*p*-nitroaniline, as a glutamyl donor, and glycyl-glycine as acceptor (Gunasekaran et al. 1995). The increase in absorbance at 405 nm was monitored for 2 min. One unit of gamma-GT activity is defined as formation of 1 μmol of *p*-nitroaniline for 1 min. The results were expressed as U/mg protein. Protein was determined as described by Stickland 1951, using bovine serum albumin as standard.

Cell viability and mutagenesis

Cells were grown up to middle exponential phase (about 10^6 cells/ml) in liquid YPD medium at 28°C/160 rpm. Thereafter, cells were re-inoculated into fresh liquid medium in the presence or not of 48 μM $CdSO_4$ and incubated at 28°C/160 rpm. Initial cell concentration was about 10^5 cells/ml. After 48 h, cells from control conditions were diluted with 50 mM phosphate buffer (pH 6.0) and plated on YPD plates; cells exposed to Cd stress were diluted in the same buffer and plated on both YPD and YPGly plates (1% yeast extract, 4% glycerol, 2% peptone and 2% agar). YPGly was used to test the inability of cells to grow under aerobic restrict media (glycerol) due to the mutation of mitochondrial DNA. Plates were done in triplicates. Colonies were counted after incubation at 28°C for at least 72 h. Viability was measured as percentage of viable cells, grown on YPD plates that survived after metal stress (Adamis et al. 2004, 2007). The mutation rate caused by Cd was measured as the number of colonies counted on YPGly plates in relation to colonies counted on YPD plates (Adamis et al. 2004, 2007; Jin et al. 2003).

Oxidative damage: lipid peroxidation and protein carbonylation

Lipid peroxidation and protein carbonylation were determined as previously described (Adamis et al. 2007). Lipid oxidation was measured by TBARS (thiobarbituric acid reactive species) method, which detects malondialdehyde—MDA. Protein carbonyl groups were used as biomarkers of protein oxidation. Five-microgram protein samples were slot-blotted onto the polyvinylidene difluoride (PVDF) membrane and then derivatized with 2,4-dinitrophenylhydrazine (DNPH). Slot blot detection was performed after treatment with anti-DNP (dinitrophenyl) followed by treatment with secondary antibody conjugated with peroxidase. Image-analysis of protein carbonyl blots were carried out by using VisionWorks LS software (UVP-Bioagency). In both assays, the results were expressed as a ratio between lipid/protein oxidation level of stressed cells and non-stressed cells.

Data analysis

Results were expressed as mean ± standard deviation of at least three independent experiments. Statistical differences were tested using ANOVA followed by Tukey–Kramer multiple comparisons test. The letter denotes homogeneity between experimental groups at $P < 0.05$. In all tables and figures, different letters mean statistically different results.

Results and discussion

Lap4 is involved in GSH degradation

Over the past 5 years, we have investigated the molecular basis of Cd detoxification in *S. cerevisiae.* We have used concentrations of metal similar to those found in the environment and accumulated in human body (IARC International Agency for Research on Cancer 1993). These levels result in increased mutability in yeast (Jin et al. 2003; Adamis et al. 2004, 2007). As occurs with mammals, we observed that

dead cells of *S. cerevisiae* were not able to remove Cd from the environment (Adamis et al. 2003). We showed that absorption of this non-essential metal is achieved by zinc transporters and that the accumulation of GSH–Cd complex in cytosol inhibits the uptake of Cd (Adamis et al. 2004; Gomes et al. 2002). Cells deficient in Ycf1, unable to transport GSH-Cd complex to vacuole, have a reduced capacity to absorb Cd. On the other hand, cells deficient in the formation of the complex, due to lack of GSH or the isoform 2 of glutathione transferase (Gtt2), on which the formation of the complex depends, absorbed high levels of the metal. The question that we want to address now is the destination of GSH stored in vacuole.

According to our results, Lap4 deficiency favored Cd absorption (Table 1): while *lap4Δ* mutant strain absorbed 58% of initial Cd, wt strain absorbed almost 3-fold less, indicating that this protein might be involved in the metabolism (degradation) of GSH. Similarly, cells deficient in gamma-GT showed increased capacity in removing Cd from the environment perhaps due to its involvement of GSH breakdown (Adamis et al. 2007).

In *S. cerevisiae*, degradation of GSH stored in vacuole lies on the sequential action of gamma-GT and cysteinylglycine dipeptidase (Mehdi and Penninckx 1997). Lap4 is a vacuolar aminopeptidase that possess a hydrolytical action over the N-terminal region of peptides and proteins. These characteristics make Lap4 a good candidate to act along gamma-GT in the catabolism of GSH (Chang and Smith 1989). Gamma-GT, which is also localized on the vacuolar membrane of yeasts, would break down the gamma-peptidic bond between glutamate and cysteine residues of GSH, releasing the first aminoacid, followed by Lap4 action that would cleave the dipeptide CysGly. In the cytosol, those aminoacids might be used for de novo synthesis of GSH in sequential reactions catalyzed by Gsh1 and Gsh2. Figure 1 shows a schematic presentation of formation of Cd complex, its vacuolar compartmentation and GSH recycle.

Thus, in the absence of Lap4, part of GSH (the dipeptide CysGly) could be compartmentalized in vacuole, reducing the availability of cysteine and glycine for GSH synthesis and decreasing the formation of Cd–GSH complex in cytosol. Consequently, less complex in cytosol does not function to inhibit Cd absorption, explaining the increased Cd uptake shown by *lap4* cells.

Corroborating the hypothesis that the mutant possesses reduced levels of Cd–GSH complex, the presence of metal did not change GSSG/GSH ratio (Fig. 2). As expected, after Cd exposure, it was observed almost 2.5-fold increase in GSSG/GSH ratio of wt strain, since the metal mobilizes high concentrations of GSH (Fauchon et al. 2002).

In mammals, under physiological conditions, the GSSG content should be less than10% of the total GSH content. However, each species has its own genetic background, so it is difficult to compare

Table 1 Metal absorption and enzyme activities (Glutathione reductase, -GR; glutathione transferase, GTT; and gamma-glutamyl transferase, Gamma-GT)

	BY4741 (wt)		*lap4* strain	
Cd absorption (%)	22 ± 3		58 ± 2	
Activities (mU/mg ptn)	Non-stressed cells	Cd-stressed cells	Non-stressed cells	Cd-stressed cells
GR	5.7 ± 1.6 (a)	14.0 ± 1.1 (b)	7.1 ± 0.6 (a)	24.0 ± 0.9 (c)
GTT	1.4 ± 0.3 (a)	2.9 ± 0.1 (b)	2.0 ± 0.1 (c)	2.0 ± 0.2 (c)
Gamma-GT	54 ± 11 (a)	84 ± 6 (b)	15 ± 2 (c)	96 ± 4 (d)

Cadmium absorption was calculated by determining the difference in metal content between the control medium without cells and the test medium containing cells. Percentages of cadmium accumulation were calculated through the equation: $\text{Absorption}(\%) = \{[(\text{initial concentration}) - (\text{final concentration})]/(\text{initial concentration})\} \times 100$

Activities were measured in cells not exposed to Cd (non-stressed cells) and after exposure to 48 μM $CdSO_4$ during 24 h (Cd-stressed cells). One unit (U) of each enzyme is defined in Material and methods. The results represent the mean ± standard deviation of at least three independent experiments. Each group of results (Metal absorption; GR, GTT, gamma-GT activities) was analyzed separately to determine statistical differences (different letters mean statistically different results at $P < 0.05$)

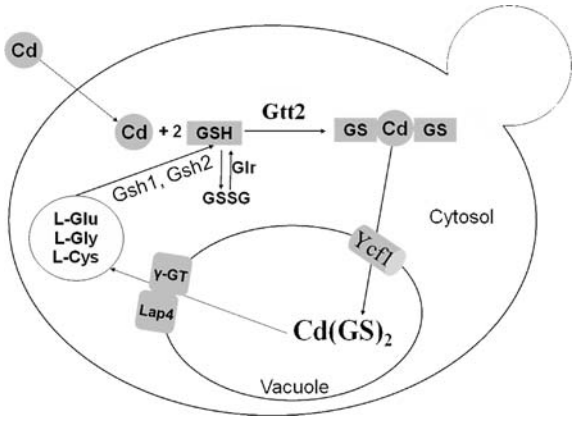

Fig. 1 Model of the mechanism of Cd detoxification in *S. cerevisiae* dependent on glutathione. In this figure: Cd, cadmium; L-Glu, L-Cys and L-Gly, L-aminoacids glutamate, cysteine and glycine, respectively; GSH and GSSG, reduced and oxidized form of glutathione, respectively; GS-Cd-GS and $Cd(GS)_2$, complex formed between cadmium and glutathione; Gtt2, glutathione transferase 2; Ycf1 transports the Cd–GSH complex to the vacuole; Gsh1 and Gsh2, enzymes responsible to GSH synthesis; Glr, glutathione reductase; γ-GT, gamma-glutamyl transferase; Lap4, cysteinylglycine dipeptidase

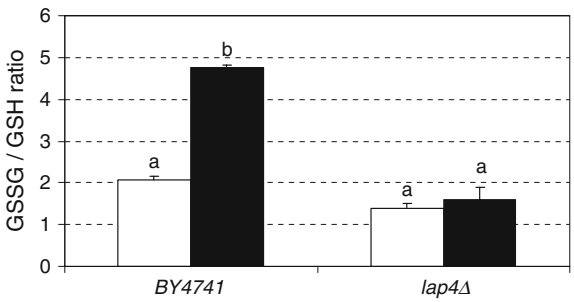

Fig. 2 Glutathione determinations. GSH and GSSG were measured in cells not exposed to Cd (white bars) and after exposure to 48 μM $CdSO_4$ during 24 h (black bars). The experiments were done as described in Material and methods. The results are expressed as ratios between the levels of GSSG and GSH (GSSG/GSH). The results represent the mean ± standard deviation of at least three independent experiments (different letters mean statistically different results at $P < 0.05$)

GSSG/GSH ratios in different biologic systems. Furthermore, in our experiments, cells were harvested in stationary phase. In this growth phase cells repire and the level of intracellular oxidation is higher than in first exponential phase, resulting in increased GSSG/GSH ratios.

To confirm that the maintenance of GSSG/GSH ratio in the *lap4* mutant is related to low levels of Cd–GSH complex, next, the activities of GR and glutathione transferase were measured (Table 1). The enzyme GR plays a crucial role in the antioxidant mechanism of defense by reducing the oxidized form of glutathione (GSSG), therefore affecting the GSSG/GSH ratio. However, GR activity was induced in both mutant and wt strains in response to Cd. On the other hand, the activity of glutathione transferase was activated by Cd only in wt strain. The formation of Cd–GSH complex requires the isoform Gtt2, a glutathione *S*-transferase (Adamis et al. 2004). Taken together, these results indicate that the *lap4Δ* strain is defective in the formation of the Cd–GSH complex, which activates Cd absorption, probably due to its deficiency in degrading GSH stored in vacuole.

Toxic effect of cadmium under lap4 deficiency

The increase of Cd absorption leads to higher intracellular oxidative damage and, consequently, to a reduction in cell viability (Adamis et al. 2003). Besides to causing nuclear mutations, exposure to Cd also induces petite mutants—unable to grow on a non-fermentable carbon source, like glycerol, owing to loss of mitochondrial function (Jin et al. 2003).

However, as can be seen in Fig. 3, Lap4 null mutant strain exhibited the same level of tolerance to Cd as wt, although it had absorbed almost 3-fold more metal (Table 1). Mitochondrial mutagenesis rate was lower in the mutant strain suggesting a mechanism independent of Lap4.

To measure the magnitude of oxidative damage caused by Cd, we analyzed the levels of protein carbonyls (as a by-product of protein oxidation) and

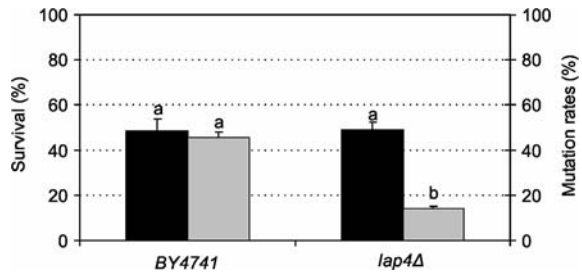

Fig. 3 Survival (black bars) and mutation rates (gray bars) after Cd exposure. BY4741 and *lap4Δ* cells were submitted to 48 μM $CdSO_4$ during 48 h. The experiments were done as described in Material and methods. The results represent the mean ± standard deviation of at least three independent experiments. Each group of results (Survival and mutation rates) was analyzed separately to determine statistical differences (different letters mean statistically different results at $P < 0.05$)

Table 2 Oxidative stress caused by Cd, measured as fold increase in lipid peroxidation and protein carbonylation

Strains	Lipid peroxidation	Protein carbonylation
BY4741	1.80 ± 0.10 (a)	1.20 ± 0.05 (a)
lap4Δ	0.70 ± 0.20 (b)	1.00 ± 0.05 (b)

Ratio between lipid peroxidation and protein carbonylation of Cd stressed and non-stressed cells. The results represent the mean ± standard deviation of at least three independent experiments. The letter denotes homogeneity between experimental groups at $P < 0.05$. Different letters mean statistically different results. Each group of results (lipid and protein) was analyzed seperately

lipid peroxidation (Table 2). Wt strain was more oxidized after Cd stress than Lap4 null mutant. These results emphasize the lower toxicity produced by Cd in *lap4Δ* strain, contrary to what had been found in cells deficient in gamma-GT (Adamis et al. 2007). The difficulty in maintaining the GSH levels by gamma-GT mutant strain render it unprotected against the oxidative stress caused by the metal.

In gamma-GT null mutant the recycle of GSH stored in vacuole can not be initialized, since GSH degradation begins with the hydrolysis of gamma-peptidic bond between glutamate and cysteine. In the absence of gamma-GT, the metal, complexed with GSH, is transported to vacuole but degradation of GSH is impaired generating a decrease in GSH levels and, consequently, an increase in the oxidative stress caused by Cd (Adamis et al. 2007). On the other hand, under Lap4 deficiency, part of GSH might be degraded in the reaction catalyzed by gamma-GT, releasing glutamate in the cytosol and the peptide CysGly, presumably retained in vacuole. Interestingly, in *lap4* mutant strain, the induction of gamma-GT activity caused by Cd was 3.5 times higher than in the wt (Table 1). This result might be explained as a form to compensate the absence of Lap4. Considering the importance of GSH recycle under Cd stress, the lack in Lap4 would be overcome by an increase in the remaining enzyme involved in GSH degradation, gamma-GT. Thus, in the mutant deficient in Lap4, high levels of glutamate from GSH compartmentalized in vacuole would be released in cytosol, enhancing cell antioxidant capacity. Glutamate activates its catabolic pathway, which increases NADPH (Coleman et al. 2001). This raise in NADPH pool could explain the low levels of oxidative damage in *lap4* cells after metal stress (Table 2). Interestingly, the activity of GR in *lap4* strain after Cd was almost 2-fold higher in the mutant than in wt (Table 1). NADPH is consumed by GR. Therefore, increased NADPH levels are required to support the increase of GR activity, which might be supplied by the higher gamma-GT activity found in *lap4* mutant. A higher GR activity might also contribute to more reduced intracellular environment in the mutant.

The remaining dipeptide CysGly in *lap4* null mutant strain cannot be used to synthesize another GSH molecule, since the enzyme Gsh1 acts on glutamate and cysteine residues and Gsh2 incorporates the glycine residue to the dipeptide glutamylcysteine. Furthermore, CysGly, apparently, does not leave the vacuole, standing bonded to Cd. Since this dipeptide neither forms new molecules of GSH nor complexes with Cd, the synthesis of Cd–GSH would be impaired in *lap4* mutant strain, favoring Cd absorption. Even presenting a high Cd absorption rate, lap4 cells remained alive, showing low mutagenic rates and oxidation levels. In the absence of Lap4, the compartmentation of the Cd–GSH complex is not affected, which protects the cells against mutations. The removal of Cd–GSH complex from the cytosol is vital to Cd tolerance (Adamis et al. 2007; Gomes et al. 2002). It is necessary to conduct additional studies to evaluate if continued exposure to Cd would alter tolerance of *lap4* null mutant strain.

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