UNIVERSITY OF PÉCS

PhD program for Biology

Molecular analysis of microorganisms life processes

Study of *Schizosaccharomyces pombe* strains under Cd^{2+} stress

PhD Thesis

Krisztina Takács

Supervisor:
Prof. Dr. Miklós Pesti

PÉCS, 2008

INTRODUCTION

All living creatures have the ability to respond to changes in the environmental conditions. The main general stress response pathway of *Schizosaccharomyces pombe* (*S. pombe*), the MAPK (mitogen-activated protein kinase) system, is responsible for eliminating diverse environmental stress stimuli such as osmolarity stress, heat shock and nutritional starvation. The Pap1 transcription factor is activated via Wis1 and Sty1 pathway in different stress responses. The transcription factor Pap1, a *S. pombe* bZIP protein homologous to mammalian AP1, plays a crucial role in defense against oxidative stress, and a variety of cytotoxic agents.

Cells cope with heavy metal stress by using different mechanisms. Cd^{2+}, a nonessential element, enters the cell through the same transport system as used by essential heavy metals. The toxicity of Cd^{2+} and other heavy metal ions lies in the strong binding affinity to metal-sensitive groups, resulting in the blockade of functional groups of biologically important molecules, the displacement and/or substitution of essential metal ions such as Ca^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} of enzymes, conformational modification, denaturation and inactivation of enzymes, and the disruption of cellular and organelle integrity. Investigation of the processes initiated by Cd^{2+} necessitates a versatile summary of molecular and enzymatic analysis, and an overview of the genes of interest. The processes and the related genes can be arranged in three groups: i) glutathione (GSH) and sulfur metabolism; ii) Cd^{2+} compartmentalization; iii) ROS (reactive oxygen species) detoxification.

Biomolecules which are involved in Cd^{2+} detoxification contain much sulfur, as they are cysteine-rich proteins. The processes of sulfur assimilation and cysteine biosynthesis have therefore been well examined and this knowledge is essential for an understanding of the cell response to Cd^{2+} stress. The glutathione S-transferases (GSTs) are important in the elimination of many xenobiotic compounds and in protecting cells from oxidative stress by detoxifying certain secondary ROS produced when Cd^{2+} attacks the cellular components. GSH is present in all organisms participating in multiple metabolic processes, such as intracellular redox state regulation, inactivation of ROS. The production of ROS is known to be one of the major mechanisms of the toxicities exerted by Cd^{2+} in many different organisms. Superoxid dismutases (SODs), peroxidases, catalase and GSH play role in the elimination of the ROS.

THE AIM OF THE STUDY

1. In our experiments we examined the Cd^{2+} sensitivity of the *S. pombe* Cr(VI) tolerant and the parental strains. We also measured those enzymes' specific activities which could be play an important role in heavy metal detoxification.

2. In the second part of the study, the sensitivity of *S. pombe* MAPK and transcription factor mutant strains were examined during the treatments of different stressors.

3. We investigated the molecular and enzymatic changes in the transcription factor mutant strain *Δpap1* in response to Cd^{2+}-stress. Recent studies have focused on the processes occurring in short-term Cd^{2+} exposure (up to 60 minutes). Indeed, we have now demonstrated the effect of long-term Cd^{2+} treatment on:
   - Cd^{2+} uptake
   - ROS production
   - enzyme activities with function in Cd^{2+} detoxification
   - and the changes of several genes expression.

MATERIALS AND METHODS

The following strains were used in our experiments:

I) *Schizosaccharomyces pombe* Cr(VI) tolerant mutant, which was originated from an auxotrof parental strain by induced mutagenesis.

II) *Schizosaccharomyces pombe* MAPK and transcription factor deletion mutant strains:

- *Δwis1*, *Δsty1, Δatf1, Δsin1, Δpap1* and a wild-type.

I. Investigation of the Cr(VI) tolerant strain

- The survival rates of the strains under Cd^{2+} stress were determined by colony counting.
- The specific SODs and GST enzyme activities were measured by spectrofotometry.

II. Investigation of the MAPK and the transcription factor mutant strains

- The minimal inhibitory concentrations were determined by spot test assay.
- The Cd^{2+} uptake of the cells were investigated by flame atomic absorption spectrometry.
- The conversion of dihydroethidium to ethidium was detected by a fluorimeter during superoxid level experiments.
- The peroxide levels of the cells were measured by flow cytometry.
- The specific SODs, GST enzyme activities and the GSH, GSSG levels were determined by spectrofotometry.
- The analysis of the RNA contents were determined by reverse transcriptase PCR and Northern blot.

RESULTS AND DISCUSSION

I. The Cr(VI)-tolerant strain was investigated to gain more information about its phenotype. The Cr(VI)-tolerant mutant was found to be sensitive to Cd^{2+}. It was demonstrated that GST and SODs enzyme activities did not influence the Cr(VI)-tolerant and the Cd^{2+}-sensitive phenotype. Further on it was stated that the Cr(VI)-tolerant phenotype of the strain were caused by the decreased reduction capacity, GSH content and GR activity of the cell.

II. Spot test assay was used to determine the sensitivity, *i.e.* the minimal inhibitory concentration of $CdSO_4$ for the MAPK signal transduction mutant and the transcription factor mutant strains. The *Δatf1* and *Δsin1* mutants exhibited similar resistance to that of the wild-type cells. The *Δwis1* and the *Δsty1* MAPK mutants were sensitive; however, of the strains carrying the deletion of different transcription factors, only the *Δpap1* mutant proved to be the most sensitive to Cd^{2+}. The spot test assay of MAPK signal transduction mutant strains and the transcription factor mutants demonstrated that the transcription factor Pap1 plays the main role in the response to Cd^{2+} stress. The genes involved in the Cd^{2+} detoxification processes should be induced and regulated in a Wis1-Sty1 SAPK-dependent manner, and the Pap1 transcription factor was found to be required for normal resistance to Cd^{2+}.

The Cd^{2+} uptakes of the *Δpap1* and the wild-type strains were determined at 0, 30, 60, 120 and 240 minutes. The Cd^{2+} uptake of the two strains was the same The kinetics of Cd^{2+} uptake was also the same in both strains, showing that the Cd^{2+} uptake system was not damaged in the *Δpap1* mutant, so that this could not influence the subsequent results.

We examined whether Cd^{2+} treatment did or did not cause oxidative stress in *S. pombe*. Significantly higher superoxide anion levels were measured in the *Δpap1* mutant strain than in the wild-type strain compared to the untreated 0 and 120-minute samples. There were no differences in the superoxide level in the samples at 240 minutes. Cd^{2+} treatment resulted in an increased superoxide level in the wild-type strain, but no change could be seen in the *Δpap1* mutant. Determination of the superoxide level in the wild-type strain proved that superoxide was generated during long-term Cd^{2+} treatment, suggesting that Cd^{2+} could trigger oxidative stress. The significantly higher amounts of superoxide measured in the untreated *Δpap1* mutant can be explained by the fact that the Cu,Zn-SOD could not catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen, and this resulted in a lower amount of peroxide. A significantly lower peroxide level was observed in the *Δpap1* mutant strain than in the wild-type strain as regards the untreated 0 and 120-minute samples. Both strains demonstrated a significantly decreased peroxide level during Cd^{2+} treatment. The

low amounts of peroxide in the Cd^{2+}-treated samples could be explained by the induction of catalase and peroxidases, which eliminated the peroxides.

Specific enzyme activities, which can be important in Cd^{2+} triggered oxidative stress, were determined to acquire more information on their role. The total SOD activity increased in the wild-type strain, but decreased in the *Δpap1* mutant during Cd^{2+} treatment. The difference is probably explained by the changes in the Cu,Zn-SOD level in the strains. The increased SOD activity can be explained by that the cell needs to eliminate the ROS generated during Cd^{2+} caused oxidative stress. A significantly lower SOD activity was measured in the *Δpap1* mutant during Cd^{2+} treatment, suggesting that the induction in enzyme activity of the Cu,Zn-SOD or/and Mn-SOD depends on Pap1 during Cd^{2+} stress. On the other hand, a previous study indicated that Cu,Zn-SOD was related to the sulfate assimilation pathway. The absence of Pap1 can result in a lower SODs activity (probably Cu,Zn-SOD) and the disturbance of the sulfate assimilation pathway. Therefore, the ROS generated during Cd^{2+} treatment can not be eliminated properly and can cause a Cd^{2+}-sensitive phenotype.

The regulation of expression *gst1* and *gst2* is Pap1-dependent. Consequently, the *Δpap1* mutant does not produce GST I and II. As the *Δpap1* cells possess no GST I and II, and there was a lower overall GST activity in the *Δpap1* mutant, suggesting that GST III cannot take over the function of the missing GST I and II. Cd^{2+} treatment caused no significant changes in GST specific activity in the wild-type strain relative to the control, suggesting that the GSH depletion can be replaced by the subsequent production; the GST therefore has sufficient substrate and all three isoenzymes can catalyze the reaction of Cd^{2+} binding to GSH. In contrast, the GST activity decreased further in the *Δpap1* mutant exposed to Cd^{2+}. These data strongly indicate that the GST III activity alone is not sufficient for the cell to cope with Cd^{2+}. We further measured the amounts of GSH and GSSG and the GSH/GSSG ratio. Our results confirmed the previous theory, with detection of a significantly lower GSH level in the *Δpap1* mutant. In the *Δpap1* mutant, the GSH level remains low because of the Pap1-dependent regulation of GR and the decrease of sulfide assimilation.

To gain further insight into the cellular function of long-term Cd^{2+} treatment, we investigated the expressions of genes involved in oxidative stress and Cd^{2+} detoxification. The expressions of twelve genes (*gpx1* - glutathione peroxidase [GP], *gsh2* - GS, *gst2* – GST, *hmt1* – ABC-type vacuolar transporter protein, *hmt2* - sulfide-quinone oxidoreductase, *sod1* – Cu,Zn-SOD, *sod2* – Mn-SOD, *SPAC3H1.10* - PCS, *SPCC965.06* - putative K^+ channel subunit, *SPCC794.01* – predicted glucose-6-phosphate 1-dehydrogenase, *SPC3C7.13* - predicted glucose-6-phosphate 1-dehydrogenase, *trx2* – thioredoxin) which encode proteins involved in the cellular Cd^{2+} detoxification processes were studied by RT-PCR. The RT-PCR

analysis proved that the *gpx1, hmt1, hmt2, pcs, sod1, sod2* SPAC3C7.13, SPCC965.06, SPCC794.01 and *trx2* expressions are independent of the Pap1 transcription factor in untreated and Cd^{2+}-treated *S. pombe* cells.

The $CdSO_4$-induced alterations in the mRNA levels of the strains were further investigated by Northern blot hybridization. We could not observe any changes in expression rates for the genes of *gpx1, hmt2, sod1, sod2* and *trx2* in the control and Cd^{2+}-treated *S. pombe* cells. However, our Northern blot analyses provided new information on Cd^{2+} stress in the wild-type strain when long-term (120-240 minutes) Cd^{2+} treatment was investigated. This study is the first report on long-term Cd^{2+}-treated *Δpap1* strain. It was shown that the putative K^+ channel subunit (SPCC965.05) was induced by long-term Cd^{2+} stress. The same induction could be observed in the *Δpap1* strain, indicating that the SPCC965.05 gene is not regulated by Pap1 and is not the cause of the Cd^{2+}-sensitive phenotype in this mutant. The *hmt1* gene was repressed after a 60-minute (long-term) Cd^{2+} treatment in both strains. The GST II gene (*gst2*) expression level was constant during the Cd^{2+} treatment in the wild-type strain, but there was no expression in the *Δpap1* mutant. New data were obtained on *SPCC794.01* (glucose-6-phosphate1-dehydrogenase) and *SPAC3H1.10* (PCS) by RT-PCR and Northern analysis: no expression profile was found in the microarray database for these two genes in either the wild type or the *Δpap1* strain. It is worth mentioning that the induction of Cu,Zn-SOD and Mn-SOD genes cannot be observed at a transcription level, but we demonstrated a significantly lower SODs activity in *Δpap1* mutant, suggesting that Pap1 effects on the SODs enzyme activity and might take part in some post-translational induction. These observations provide new information and support previous results on the post-translational regulation of Cu,Zn-SOD.

In summary, the Cd^{2+}-sensitive phenotype of the *Δpap1* mutant can be explained as follows. The *Δpap1* mutant accumulates the same amount of Cd^{2+} as the wild-type strain, although the *Δpap1* mutant cannot eliminate the effect of the intracellular Cd^{2+} and the generated ROS. The low GSH and sulfide production results in decreased GST activity in the *Δpap1* mutant; therefore the scavenging of Cd^{2+} does not process accordingly. No induction of SODs activity could be observed in the *Δpap1* strain, suggesting that the presence of Pap1 protein is essential for normal enzyme activation in cellular responses to Cd^{2+}. Not any changes in *sod1* and *sod2* expression were observed by Northern blotting, indicating that Pap1 might be involved in the post-transcriptional/post- translational induction of Cu,Zn-SOD and/or Mn-SOD in response to long-term Cd^{2+} stress.

PUBLICATIONS

Significant contributions to Thesis:

Takács, K., Gazdag, Z., Raspor, P., Pesti, M. (2007): Gene expressions and enzyme analyses in the *Schizosaccharomyces pombe Δpap1* transcription factor mutant exposed to Cd^{2+}. *J. Basic Microbiol.* 47(4):74-83.

Gazdag, Z., Pócsi, I., Belágyi, J., Emri, T., Blaskó, Á., **Takács, K.** and Pesti, M. (2003): Chromate tolerance caused by reduced hydroxyl radical production and decreased glutathione reductase activity in *Schizosaccharomyces pombe*. *J. Basic Microbiol.,* 43: 96-103.

Abstracts used in preparation of Thesis:

Takács, K., Blaskó, Á., Gazdag, Z., Pesti, M. (2002): *Schizosaccharomyces pombe* jelátviteli mutánsainak nehézfém és oxidatív stressz érzékenysége. A Magyar Mikrobiológiai Társaság 2002. évi Nagygyűlése Balatonfüred, Hungary, pp. 19.

Takács K., Pesti M. (2004): *Schizosaccharomyces pombe Δpap1* jelátviteli mutánsának génexpressziós vizsgálatai kadmium kezelés hatására. A Magyar Mikrobiológiai Társaság 2004. évi Nagygyűlése. Keszthely, Hungary

Takács, K., Pesti, M. (2005): Gene expressions in *Schizosaccharomyces pombe Δpap1* signal transduction mutant exposed to cadmium. 33th Annual Coference On Yeasts, Smolenice, Slovakia

Other contribution:

Gyetvai, Á., Emri, T., **Takács, K.**, Dergez, T., Fekete, A., Pesti, M., Pócsi, I. Lenkey, B. (2006): Lovastatin posesses a fungistatic effect against *Candida albibans,* but does not trigger apoptosis in this opportunistic human pathogen. *FEMS Yeast Res.*, 6(8):1140-8.

Other abstracts:

Gazdag, Z., Farkas, N., Belágyi, J., Papp, G., Rácz, T., **Takács, K.** and Pesti, M. (2003): Altered cadmium sensitivity of respiratory-deficient *Schizosaccharomyces pombe* mutant. 14th International Congress of the Hungarian Society for Microbiology, Balatonfüred, Hungary

Pesti, M., Gazdag, Z., **Takács, K.**, Czakó-Vér, K., Koósz, Zs., Antal, J., Rácz, T. (2004): A krómvegyületek hatásmechanizmusa élesztősejteken. A Magyar Mikrobiológiai Társaság 2004. évi Nagygyűlése. Keszthely, Hungary

Czéh, Á., Gazdag, Z., Vér, Cs., Rudolf, P., Kulik, Z., Nagy, K., Őss, M., **Takács, K.**, Borsodi, A., Márialigeti, K., Pesti, M. (2005): The role of alkaliphilic bacterim species in the compost production enriched with wood ashes. Magyar Mikrobiológiai Társaság 2005. évi Nagygyűlése és a 1st Central European Forum for Microbiology (CEFORM), Keszthely, Hungary