UNIVERSITY OF PÉCS

Ph.D. program for Biology

Molecular analysis of microorganisms' life processes

The analysis of oxidative stress processes in *Schizosaccharomyces pombe* fission yeast cells

Ph.D. thesis

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PÉCS, 2013

Introduction

All organisms must face various types of stress from the environment and also from the intracellular space. This **external stress** can be: osmotic stress, heat stress, stress caused by starvation or by toxins, DNA damaging and oxidative stress. Aerobic organisms produce reactive oxigen species (ROS) through life processes but always try to reach an optimal balance of homeostasis by antoxidant defense systems as effectively possible. The most important parts of this defense system are the antioxidants, antioxidant enzymes and metal-binding peptides.

Some metals play an essential role in living organisms. These are the so called essential metals and can usually be found as a component of the enzyme. Whether essential or non-essential, any metal above a certain concentration in the environment can be lethal to an organism. In several industrial production procedures different metals are used. The most toxic of these is chromium. Chromate anion can be reduced to Cr(III) in cells meanwhile reactive oxigene species (ROS) are produced. The resulting compounds are neuro- and genotoxic as well as carcinogenic and damage the normal cell functions.

Living organisms can absorb chromium compounds in different quantities and chromate ions affect them in different ways. Some varieties of plants or microbes are able to survive despite of the high heavy metal concentration in the industrial environment. This fact could provide for the protection against metal pollution if we had exact knowledge of inheritance of metal sensitivity and tolerance and knew the stress responses induced by metal pollution.

Investigation of the effects of chromium and other heavy metals and their effects on living cells remain actual nowadays because several processes are still unknown. The antioxidant system induced by external chromium compound stressors is very complex (enzymes, catalysators, transcription factors). Studying the genes responsible for chromium tolerance and chromium sensitivity is important for discovering the chromium effects' mechanisms.

Besides the external stress effects induced by heavy metals, so called internal stress responses can be caused by mutations and viral proteins. Proteins coded by different infective agents can also induce oxidative stress in cells. The disease called AIDS is caused by HIV-1 and HIV-2 viruses. Nowadays 50 million people are suffer from it, but the number of patients is growing day by day.

HIV first damages $CD4^+$ T-lymphocytes directly or by integration of provirus into the lymphocyte's DNA and integrates into it until virus production starts due to some triggers. Infection is followed by a long term incubation period. During the incubation period the volume of $CD4^+$ T lymphocytes decreases until it reaches a critical level, so the immune system is not able to stop the infectional processes of pathogens. Consequently many diseases may appear such as opportunist infections, tumors, direct damages and the nerve system's complications due to systematic diseases.

Most of the studies search for the solution to prevent the viral infection or to prevent diseases. Recent years investigations focus on a viral protein R (Vpr) that can be found in HIV-1, HIV-2 and SIV viruses linked to virion and plays a role in viral infection processes. The main role of Vpr protein is still not clear but there are evidences that it is responsible for many process during the infection: it blocks proliferation in G2/M phase, it is responsible for breaking the nucleus membrane and leads to apoptosis.

We studied the stress responses to both chromium and Vpr protein on the same model organism, on fission yeast *Schizosaccharomyces pombe*. Most of its genes show high similarity to eucaryotic cell genes. Heterothallyc strains allow to use the classical genetic analysis (tetrade analysis). Mutations in genes are appearing phenotypically due its haploid chromatine. Cell cycle and stress responses are also similar to other eucariotic organisms. That is why *Schizosaccharomyces pombe* is a perfect model organism.

Specific Aims

Oxidative stress is one of the main fields of research at the Department of General and Environmental Microbiology at the University of Pécs, Faculty of Sciences. To analyze the mechanism of action of various agents and their oxidative stress inducing properties, the eukaryotic unicellular fission yeast called *Schizosaccharomyces pombe* has been used.

A priority field has been the study of mechanism of action of chromium compounds, their stress inducing properties as well as the functional characterization of the gene or genes responsible for chromium sensitivity and tolerance. During our research, chromium-sensitive and tolerant strains of *S. pombe* were used that were isolated by mutagenesis at our department (Czakó et al. 1999). In the case of the selected mutant strains, those mutants were selected first in which the altered chromium sensitivity was resulted by the mutation of a single gene.

According to our hypothesis different proteins with viral origin may or do function as inner stressors. During HIV-1 infection, the measurable level of GSH is decreased in both the plasma and cerebrospinal fluid that may be the result of the altered oxidoreductase system, thus our research focused on the proteins of HIV-1. Our department possesses *S. pombe* strains with integrated genes encoding either the wild-type Vpr protein (NL43) (RE007 strain) or one of the mutant Vpr proteins (F34I) (RE076). These constructs enabled studies to focus on the stress inducing effects of the Vpr protein on eukaryotic cells.

While studying the effects of different inner and outer oxidative stress mechanisms on the cells of *Schizosaccharomyces pombe,* the following specific aims were proposed:

1.) To analyze spore clones derived by crossing *chr1-66T* and *chr2-046T* strains as well as *chr1-66T* and *chr1-14T* strains using random spore analysis and to determine the ratio of the chromium-tolerant and intolerant progeny. It was important to isolate a gene-damaged, chromium-sensitive or chromium-tolerant mutant for subsequent studies.

2.) To measure at what rate ROS increases in the RE0076 strain expressing mutant Vpr protein as well as to study how the cell regulates the antioxidant defense system in the presence of the Vpr protein.

Method

89chr^{+}(*89chr*$^{+}$*ura4-D18 h*$^{+}$*), 90chr*$^{+}$*(90chr*$^{+}$*ura4-D18 h*$^{-}$*), chr1-14T (chr1-14Tleu1-32 h*$^{-}$*), chr2-046T (chr2-046Tleu1-32 h*$^{-}$*), 9chr*$^{+}$ *(9chr*$^{+}$ *leu1-32 h*$^{-}$*), chr1-661T (chr1-661Tlys1-31 h*$^{+}$*), chr-63T (chr-63Tlys1-131 h*$^{-}$*), and* SP223 *(ade6-216 leu1-32 ura4-294 h*$^{-}$*), and* RE076 *(leu1-32 ura4-249 ::vpr(F34I)::ura4*$^{+}$ *ade6-216 h*$^{-}$*)*. F34I*vpr* gene-carrying plasmid, which is under the control of an inducible *nmt1* (no-message thiamine) promoter, was integrated as a single copy gene at the *ura4* gene locus in the *S. pombe* chromosome of a wild type *S. pombe* strain SP223.

Cell numbers were determined with a hemocytometer.

The minimal inhibitory concentrations were determined by spot test assay.

Spore clones obtained from the crosses were tested for chromium tolerance by spot test assay by using YEA.

The cells were stained with calcofluor white M2R to enhance the visualization of the septa. After staining, the number of cells passing mitosis was estimated in triplicate by

counting the proportion of septated cells in random fields of approximately 500 cells. The number of living cells was assessed by using propidium iodide.

Cell-free extracts were prepared by using X-pressing (Dybecksgatan 10, S-412 70 Göteborg, Sweden). The protein content of the cell-free extract was measured by a Lowry method with modification.

The specific intracellular activities of glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase G6PD, catalase, superoxide dismutases (Cu/Zn-SOD and Mn-SOD), and the specific intracellular concentrations of glutathione (GSH) and glutathione disulphide (GSSG), were determined by using well-established colorimetric assays.

To estimate the intracellular peroxide and superoxide levels, the indicators dihydrorhodamine 123 (DHR 123) and dihydroethidium were used, the formation of rhodamine and ethidium (ET) was quantified spectrofluorimetrically and by flow cytometry, respectively. The *in vivo* generation and reduction of Cr(V) in *S. pombe* was followed by electron paramagnetic resonance (EPR) spectroscopy. The *in vivo* and *in vitro* formation of $^{\bullet}OH$ was measured by using the spin trap 0.1 M N-*tert*-butyl-*α*-phenyl nitrone (PBN).

Data represent the mean of four independent experiments. *p* values were calculated using the Student's t-test.

Results

Analysis of the inheritance of chromium tolerance and sensitivity

Chromium-sensitive and tolerant mutants were isolated from wild-type *S. pombe* strain by NTG (N-metil-N-nitro-N-nitrozoguanidin) mutagenesis and UV irradiation (Czakó el al., 1999). Eleven chromium-sensitive or tolerant strains were crossed with the *ura4D18* marker expressing *89chr*$^{+}$*h*$^{+}$ and *90chr*$^{+}$*h*$^{-}$ strains considering the adequate mating types. Among the mutants tested, only crossing of the *chr1-661T*, *chr2-046T* and *chr1-14T* strains with the *89chr*$^{+}$*h*$^{+}$ and *90 chr*$^{+}$*h*$^{-}$ resulted in zygotic asci that were further analyzed by tetrad analysis and random spore analysis. Strains of *chr1-661T* (lys1-131 h^{+}, $MIC_{Cr(VI)}$:275μM) and *chr2-046T* (leu1-32 h-, $MIC_{Cr(VI)}$:300 μM) were crossed. The production of asci as well as the subsequent random spore analysis were performed by protoplast fusion. We have tested 104 spores; 44 out of 104 were chromium tolerant ($MIC_{Cr(VI)}$:275 μM), while 60 were chromium sensitive. These results suggest that the single gene mutations are not allelic in the two strains, i.e. they occurred in two different genes. When crossing the *chr1-661T* (lys1-131 h^{+}, $MIC_{Cr(VI)}$:275μM) and *chr1-14T* (leu1-32 h^{-} $MIC_{Cr(VI)}$:275μM) strains, 84 spore clones were

isolated. 73 out of 84 showed similar chromium tolerance to parents ($MIC_{Cr(VI)}$:275µM). Although eleven clones were chromium tolerant, the level of tolerance slightly decreased ($MIC_{Cr(VI)}$:250µM). These results demonstrate that the mutation resulting in chromium tolerance occurred in the same gene in the two strains. Both the tetrad and random spore analyses confirmed that the *chr1-661T* strain has stable, single gene Cr(VI) tolerance. Later it has been shown that the Cr(VI) tolerant phenotype of the *chr1-66T* mutant is associated with oxidative stress sensitivity. This enabled the separation of the tolerant and parent strains at the level of a single cell as well as the development of a direct selection method for subsequent transformation experiments.

Cross-resistance experiments for chromium, cadmium, copper, zinc, and nickel containing metallic compounds were performed on the *9chr*$^+$ and *chr2-04T* strains, as well as on the *89chr*$^+$ and *90chr*$^+$ *ura4D18* strains with auxotroph properties. We have demonstrated a wide range of cross-sensitivity in the mutants. In case a mutant strain is tolerant to chromium, it does not mean resistance to other metals. The reproduction rate of the mutants showing different sensitivity was also altered. This is because the alteration of the growth curve depends on the antioxidant defense system that is also influenced by chromium-sensitivity.

The effect of the Vpr protein:

First we focused on the reproduction of S. pombe RE076 mutant Vpr protein expressing strain in the presence of thiamine (gene repression) and without thiamine (gene expression) and the parental Sp223 strain, too. We found that cells of the F34Ivpr-expressing, F34Ivpr-repressing and parental strains exhibited normal growth up to 20 h at 30 °C. Under vpr expressing conditions (in cultures without thiamine), multiplication of the cells of strain RE076 stopped at 20 h and started again at 26 h. The reason is that the Vpr induced G2 blocks the cell cycle of the F34Ivpr expressing cells. The growth of F34Ivpr repressed strain RE076 became slower after 20 h, and at the end of cultivation (35 h) its cell number was 20% less than that of its parental strain. Vpr-induced G2 inhibition was confirmed by determination of the septation index. Vpr-induced G2 inhibition was confirmed by the determination of the septation index. Cells in the logarithmic phase contain approximately 10–20% septa. In contrast to the parental strain SP223, Vpr expression in strain RE076 exhibited a rapid decrease in septum formation, suggesting that the cells were unable to enter mitosis but were arrested at the G2/M boundary; the vpr-repressing cells were only slightly different from

those of the parental strain SP233. Septa were observed in almost 25% of Sp223 parental strains, 17% of RE076 F34Ivpr repressing strains. Vpr induced morphological change of the F34Ivpr expressing cells (the cells exhibited elongated 'cdc' phenotype) in the 35-h culture, but not in the 14 h culture. The reason of difference between Sp223 and RE076 Vpr repressing strains is the 97-98% effectiveness of nmt1 promoter region. For control strain the SP223 was chosen.

Based on our preliminary results and earlier published data, we decided to investigate the effect of F34Ivpr on oxidative stress at early (14-h) and late (35-h) log phase cultures. We were searching for the effect of mutant Vpr protein indicating physiological changes in cells.

Significant decrease of GSH concentrations was detected in both the 14- and 35 h cultures of the F34Ivpr expressing cells. The GSSG concentration of the F34Ivpr expressing cells was only decreased at 14 h but increased at 35 h. The decreased GSH level showed that ROS level probably increased. Superoxide ($O_2^{\bullet-}$) concentration of F34Ivpr expressing strain, calibrated to ethidium bromide (ET) significantly decreased in 14 h culture and increased in 35 h one as opposed to its parental strain SP223 despite of the total decrease of the SOD activities at both timepoints. Significanlyt increasing level of peroxides was observed in mutant RE076 strains at 14 and 35h cultures as compared to Sp223 parental strain. Intensity of peroxides was much lower in 14h cultures than in 35h cultures but it depended on the age of strains. The increased level of peroxides together with lower level of GSH leads to decreasing cell number.

No differences in Cr(V) concentrations under F34Ivpr inducing conditions of 14 h and 35 h cultures were detected, however addition of 2 mM NADPH resulted in significant increase of Cr(VI) reducing ability of parental strain SP223, especially in 14h culture. Significantly decreased $^{\bullet}OH$ radical production of RE076 strain was detected after addition of Cr(VI) or Cr(V) and NADPH both in 14 h and 35 h cultures. Level of Cr(V) was almost four times higher in parental strains than in RE076. The same process was observed in 35h cultures with a lower increasing tendency. Significantly decreased hidroxil-radical concentration was experienced in RE076 after adding Cr(VI) to Cr(V)+NADPH both in 14 and 35h cultures despite of a high level of H_2O_2 concentration and increased level of superoxide ($O_2^{\bullet-}$) in 35h culture. These data show the oxidative status of RE076 cells.

At the early log phase of F34Ivpr expressing cells, decreased specific activities of SOD, CAT, GR and G6PD were detected. No changes were seen in the specific activities of GPx and GST. These alterations in the specific activities in the F34I vpr-expressing cells might be the result of decreased concentration of GSH and elevated concentration of H_2O_2.

At the late log phase, in the 35-h culture, the F34Ivpr expressing cells exhibited the same decreased levels of the above mentioned enzymes (i.e., GST, SOD, CAT and G6PD) and increased GPx activity and superoxide.

Our results support that the increasing level of ROS is connected to ROS-regulated redox-sensitive transcription, as ROS determine the survival of cells both in human and in fission yeast cells.

Summary

1. The research program of our General and Environmental Microbiology Department is the effect mechanism of chromium in *S. pombe* cells regarding gene analysis for chromium tolerance and chromium sensitivity. From the 11 mutant strings produced by induced mutation those chromium tolerant mutants had to be selected which had only one damaged gene. Not all strains to be crossed were able to form zigotic asci. Therefore, our aim was randomspora analysis besides tetrad analysis of the derived crossed spore from *chr1-661T* , *chr2-046T, chr1-661T* and *chr1-14T* strains to examine the proportions of chromium-tolerant and chromium sensitive offsprings. For the rest of the research was necessary to isolate a one-gene damaged chromium sensitive and a one-gene damaged chromium tolerant mutant. The *chr1-661T* and *chr2-046T, the chr1-661T and* chr1-*14T* strains were crossed, spore clones were isolated, and chromium tolerance tests were done on each isolated spore clone.

I found that after crossing the *chr1-661T* and *chr2-046T* strains, from the separated 104 clones 60 were chromium sensitive and 44 were chromium tolerant. These results proved that two different genes are responsible for chromium tolerance. From the isolated 84 spore clones of *chr1-661T* and *chr1-14T* strains 73 showed tolerance to chromium, so the tested mutants had to have the same gene mutations. I determined the cross resistance of *9chr*$^{+}$*, chr2-04T*, *89chr*$^{+}$ and *90chr*$^{+}$ strains with *ura4D18* auxotrophy for cadmium, iron, zinc, vanadium, nickel, copper and selenium. I found that chromium tolerance does not mean automatically tolerance for other metals as well.

The results of this work led to further tests, to Koósz et al., (2008) pUR18N vector transformed experiments, which later proved the GR plays a decisive role in reduction of Cr(VI).

2. In previous studies it was found that viral proteins may operate as internal stressors in cells. We examined the effects of HIV-1 Vpr protein on *S. pombe* cells which expresses the

protein under certain circumstances (regulated by promoter region). For this we used a mutant Vpr protein in which there is only one amino acid difference from the wild type Vpr protein, but does not kill the cells after the G2/M block, allowing them to restart the reproduction cycle. This feature allowed us to examine the oxido-reductant mechanisms of older cultures. Our aim was to determine the growing of ROS level in mutant Vpr protein-expressing cells, and how the affected cell regulates the antioxidant defense system in the presence of the Vpr protein. Comparing the mutant F34Ivpr-expressing strain (RE076) to the parental strain (SP223) in an early log (14 hour culture) and a later log (35 hour culture) growth phase we proved first that F34IVpr caused oxidative stress in the cells. By generating ROS, increased the concentrations of superoxide and peroxide, resulting reduced intracellular GSH and hydroxyl radical that led to significant lower levels of SODs, CAT and GSH-dependent GPx, GR, GST and G6PD antioxidant enzymes specific activity.

Our results clearly show that this oxidative stress response is atypical, which is explained with the Vpr protein, by inducing major changes in other main processes parallel with the oxidative stress process, resulting the cells can not regulate anymore the changes caused by the oxidative stress to restore the redox homeostasis. These results and observations highlight the role of Vpr protein in HIV-1 infection which can be foundations for future studies of oxidative stress caused by Vpr protein.

List of publications

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Impact Factors: 7,336