GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Genes involved in glucose repression and oxidative stress response in the fission yeast *Schizosaccharomyces pombe*

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Genet. Mol. Res. 10 (4): 4041-4047 (2011)
Received October 13, 2011
Accepted October 27, 2011
Published November 8, 2011
DOI http://dx.doi.org/10.4238/2011.November.8.4

ABSTRACT. We looked for changes in gene expression and novel genes that could be involved in the interaction between glucose repression and oxidative stress response in the fission yeast, *Schizosaccharomyces pombe*, using a constitutive invertase mutant, *ird11*, which is resistant to glucose. BLAST analysis was made of the *S. pombe* genome database of cDNAs whose expression ratios differentially decreased or increased upon exposure to mild oxidative stress in this mutant compared to the wild type. Genes with this type of activity were identified as *rpl302*, encoding 60S ribosomal protein L3, and *mpg1*, encoding mannose-1-phosphate guanyltransferase; their expression patterns were measured using quantitative real-time PCR. We found that the expression levels of *rpl302* and *mpg1* genes in *ird11* under unstressed conditions were increased compared to those of the wild type. Under stress conditions, the expression levels of the *rpl302* gene were decreased in both strains, while *mpg1* expression levels remained unchanged. These results suggest that these genes play a role in the response to oxidative stress in this mutant strain.

Key words: *Schizosaccharomyces pombe*; Glucose signalling; Oxidative stress response; Glucose repression; Differential display

INTRODUCTION

Glucose is the most preferred and efficient carbon and energy source for almost all organisms. This simple sugar also regulates, via glucose sensing and signaling, a variety of metabolic pathways such as the respiratory pathway, stress response element and glycolysis enzymes for utilization of alternative carbon sources, glucose transporters, etc., resulting in opportunities to act as a metabolic intermediate, cofactor and end-product (Voet et al., 1999; Johnston, 1999; Flores et al., 2000; Rolland et al., 2001).

Eukaryotic organisms have myriad complex strategies for responding and regulating glucose sensing and signaling pathways. Studies for revealing these mechanisms have been done extensively in budding yeast *Saccharomyces cerevisiae*, one of the most important eukaryotic model organisms (Rolland et al., 2001; Gancedo, 2008). Another yeast *Schizosaccharomyces pombe*, which has shown significant similarities withmammalian cells in terms of features at the genetic, biochemical and molecular level (Schmidt et al., 2007), is also commonly used for studies focusing on glucose metabolism (Lin et al., 2000; Janoo et al., 2001; Roux et al., 2009) and oxidative stress response (Toone et al., 2001; Chen et al., 2003, 2008; Ikner and Shiozaki, 2005).

Glucose is primarily detected by membrane receptors (G protein-coupled receptors; GPCRs) and generates a signal via the cAMP-dependent protein kinase A (PKA) signal pathway in *S. pombe*. (Welton and Hoffmann, 2000; Hoffman, 2005). It is known that glucose depletion or respiratory growth on a non-fermentable carbon source leads to increased oxidative stress response in *S. pombe* (Moradas-Ferreira et al., 1996; Quinn et al., 2002; Chen et al., 2003; Madrid et al., 2004; Kim et al. 2006). The central element of the mitogen-activated protein kinase (MAPK) cascade directing the transcriptional response to oxidative stress in this fission yeast is MAPK Sty1, and this protein kinase activates the transcription factors Atf1p and Pap1p (Ikner and Shiozaki, 2005). Additionally, glucose sensing and signaling regulates the increased expression of various stress response genes through the activation of the Atf1p and Pap1p transcription factors in *S. pombe* (Madrid et al., 2004; Stiefel et al., 2004). The activation of a Sty1 MAP kinase by calorie restriction participates in life span extension, which was shown for the first time by Zuin et al., (2010). In the same study, it was reported that either low-glucose media or activation of the nutrient-dependent Pka1 pathway promoted life span extension in fission yeast, including high respiratory rates, elevated ROS production, activation of the MAP kinase Sty1 pathway and expression of survival genes.

Although there is a wide range of studies supporting the relationship between glucose repression and oxidative stress in *S. pombe*, it is not fully understood how this relationship takes place and which molecules play crucial roles in this process. We aimed to investigate the relationship between each major step of glucose metabolism and its related effect on oxidative stress response using a constitutive invertase mutant resistant to glucose repression (*ird11*) (Kig et al., 2005). This mutant, ird11, seems to be affected by glucose signaling in a different manner than that caused by glucose deprivation (Palabiyik et al., in press).

In this study, the genes responsible for resistance to oxidative stress in the mutant compared to wild-type were determined by employing the differential display technique. The characterization of each potential gene representing differentially expressed cDNA fragments was implemented by scanning the *S. pombe* genome database, and two genes were identified: *rpl302*, encoding 60S ribosomal protein L3, and *mpg1*, encoding mannose-1-phosphate guanyltransferase. Differential expression patterns of these genes were confirmed by quantitative real-time PCR.

MATERIAL AND METHODS

Yeast strains and growth media

The *S. pombe* Lindner *liquifaciens* strains wild-type (972 h^-) and a constitutive invertase mutant resistant to glucose repression (*ird11*h^-) (Kig et al., 2005) were used. Selective medium for the *ird11* mutant (0.5% yeast extract, 3% sucrose and 400 µg/mL 2-deoxy-D-glucose; Rincon et al., 2001) and standard rich yeast extract medium (YEL and YEA; Gutz et al., 1974) were used for cultivation.

Hydrogen peroxide treatment of *S. pombe* cells

Exponentially growing *S. pombe* cells (*ird11* and wild-type) were split into two tubes. In one of them, as the experimental group, the medium contained a mild level of hydrogen peroxide (Sigma, H1009) (2 mM H_2O_2, exposure for 1 h) as described by Pekmez et al. (2008). The other group had no added H_2O_2 (control group). At the end of treatment, both experimental and control cells were harvested by centrifugation and washed with sterile distilled water.

Differential expression analysis

H_2O_2-induced changes in gene expression in *ird11* and wild-type were investigated using the differential display technique, originally developed by Liang and Pardee (1992).

RNA isolation and cDNA synthesis

Total RNA was isolated from H_2O_2-treated and untreatedcells, using the High Pure RNA Isolation kit (Roche) following the manufacturer's instructions. First strand fluorescein-labeled oligo(dT)-primed cDNA synthesis was performed using the Fluorescence Differential Display kit (Takara), according to the manufacturer protocol.

PCR amplification

Twenty-four PCR reactions were performed with three anchored fluorescein-labeled oligo (dT) primers (Takara) combined with eight random primers (HAP1-8; Kig and Temizkan, 2009). A volume of 2 µL cDNA template was used directly in a 20-µL reaction mixture containing 1X LA PCR buffer II, 0.1 mM dNTP mix, 1.3 mM $MgCl_{2,\ 0.2\ U\ LA}$ Taq DNA polymerase (Takara), 0.5 µM random primer and 0.3 µM of the same anchored flourescein-labeled oligo(dT) primer used in first strand cDNA synthesis. After an initial 2 min of denaturation at 94°C, 40 amplification cycles were performed as follows: 30 s at 94°C, 2 min at 38°C, and 1 min at 72°C. This was followed by 5 min final extension at 72°C, and the reaction mix stored at 4°C.

Electrophoresis and silver staining

PCR products were separated on 6 % non-denaturing polyacrylamide gels (16.5 cm x 28.0 cm x 0.75 mm). The gels were pre-run for 30 min prior to loading, and electrophoresis was performed in 1XTBE buffer at 8 W for 15 h. cDNA bands were visualized by silver

staining as described by Waterborg (2002).

Recovery and reamplification

Bands of interest were cut out and DNA fragments from polyacrylamide gels were recovered as described by QIAEX-II. Eluted DNA was re-amplified under identical conditions as in the initial PCR (except that 35 cycles were performed) with the corresponding oligo (dT) and random primer sets. PCR products were checked on 2 % agarose gels and then purified with the High Pure PCR Product Purification kit (Roche).

Sequence analysis

Re-amplified PCR products were sequenced using the corresponding HAP primers. The sequencing was performed commercially (Refgen Ltd., Turkey), on an ABI 3130XL Genetic Analyzer using the BigDye Cycle Sequencing kit v3.1 (Applied Biosystems). Sequence data were subjected to *BLASTX (Swiss-Prot) and BLASTN (*RefSeq mRNA) searches of the *S. pombe* genome database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). BLAST hits with *E value* less than 10^{-4} and with *bit score* greater than 50 (for BLASTX) or 100 (for *BLASTN*) were considered significant.

Real-time PCR

Quantitative real-time PCR was performed with the Mx3005P system (Stratagene), using the FastStart SYBR Green Master kit (Roche). The gene-specific primers used were (*act1*: Forward 5'-ACTATGTATCCCGGTATTGCC-3', Reverse 5'-GACAGAGTATTTACGCTCAGG-3'; *rpl302*: Forward 5'- F: ATGATGCCAAGAATGCTTCC-3', Reverse 5'-AGTGGCACCA TTCAACATGA-3'; *mpg1*: Forward 5'-F:CGACCTTGAGGGTTATTGGA-3', Reverse 5'-ACTAGCTGGGGCCAAGATTT-3'). The synthetic first-strand cDNA used as template was synthesized with following manufacturer instructions: The reaction mixtures consisting of 25 mL FastStart SYBR Green Master, forward and reverse primers (0.2 µM each) and 0.1 µg cDNA were made up to 50 µL with ultra-pure nuclease-free water. The PCR conditions were set as follows: 95°C for 10 min (pre-incubation), followed by 40 cycles of 95°C for 10 s, 53°C for 10 s, and 72°C for 20 s. The final step included a gradual temperature increase from 55°C to 95°C at a rate of 1°C/10 s to enable melting curve data collection. A non-template control was run and serial dilutions (1, 1:10 and 1:100) of the reference (actin gene, *act1*) and the target genes were included in every assay. Amplification specificity of each reaction was verified by melting curve analysis. Expression levels were normalized against the reference gene, *act1*. Relative gene expression levels were determined according to the method of Pfaffle (2001).

RESULTS AND DISCUSSION

It is known that calorie restriction increases tolerance against oxidative stress and life span in *S. pombe* (Lin et al., 2000; Madrid et al., 2004; Roux et al., 2010). The increased resistance to oxidative stress, caused by glucose depletion (Madrid et al., 2004), gives rise to either carbon stress or accumulation of reactive oxygen species, which promotes aerobic respiration (Roux et al., 2009).

Because *ird11* is resistant to glucose repression (Kig et al., 2005) and to oxidative stress, differing from that caused by glucose deprivation, which triggers aerobic metabolism (Palabiyik et al., in press), it indicates that this mutant may provide a convenient tool to use in studies related to glucose sensing/signaling and oxidative stress.

In this study, we screened for genes whose expression decreased or increased upon exposure to mild oxidative stress in *ird11* and wild-type, using the differential display technique. We isolated 17 different fragments from polyacrylamide gels. Two of them were sequenced and subjected to BLAST searches. According to *S. pombe* genome database query, we found that these sequences belong to the genes of *rpl302* (SPAPB8E5.06c), encoding 60S ribosomal protein L3 (Liebich et al., 1994), and *mpg1* (SPCC1906.01), encoding mannose-1-phosphate guanyltransferase. Ribosomal protein L3 is a member of a general gene expression system, and there are numerous ribosomal proteins displaying a unique promoter type in *S. pombe* (Gross and Käufer, 1998; Gasch, 2007). Mannose-1-phosphate guanyltransferase is an essential protein that is involved in glycosylation of cell wall proteins (Kukuruzinska et al., 1987; Huang and Snider 1995; Warit et al., 2000), and is required for the formation of a correct septum structure and in cell growth (Donoso et al., 2005). In addition, *mpg1* is a conserved gene from fungi to higher eukaryotes (Donoso et al., 2005)

Differentially expressed genes were confirmed by quantitative real-time PCR using gene-specific primer sets. It was shown that expression profiles of *rpl302* and *mpg1* genes in quantitative real-time PCR were parallel to band intensities of these genes observed on polyacrylamide gels. The *mpg1* expression seemed to be similar in H_2O_2-treated *ird11* and wild-type (Figure 1).

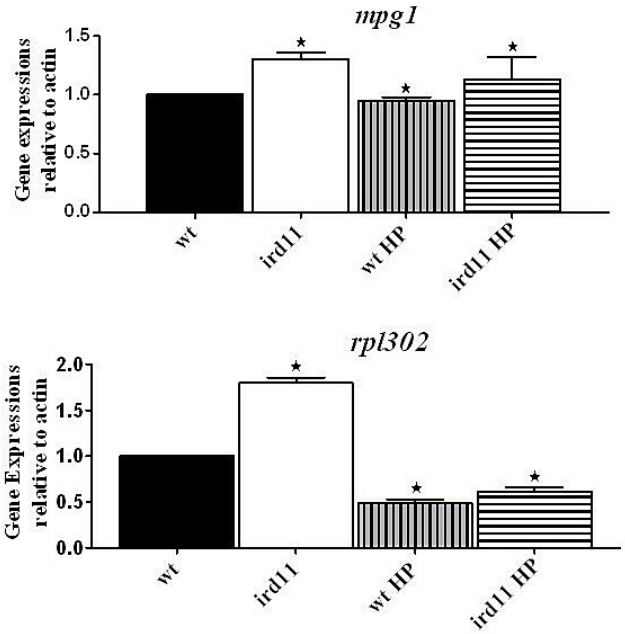

Figure 1. Expression levels of *rpl302* and *mpg1* genes relative to *act1* gene. The expression levels of the *rpl302* and *mpg1* genes in treated wild-type and *ird11* (wt HP and *ird11* HP) and un-treated cells (wt and *ird11*) were determined according to the Pfaffl method relative to actin gene. Un-treated wild type (wt) was used as a calibrator. Significant differences between calibrator (wt) and other cells (*ird11*, wt HP and *ird11*HP) were evaluated by Student's *t*-test. *P < 0.05. (wt: wild type, HP: treated with H_2O_2).

Because ribosomal protein L3 is a member of a general gene expression system (Gasch, 2007), we estimated that the expression level of *rpl302* gene under stressed conditions was decreased. In addition, as expected, the expression level of *rpl302* gene in H_2O_2-treated *ird11*and wild-type cells was found to be lower than that of un-treated wild type (0.6 and 0.5 times, respectively). This indicated that the expression level of *rpl302* gene was sharply reduced in both cells under stressed conditions. Although the ribosomal protein genes are among the most tightly co-regulated genes in the yeast genome (Gasch et al., 2000; Causton et al., 2001), interestingly, *rpl302* expression in un-treated *ird11* was found to be increased 1.8 fold compared to un-treated wild-type (Figure 1). Similarly, the expression level of the *mpg1* gene in un-treated *ird11* was found to be statistically higher (1.3 times) compared to that of wild-type. These findings suggest that both genes may play a role in the oxidative stress tolerance in *ird11*. The findings obtained from this study provided additional evidence which may be used in a convenient model cell for studies on glucose sensing/signaling and the oxidative stress response pathways.

ACKNOWLEDGMENTS

Research supported by Istanbul University Research Fund (project #BAP1477 and #4152). We thank Dr. Cenk Kig, from the Department of Molecular Cell Biology, Catholic University of Leuven, Belgium, for the gift of the arbitrary primers.

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