Importance of glucose-6-phosphate dehydrogenase in the adaptive response to hydrogen peroxide in *Saccharomyces cerevisiae*

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Glucose-6-phosphate dehydrogenase (G6PDH)-deficient cells of *Saccharomyces cerevisiae* showed increased susceptibility and were unable to induce adaptation to oxidative stress. Historically, mainly in human erythrocytes, it has been suggested and accepted that decreased cellular GSH, due to loss of the NADPH-dependent activity of glutathione reductase (GR), is responsible for the increased sensitivity to oxidative stress in G6PDH-deficient cells. In the present study we investigated whether the increased susceptibility and the inability to induce adaptation to H₂O₂ stress of G6PDH-deficient yeast is caused by incompleteness of glutathione recycling. We constructed G6PDH- and GR-deficient mutants and analysed their adaptive response to H₂O₂ stress. Although G6PDH-deficient cells contained comparable amounts of GSH and GR activity to wild-type cells, GSSG was

not reduced efficiently, and intracellular GSSG levels and the ratio of GSSG to total glutathione (GSSG/tGSH) were higher in G6PDH-deficient cells than in wild-type. On the other hand, GR-deficient cells showed a susceptibility identical with that of wild-type cells and induced adaptation to H₂O₂ stress, even though the GSSG/tGSH ratio in GR-deficient cells was higher than in G6PDH-deficient cells. These results indicate that incompleteness of glutathione recycling alone is not sufficient to account for the increased sensitivity and inability to induce adaptation to H₂O₂ stress of G6PDH-deficient yeast cells. In *S. cerevisiae*, G6PDH appears to play other important roles in the adaptive response to H₂O₂ stress besides supplying NADPH to the GR reaction.

INTRODUCTION

Cells pre-exposed to comparatively mild and sublethal stress situations activate adaptive responses and acquire tolerance to subsequent more lethal stress. Such responses, named adaptation, are observed not only in bacterial cells but also in eukaryotic organisms, including *Saccharomyces cerevisiae* [1]. Detailed mechanisms of adaptation to oxidative stress have been most extensively characterized in bacterial cells [2], but have also been studied in yeast. In *S. cerevisiae*, at least 21 proteins are increased in response to exposure to H₂O₂, and a number of genes are transcriptionally regulated by H₂O₂ stress [3–5]. Some investigators, including us, have shown that glutathione is essential as an antioxidant in the adaptation to H₂O₂ in *S. cerevisiae* [6–9]. Furthermore glutathione-synthesis and glutathione-recycling activities are increased, and expression of the *GSH1* (encoding γ -glutamylcysteine synthetase) and *GLR1* (glutathione reductase) genes is elevated under the control of yAP-1, a transcriptional activator in *S. cerevisiae* [10–12].

Glutathione (γ -glutamylcysteinylglycine) is a low-molecular-mass thiol that is widely distributed in micro-organisms, plants and animals. It has various functions in the defence against oxidative stress and xenobiotic toxicity [13]. It can act as the electron donor for glutathione peroxidase in animal cells, and also directly reacts with reactive oxygen species. The reduced form (GSH) is oxidized to glutathione disulphide (GSSG) by the glutathione peroxidase reaction as well as the reaction with reactive oxygen species. GSSG is reduced and recycled by an NADPH-dependent reaction catalysed by glutathione reductase (GR). GR is necessary for the maintenance of GSH levels in cells. NADPH, the reducing agent, is mainly generated by

glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) via the pentose phosphate pathway. G6PDH catalyses the rate-limiting NADPH-producing step in the pentose phosphate pathway [14].

We have previously reported that G6PDH and GR were induced simultaneously by oxidative stress in *S. cerevisiae* [6], whereas in another yeast, *Hansenula mrakii*, only G6PDH was induced [15]. It has also been reported that G6PDH-deficient *S. cerevisiae* showed hypersensitivity to oxidative stress [16–18]. These data strongly indicate that G6PDH plays an important role in the acquisition of tolerance against oxidative stress. However, the intrinsic function of G6PDH in the oxidative stress response in yeast is still obscure.

In humans, G6PDH deficiency is a common genetic disorder that can result in haemolytic anaemia and enhanced oxidant sensitivity of erythrocytes [19]. In the absence of this enzyme, erythrocytes are unable to reduce GSSG using GR because of the lack of NADPH, and thus have low concentrations of GSH. Historically, the decrease in cellular GSH was thought to account for the increased oxidant-sensitivity of G6PDH-deficient erythrocytes. However, Scott et al. [20–22] have cast substantial doubts on the pre-eminent importance of GSH in the defence against H₂O₂ stress. They demonstrated that GSH levels did not affect susceptibility to H₂O₂ stress using G6PDH-deficient human erythrocytes and acatalasaemic mice. No statistical correlation was found between GSH level and oxidant sensitivity of erythrocytes, but a strong correlation between NADPH level and oxidant sensitivity was observed.

In *Escherichia coli*, G6PDH is induced by the *soxRS* regulon in oxidative stress response [23]. However, it has been reported that glutathione and glutathione recycling are not necessary for

Abbreviations used: G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; GR, glutathione reductase; tGSH, total glutathione (GSH + GSSG); TRX, thioredoxin.

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protection of *E. coli* cells against oxidative stress, because the *gshA::Tn10* mutant showed normal resistance to H_2O_2 and cumene hydroperoxide [24]. The *gshA* gene encodes γ -glutamyl-cysteine synthetase, which catalyses the rate-limiting step in GSH biosynthesis, and such a mutant has less than 0.4% of wild-type GSH levels. Furthermore the GR-deficient mutant (*gor*⁻) can keep intracellular glutathione highly reduced, suggesting that GR is not required for maintenance of GSH in *E. coli* [25]. The roles of glutathione and glutathione recycling in the resistance to oxidative stress may be different among yeasts, animals and bacterial cells.

We are interested in the roles of G6PDH and glutathione recycling in the adaptive response to oxidative stress in *S. cerevisiae*. In this study we investigated, by analysing phenotypes of GR- and G6PDH-deficient mutants, whether the increased sensitivity of G6PDH-deficient yeast is caused by insufficient recycling of glutathione.

EXPERIMENTAL

Yeast strains and medium

Strains of *S. cerevisiae* used in this study were as follows: YPH250 (*MATa trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*), GRK1 (*MATa trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 glr1Δ::HIS3*), ZWK7 (*MATa trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 zwf1Δ::URA3*), ZGK2 (*MATa trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 zwf1Δ::URA3 gnd1Δ::HIS3*). *S. cerevisiae* YPH250 was obtained from the Yeast Genetic Stock Center, University of California at Berkeley. Cells of *S. cerevisiae* were cultured in 50 ml of YPD medium (1% yeast extract, 2% peptone, 2% glucose, pH 5.5) or SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids, pH 5.5) with appropriate amino acids and bases at 28 °C with reciprocal shaking (200 rev./min) in 300 ml Erlenmeyer flasks. Exponentially growing cells were harvested at $A_{610} = 0.2$ – 0.3 .

Construction of disruption mutants and plasmids

Disruption of the *ZWF1* gene, which encodes G6PDH, was achieved using pBM2720 (kindly provided by Dr. M. Johnston [16]). pBM2720 was digested with *EcoRI* and *BamHI*, and the resulting fragment containing *zwf1::URA3* was introduced into *S. cerevisiae* YPH250 to yield a strain ZWK7 (*zwf1Δ*). The *GND1* gene, which encodes 6PGDH, was disrupted by the use of pBSK-*gnd1::HIS3*, which was kindly donated by Dr. H. Juhnke and Dr. K.-D. Entian [17]. The plasmid was digested with *ClaI* and *SacII*, and the resultant fragment carrying the *gnd1::HIS3* was introduced into the strain ZWK7 to produce strain ZGK2 (*zwf1Δ/gnd1Δ*). Since a fission yeast *Schizosaccharomyces pombe* was reported to have a pathway that can skip the G6PDH reaction, and NADPH can be generated by the 6PGDH reaction [26,27], we then constructed a *zwf1 Δ/gnd1Δ* double mutant to completely disrupt generation of NADPH via the pentose phosphate pathway. To disrupt the *GLR1* gene which encodes GR, the following oligonucleotide primers were used in PCR to amplify a 1.95 kb fragment containing the open reading frame of the *GLR1* gene from genomic DNA: 5'-TACGCATGCGG-CAAGCATTTTCATGTAGGAG-3' and 5'-ATTGTGCGACGA-GAGTCGGAATGTATCCTCA-3' [28]. These primers were designed to contain the *SphI* and *SalI* sites respectively (shown by italic letters). PCR was carried out with the following conditions: denaturation at 92 °C for 30 s, annealing at 55 °C for

1 min, extension at 72 °C for 2 min; 30 cycles. The PCR fragment was digested with *SphI* and *SalI*, and cloned into the *SphI*–*SalI* site of pUC19 (pGR19). A 1.7 kb *BamHI* fragment containing the *HIS3* gene was inserted between the *ClaI* and *NheI* sites internal to the *GLR1* gene in pGR19, to construct a plasmid pGRK3. Plasmid pGRK3 was digested with *SphI* and *SalI*, and a fragment containing *glr1::HIS3* was transformed to YPH250 to construct a strain GRK1 (*glr1Δ*). Disruption of each gene was verified by PCR. To construct the high-copy-number plasmid of the *ZWF1* gene, the following oligonucleotide primers were used in PCR to amplify a 2.1 kb fragment containing the open reading frame of the *ZWF1* gene from genomic DNA: 5'-TGGGG-ATCCCCAAATGTCCTGACCGCGGC-3' and 5'-AAAGC-ATGCATAAGATGCATACTCCGGCGG-3' [16]. The amplified DNA fragment was treated by Klenow fragment, and then cloned to the *BamHI* site of YEpl3, which was also treated by Klenow fragment. The resultant plasmid was named YEplZWF1. The DNA fragment of the *GLR1* gene amplified by PCR was treated by Klenow fragment, then cloned into the *BamHI* site of YEpl3, which was also treated by Klenow fragment, to yield YEplGLR1. Yeast transformation was performed by the method of electroporation (Bio-Rad; Gene pulser II).

H_2O_2 treatment

To observe the susceptibility to H_2O_2 , cells were harvested and resuspended in 100 mM potassium phosphate buffer, pH 7.4, to obtain the initial $A_{610} = 0.1$ [29]. This represented approx. 2×10^6 cells/ml. Various concentrations of H_2O_2 were added to 5 ml samples, and cell survival was monitored by taking samples at 20 min intervals, diluting them with the same buffer and plating aliquots on YPD agar. For adaptation experiments, cells were pretreated by resuspending them in fresh YPD or SD medium containing a sublethal concentration of H_2O_2 (0.2 mM) and incubating them with shaking at 28 °C for 1 h. Pretreated cells were harvested and resuspended in 100 mM potassium phosphate buffer, pH 7.4, and challenged with the lethal concentration of H_2O_2 (2 mM).

Enzyme assays

Cells were disrupted by vortex-mixing with glass beads in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM PMSF and 1 μ g/ml pepstatin A. GR activity was determined by the method of Miki et al. [30]. G6PDH activity was determined by the method of Kuby and Noltman [31], and 6PGDH activity by the method of Rippa and Signorini [32]. Protein was determined by the method of Lowry et al. [33].

Determination of glutathione

tGSH was determined by the method of Tietze [34], and GSSG was measured by the method of Anderson [35].

RESULTS

Susceptibility to H_2O_2 stress

Wild-type and mutant cells in exponential growth phase were treated with various concentrations of H_2O_2 (0.2–5 mM). G6PDH-deficient cells (ZWK7 and ZGK2) displayed greater susceptibility to H_2O_2 than wild-type cells (Figure 1). Juhnke et

Figure 1 Susceptibility of exponential-phase cells to H₂O₂ stress

Cells growing exponentially in YPD medium were collected and resuspended in 100 mM potassium phosphate buffer, pH 7.4, to obtain the initial $A_{610} = 0.1$ (2×10^6 cells/ml), and treated with various concentrations of H₂O₂. Samples were diluted and plated on YPD agar to monitor the cell viability. H₂O₂ concentrations were 0 (○), 0.2 (▲), 1 (△), 2 (■) and 5 (□) mM. Results represent the mean from four independent experiments. Standard deviation did not exceed 5% in the range 100–10%, 1% in the range 10–1%, and 0.3% in the range 1–0.1%.

Figure 2 Induction of adaptation to H₂O₂ stress

Details of conditions for the experiments are described in the text. ●, Cells were pretreated with 0.2 mM H₂O₂ in YPD medium for 60 min, and then challenged with 2 mM H₂O₂. ○, Cells were challenged directly with 2 mM H₂O₂. Results represent the mean from four independent experiments. Standard deviation did not exceed 5% in the range 100–10% and 1% in the range 10–1%.

al. [17] also reported that G6PDH-deficient mutants showed increased susceptibility to H₂O₂. We therefore confirm the importance of G6PDH in the protection of *S. cerevisiae* against H₂O₂ stress. In contrast, GR-deficient cells (GRK1) showed an almost identical susceptibility with that of wild-type cells. Grant

Table 1 Changes in glutathione-recycling enzyme activities in the adaptive response

Cells growing exponentially in YPD medium were collected, and resuspended in fresh YPD medium with or without 0.2 mM H₂O₂ for 60 min. Values are means $\pm$ S.E.M. from four independent experiments. N.D., not detected. One unit of GR activity is defined as the amount of enzyme oxidizing 1.0 μ mol of GSSG/min at 25 °C. One unit of G6PDH activity is defined as the amount of enzyme reducing 1.0 μ mol of D-glucose 6-phosphate/min at 25 °C. One unit of 6PGDH activity is defined as the amount of enzyme oxidizing 1.0 μ mol of 6-phospho-D-glucuronate/min at 25 °C.

Strain	Presence of H ₂ O ₂	Activity (m-units/mg of protein)		
		GR	G6PDH	6PGDH
YPH250	—	27.5 $\pm$ 1.1	451 $\pm$ 9	185 $\pm$ 25
	+	39.2 $\pm$ 4.2	861 $\pm$ 39	301 $\pm$ 75
ZWK7	—	34.2 $\pm$ 0.9	N.D.	84 $\pm$ 17
	+	42.2 $\pm$ 3.2	N.D.	82 $\pm$ 11
ZGK2	—	30.3 $\pm$ 1.0	N.D.	N.D.
	+	37.4 $\pm$ 2.9	N.D.	N.D.
GRK1	—	N.D.	691 $\pm$ 21	182 $\pm$ 41
	+	N.D.	814 $\pm$ 28	272 $\pm$ 63

et al. [11,12] reported that there was no difference in susceptibility to H₂O₂ between GR-deficient and wild-type cells during the exponential phase, but GR-deficient cells in the stationary phase were more sensitive than wild-type cells. We examined the susceptibility of H₂O₂ using exponential-phase cells; therefore our data are consistent with the results of Grant et al.

Induction of adaptation to H₂O₂ stress

We investigated the effects of G6PDH and GR deficiency on the induction of adaptation to H₂O₂ stress. Adaptation was induced by pretreatment of exponential-growth-phase cells with 0.2 mM H₂O₂ for 60 min (first stress), and then the pretreated cells were challenged with 2 mM H₂O₂ (second stress). With the 2 mM H₂O₂ challenge, a difference in susceptibility was observed between wild-type cells and mutants (Figure 2). The pretreatment induced a large increase in tolerance to 2 mM H₂O₂ in wild-type cells. In G6PDH-deficient cells (ZWK7 and ZGK2), tolerance was hardly increased at all by the pretreatment, and adaptation was not induced. This result indicates that G6PDH is necessary for induction of adaptation to H₂O₂ stress in *S. cerevisiae*.

GR-deficient cells, in spite of lacking GR activity, were still able to increase tolerance to H₂O₂ stress, but not as efficiently as wild-type cells. This suggests that the GR reaction is also important in adaptation. However, G6PDH is probably more important than GR for the adaptive response to H₂O₂ stress.

Changes in glutathione-recycling enzyme activities in the adaptive response

We tried to clarify whether glutathione recycling affects induction of adaptation in G6PDH-deficient cells. Table 1 shows the changes in the enzyme activities involved in glutathione recycling (GR, G6PDH and 6PGDH). In wild-type cells, the activity of each enzyme was increased approx. 1.4–1.9-fold by the first stress (0.2 mM H₂O₂ for 60 min). GR activity of the G6PDH-deficient mutants (ZWK7 and ZGK2) was somewhat higher than that of wild-type cells under conditions of no stress, and was increased approx. 1.2-fold by the first stress treatment. 6PGDH activity of

Table 2 Changes in glutathione redox state in the adaptive response

Cells growing exponentially in YPD medium were collected, and resuspended in fresh YPD medium with or without 0.2 mM H₂O₂ for 60 min. Values are means $\pm$ S.E.M. from four independent experiments.

Strain	Presence of H ₂ O ₂	tGSH (μ mol/g)	GSSG (nmol/g)	GSSG/tGSH
YPH250	—	2.08 $\pm$ 0.03	73 $\pm$ 3	3.5
	+	2.25 $\pm$ 0.09	75 $\pm$ 4	3.3
ZWK7	—	2.47 $\pm$ 0.07	165 $\pm$ 4	6.7
	+	2.67 $\pm$ 0.08	175 $\pm$ 4	6.6
ZGK2	—	2.44 $\pm$ 0.06	153 $\pm$ 6	6.3
	+	2.51 $\pm$ 0.10	169 $\pm$ 3	6.7
GRK1	—	2.43 $\pm$ 0.04	254 $\pm$ 8	10.5
	+	2.69 $\pm$ 0.10	281 $\pm$ 5	10.4

Figure 3 Effect of *ZWF1* or *GLR1* gene overexpression on the adaptation to H₂O₂ stress in G6PDH-deficient cells

Details of conditions for the experiments are given in the text. ●, Cells were pretreated with 0.2 mM H₂O₂ in SD medium for 60 min, and then challenged with 2 mM H₂O₂. ○, Cells were challenged directly with 2 mM H₂O₂. Results represent the mean from four independent experiments. Standard deviation did not exceed 5% in the range 100–10% and 1% in the range 10–1%.

ZWK7 (*zwf1Δ*) was 55% lower than that of wild-type cells, and was not increased by the first stress treatment. Comparing the G6PDH and 6PGDH activities of the GR-deficient mutant (GRK1) and the wild-type, 6PGDH activity was almost the same in the two strains, but G6PDH activity in GRK1 cells under no stress was approx. 1.5-fold higher than that of wild-type cells. G6PDH and 6PGDH activities were increased approx. 1.2-fold and 1.5-fold respectively by the first stress treatment even in the GR-deficient mutant.

Changes in glutathione redox state in the adaptive response

Table 2 shows the changes in the glutathione redox state in the adaptive response. Both intracellular tGSH and GSSG in all strains showed the tendency to increase on exposure to the first stress. However, no significant alteration in the GSSG/tGSH ratio was observed. It seems that *S. cerevisiae* has the ability to keep the GSSG/tGSH ratio at a certain level under mild oxidative conditions. The levels of GSH (tGSH – GSSG) in the mutants

Table 3 Changes in glutathione-recycling enzyme activities in the adaptive response of G6PDH-deficient cells

Cells growing exponentially in SD medium were collected, and resuspended in fresh SD medium with or without 0.2 mM H₂O₂ for 60 min. Values are means $\pm$ S.E.M. from four independent experiments. N.D., not detected. Units are defined for each enzyme in Table 1.

Strain	Presence of H ₂ O ₂	Activity (m-units/mg of protein)		
		GR	G6PDH	6PGDH
ZWK7 + YEpl3	—	27.5 $\pm$ 1.1	N.D.	50 $\pm$ 13
	+	39.2 $\pm$ 4.2	N.D.	58 $\pm$ 18
ZWK7 + YEplZWF1	—	34.2 $\pm$ 0.9	1344 $\pm$ 71	461 $\pm$ 32
	+	42.2 $\pm$ 3.2	2634 $\pm$ 47	640 $\pm$ 22
ZWK7 + YEplGLR1	—	116.3 $\pm$ 3.4	N.D.	61 $\pm$ 14
	+	173.2 $\pm$ 6.2	N.D.	70 $\pm$ 11
ZGK2 + YEpl3	—	30.3 $\pm$ 1.0	N.D.	N.D.
	+	37.4 $\pm$ 2.9	N.D.	N.D.
ZGK2 + YEplZWF1	—	28.7 $\pm$ 3.0	912 $\pm$ 81	N.D.
	+	35.1 $\pm$ 3.4	1782 $\pm$ 98	N.D.
ZGK2 + YEplGLR1	—	108.3 $\pm$ 4.9	N.D.	N.D.
	+	161.5 $\pm$ 7.9	N.D.	N.D.
YPH250	—	24.9 $\pm$ 1.8	464 $\pm$ 7	197 $\pm$ 17
	+	37.5 $\pm$ 4.3	868 $\pm$ 32	259 $\pm$ 25
YPH250 + YEpl3	—	22.6 $\pm$ 3.6	441 $\pm$ 15	177 $\pm$ 16
	+	36.4 $\pm$ 3.3	803 $\pm$ 27	244 $\pm$ 22

were slightly higher than that of the wild-type. GR-deficient cells contain larger amounts of GSSG than wild-type cells because of the lack of GR activity. The GSSG/tGSH ratio was also higher in GR-deficient cells than wild-type cells (GRK1, 10.5; wild-type, 3.5). In G6PDH-deficient cells (ZWK7 and ZGK2), GR activity under conditions of no stress was slightly higher than that of wild-type cells (Table 1); however, GSSG was not reduced as efficiently as in wild-type cells. The GSSG/tGSH ratio of these mutants were lower than that of GR-deficient cells, but higher than that of wild-type cells (ZWK7, 6.7; ZGK2, 6.3). Although both GSSG level and the GSSG/tGSH ratio of GR-deficient cells were higher than those of G6PDH-deficient cells, GR-deficient cells still showed induction of adaptation and higher resistance to H₂O₂ stress than G6PDH-deficient cells (Figures 1 and 2). These results indicate that the increased susceptibility and inability to induce adaptation to H₂O₂ stress in G6PDH-deficient cells were not caused by the insufficient recycling of glutathione alone.

Overexpression of the *ZWF1* or *GLR1* gene in G6PDH-deficient cells

We next tested whether overexpression of the *ZWF1* or *GLR1* gene in G6PDH-deficient cells improves the glutathione redox state and reinstates the ability to induce adaptation to H₂O₂ stress. Overexpression of the *ZWF1* gene in G6PDH-deficient cells (ZWK7 + YEplZWF1 and ZGK2 + YEplZWF1) rendered these mutants more resistant to H₂O₂ than the mutants carrying the vector alone (ZWK7 + YEpl3 and ZGK2 + YEpl3). The induction of adaptation to H₂O₂ was recovered by overexpression of the *ZWF1* gene in G6PDH-deficient cells (Figure 3). In ZWK7 + YEplZWF1 and ZGK2 + YEplZWF1, by carrying a high-copy-number plasmid YEplZWF1, G6PDH activities were approx. 2–3-fold higher than in wild-type cells under conditions

Table 4 Changes in glutathione redox state in the adaptive response of G6PDH-deficient cells

Cells growing exponentially in SD medium were collected, and resuspended in fresh SD medium with or without 0.2 mM H₂O₂ for 60 min. Values are means $\pm$ S.E.M. from four independent experiments.

Strain	Presence of H ₂ O ₂	tGSH (μ mol/g)	GSSG (nmol/g)	GSSG/tGSH
ZWK + YEp13	—	2.43 $\pm$ 0.04	154 $\pm$ 8	6.3
	+	2.69 $\pm$ 0.10	171 $\pm$ 5	6.4
ZWK7 + YEpZWF1	—	2.27 $\pm$ 0.07	85 $\pm$ 5	3.7
	+	2.57 $\pm$ 0.08	86 $\pm$ 3	3.3
ZWK7 + YEpGLR1	—	2.03 $\pm$ 0.11	164 $\pm$ 8	8.1
	+	2.21 $\pm$ 0.10	168 $\pm$ 7	7.6
ZGK2 + YEp13	—	2.57 $\pm$ 0.06	182 $\pm$ 10	7.1
	+	2.71 $\pm$ 0.05	188 $\pm$ 7	6.9
ZGK2 + YEpZWF1	—	2.44 $\pm$ 0.06	81 $\pm$ 7	3.3
	+	2.52 $\pm$ 0.05	83 $\pm$ 5	3.3
ZGK2 + YEpGLR1	—	2.11 $\pm$ 0.14	148 $\pm$ 8	7.0
	+	2.36 $\pm$ 0.12	156 $\pm$ 7	6.6
YPH250	—	2.12 $\pm$ 0.08	79 $\pm$ 4	3.7
	+	2.31 $\pm$ 0.09	72 $\pm$ 4	3.1
YPH250 + YEp13	—	2.06 $\pm$ 0.11	70 $\pm$ 6	3.5
	+	2.30 $\pm$ 0.07	78 $\pm$ 4	3.4

of no stress (Table 3). G6PDH activities in ZWK7 + YEpZWF1 and ZGK2 + YEpZWF1 were increased approx. 2-fold by the H₂O₂ treatment, as well as in the wild-type (Table 3). It has been reported that the *ZWF1* gene contains a similar sequence to the yAP-1-recognition element in the 5'-flanking region [36]. The increase in G6PDH activity caused by the H₂O₂ treatment may be due to induction of *ZWF1* gene expression under the control of yAP-1.

As observed in Table 3, 6PGDH activity was lower in strain ZWK7 (*zwf1Δ*) carrying a vector YEp13 (ZWK7 + YEp13; 50 $\pm$ 13 m-units/mg) and *GLR1*-overexpressing plasmid YEp-GLR1 (ZWK7 + YEpGLR1; 61 $\pm$ 14 m-units/mg) than in the wild-type strain (YPH250 + YEp13; 177 $\pm$ 16 m-units/mg); however, surprisingly, 6PGDH activity was increased approx. 8–9-fold by overproduction of G6PDH in strain ZWK7 (ZWK7 + YEpZWF1; 461 $\pm$ 32 m-units/mg). 6PGDH activity increased in proportion to the increase in G6PDH activity in wild-type cells and ZWK7 + YEpZWF1, but not in G6PDH-deficient cells (ZWK7 and ZWK7 + YEp13) under conditions of stress (Tables 1 and 3). Sinha and Maitra [37] reported that 6PGDH was induced by 6-phosphogluconolactone and 6-phosphogluconate in *S. cerevisiae*. 6-Phosphogluconolactone is one of the products of the G6PDH reaction, and is hydrolysed to 6-phosphogluconate by 6-phosphogluconolactonase. 6PGDH may be induced in yeast cells overexpressing the *ZWF1* gene because of a good supply of the substrate for the enzyme. In contrast, expression of the *GND1* gene encoding 6PGDH may be repressed in the *zwf1Δ* mutant because of an insufficient supply of the substrate, 6-phosphogluconate. At any rate, a deficiency in G6PDH appears to affect the generation of NADPH via the pentose phosphate pathway.

In the case of GR activity, it was not affected significantly by the overexpression of the *ZWF1* gene. Both the quantity of GSSG and the GSSG/tGSH ratio were reduced to the level of the wild-type cells by overexpression of the *ZWF1* gene in G6PDH-deficient cells (Table 4). These results confirm that

G6PDH is essential for efficient glutathione recycling and induction of adaptation.

On the other hand, overexpression of the *GLR1* gene in G6PDH-deficient cells (ZWK7 + YEpGLR1 and ZGK2 + YEpGLR1) did not allow these mutants to recover the ability to adapt to H₂O₂ stress (Figure 3). Neither the quantity of GSSG nor the GSSG/tGSH ratio were reduced in ZWK7 + YEpGLR1 and ZGK2 + YEpGLR1, although these mutants had approx. 5-fold higher GR activity than wild-type cells and GR activity was increased by the H₂O₂ treatment (Tables 3 and 4). These results indicate that GR uncoupled from G6PDH may be unable to function efficiently to reduce GSSG.

DISCUSSION

The focus of our study was to evaluate whether insufficient recycling of glutathione, as the result of insufficient GR activity, is responsible for the increased susceptibility to H₂O₂ stress of G6PDH-deficient yeast cells. To accomplish this, we constructed G6PDH- and GR-deficient mutants and analysed their phenotypes. G6PDH-deficient cells showed higher susceptibility to H₂O₂ stress (Figure 1), and were unable to induce adaptation to such stress (Figure 2). In addition, overexpression of the *ZWF1* gene in G6PDH-deficient cells reinstated the ability to induce adaptation to H₂O₂ stress (Figure 3). G6PDH is the key enzyme in the generation of NADPH in the cytosol, and its activity could affect 6PGDH activity (Tables 1 and 3). It has been reported that total NADPH levels in G6PDH-deficient cells are reduced to 59–67% of those in isogenic wild-type cells [18]. These results indicate that the NADPH generation by G6PDH is necessary for the acquisition of tolerance against H₂O₂ stress in *S. cerevisiae*. It is well known that G6PDH deficiency in humans is characterized by an increased sensitivity to H₂O₂ stress [22], and, in some tissues (e.g. liver, adipose, lung and proliferating cells), G6PDH activity is increased by oxidative stress [14]. We confirmed that G6PDH is necessary for the acquisition of tolerance against H₂O₂ in *S. cerevisiae* as well as in humans.

It has been thought for a long time that the decrease in GSH caused by loss of the NADPH-dependent activity of GR is responsible for the increased susceptibility to oxidative stress of G6PDH-deficient animal cells [20–22]. In *S. cerevisiae* also G6PDH deficiency resulted in insufficient reduction of GSSG. Even though sufficient amounts of GR exist in G6PDH-deficient cells, both the amount of intracellular GSSG and the GSSG/tGSH ratio were increased by the deficiency of G6PDH (Tables 1 and 2). These results confirm the importance of G6PDH in the recycling of glutathione in *S. cerevisiae*. However, all mutants had amounts of GSH that were equal to or higher than that of wild-type cells (Table 2). Therefore the increased susceptibility and the inability to induce adaptation to H₂O₂ in the G6PDH-deficient *S. cerevisiae* were not caused by the decrease in intracellular GSH content.

The amount of GSSG and the GSSG/tGSH ratio in G6PDH-deficient cells were higher than those in wild-type cells, but lower than those in GR-deficient cells (Table 2). Adaptation was induced in GR-deficient cells, but not in G6PDH-deficient cells (Figure 2). No correlation was found between the GSSG/tGSH ratio and H₂O₂ susceptibility, or between the GSSG/tGSH ratio and induction of adaptation to H₂O₂ stress in *S. cerevisiae*. These results clearly demonstrate that incompleteness of glutathione recycling alone is not sufficient to explain the increased susceptibility and inability to induce adaptation to H₂O₂ stress in G6PDH-deficient yeast cells. However, we do not conclude that glutathione recycling is not important in the adaptive response in *S. cerevisiae*. Glutathione recycling must be necessary for the

acquisition of the full increase in tolerance to H_2O_2 stress, because GR-deficient cells were unable to increase the tolerance as efficiently as wild-type cells (Figure 2). In the adaptive response to H_2O_2 stress, expression of the *GLR1* gene is induced by yAP-1, which is an oxidative-stress-response transcriptional activator [11,12]. These data strongly suggest that glutathione recycling via the GR reaction must be one of the mechanisms of the adaptive response to H_2O_2 stress.

It seems that GR contributes less than G6PDH to the increase in tolerance against H_2O_2 in adaptation, since G6PDH deficiency caused more serious effects (Figures 1 and 2). Excess GR in G6PDH-deficient cells had no effect on either glutathione redox state or tolerance against H_2O_2 (Figure 3, Table 4). Overproduction of GR in wild-type cells (YPH250 + YEpGLR1) also had no effect on the glutathione redox state and tolerance (results not shown). These results indicate that GR is unable to function at full capacity if not coupled to G6PDH, and that excess GR disproportionate with NADPH supply may be of no use in reducing GSSG. It seems that the balance between GR activity and NADPH supply is important to recycle glutathione efficiently.

In GR-deficient cells, the GSSG/tGSH ratio did not change significantly on 0.2 mM H_2O_2 treatment (Table 2). This suggests that GSSG may be reduced by other thiol-disulphide oxidoreductases such as thioredoxin (TRX). Recently, Miller [38] reported that GSSG was reduced not only by GR but also TRX in *S. cerevisiae*. It has also been reported that GR is not required for maintenance of GSH in *E. coli* [25]. The GR reaction may be compensated for or replaced to some extent by other mechanisms, such as the TRX reaction or an increase in *de novo* synthesis of GSH, in the adaptive response to H_2O_2 stress in *S. cerevisiae*.

On the other hand, G6PDH deficiency affects cell metabolism pleiotropically, since NADPH is involved in many enzyme reactions. For example, G6PDH-deficient *S. cerevisiae* shows methionine auxotrophy [39]. Several studies have clearly demonstrated that mammalian and yeast catalases contain tightly bound NADPH and require NADPH to prevent the formation of compound II (inactive form) by H_2O_2 [40–42]. Subsequently, Scott et al. [22] reported that the increased oxidant sensitivity of G6PDH-deficient erythrocytes correlated closely with NADPH levels and subsequent impaired catalase activity. These data implied that the loss of catalase activity was the mechanism underlying the increased oxidant sensitivity of G6PDH-deficient erythrocytes. Yeast catalase also contains NADPH for prevention of the formation of compound II, and plays an important role in the acquisition of tolerance against oxidative stress by the adaptive response [29]. The loss of catalase activity may be one of the mechanisms underlying the increased sensitivity of G6PDH-deficient *S. cerevisiae*, as well as in erythrocytes. In addition to catalase, TRX and thioredoxin peroxidase would be influenced by G6PDH deficiency. Oxidized TRX is reduced and recycled by an NADPH-dependent reaction catalysed by thioredoxin reductase. TRX is also important for the adaptive response to oxidative stress in *S. cerevisiae*. The *TRX2* gene, which encodes thioredoxin, is also regulated by yAP-1, and its expression is induced by H_2O_2 stress [43]. Furthermore, TRX can act as an electron donor for thioredoxin peroxidase. Thioredoxin peroxidase reduces H_2O_2 and alkyl hydroperoxides, and serves as an antioxidant enzyme in the protection against oxidative stress in *S. cerevisiae* [44].

The decrease in NADPH level caused by G6PDH deficiency affects many types of reaction in the adaptive response in *S. cerevisiae*, and glutathione recycling catalysed by GR is just one of these reactions. Thus the mechanisms involved in the increased sensitivity and the inability to induce adaptation of G6PDH-

deficient cells appear to be complicated. This implies that G6PDH plays an important role as the key enzyme in the adaptive response against oxidative stress. Schnell et al. [36] have reported that G6PDH activity was increased by overexpression of the *YAP1* gene, and that the *ZWF1* gene has a similar sequence to the yAP-1-recognition element in the 5'-flanking region. This suggests that the *ZWF1* gene may be regulated by yAP-1 at the transcriptional level in the oxidative stress response, as observed in the case of the *GSH1*, *GLR1* and *TRX2* genes, and also implies that induction of G6PDH is one of the mechanisms of adaptive response to H_2O_2 in *S. cerevisiae*.

In conclusion, the decrease in GSH content is not the reason for the increased sensitivity to H_2O_2 in G6PDH-deficient *S. cerevisiae*. Glutathione recycling is one of the mechanisms of the adaptive response to H_2O_2 , and is certainly insufficient in G6PDH-deficient cells. Incompleteness of glutathione recycling appears to be one of the causes of increased sensitivity in G6PDH-deficient cells; however, this alone is not sufficient to explain the increased sensitivity and the inability to induce adaptation to H_2O_2 . G6PDH must play other important roles in the adaptive response to H_2O_2 , besides supplying NADPH to the GR reaction. Further studies on the role of G6PDH in the adaptive response to oxidative stress are under way to clarify the function of G6PDH in *S. cerevisiae*.

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REFERENCES

- Jamieson, D. J. (1992) *J. Bacteriol.* **174**, 6678–6681
- Storz, G. and Toledano, M. B. (1994) *Methods Enzymol.* **236**, 196–207
- Davies, J. M. S., Lowry, C. V. and Davies, K. J. (1995) *Arch. Biochem. Biophys.* **317**, 1–6
- Jamieson, D. J. (1995) *Redox Report* **1**, 89–95
- Moradas-Ferreira, P., Costa, V., Piper, P. and Mager, W. (1996) *Mol. Microbiol.* **19**, 651–658
- Izawa, S., Inoue, Y. and Kimura, A. (1995) *FEBS Lett.* **368**, 73–76
- Stephen, D. W. S., Rivers, S. T. and Jamieson, D. J. (1995) *Mol. Microbiol.* **16**, 415–423
- Grant, C. M., MacIver, F. H. and Dawes, I. (1996) *Curr. Genet.* **29**, 511–515
- Stephan, D. W. S. and Jamieson, D. J. (1997) *Mol. Microbiol.* **23**, 203–210
- Wu, A.-L. and Moye-Rowley, W. S. (1994) *Mol. Cell. Biol.* **14**, 5832–5839
- Grant, C. M., Collinson, L. P., Roe, J.-H. and Dawes, I. W. (1996) *Mol. Microbiol.* **21**, 171–179
- Grant, C. M., MacIver, F. H. and Dawes, I. W. (1996) *Mol. Microbiol.* **22**, 739–746
- Meister, A. and Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52**, 711–760
- Kletzien, R. F., Harris, P. K. W. and Foellmi, L. A. (1994) *FASEB. J.* **8**, 174–181
- Tran, L.-T., Miki, T., Kamakura, M., Izawa, S., Tsujimoto, Y., Miyabe, S., Inoue, Y. and Kimura, A. (1995) *J. Ferment. Bioeng.* **80**, 606–609
- Nogae, I. and Johnston, M. (1990) *Gene* **96**, 161–169
- Juhnke, H., Krems, B., Kotter, P. and Entian, K.-D. (1996) *Mol. Gen. Genet.* **252**, 456–464
- Slekar, K. H., Kosman, D. J. and Culotta, V. C. (1996) *J. Biol. Chem.* **271**, 28831–28836
- Beutler, E. (1994) *Blood* **84**, 3613–3636
- Scott, M. D., Zuo, L., Lubin, B. H. and Chiu, D. T.-Y. (1991) *Blood* **77**, 2059–2064
- Scott, M. D., Lubin, B. H., Zuo, L. and Kuypers, F. A. (1991) *J. Lab. Clin. Med.* **118**, 7–16
- Scott, M. D., Wagner, T. C. and Chiu, D. T.-Y. (1993) *Biochim. Biophys. Acta* **1181**, 163–168
- Wu, J. and Weiss, B. (1992) *J. Bacteriol.* **174**, 3915–3920
- Greenberg, J. T. and Demple, B. (1986) *J. Bacteriol.* **168**, 1026–1029
- Tuggle, C. K. and Fuchs, J. (1985) *J. Bacteriol.* **162**, 448–450
- Tsai, C. S., Ye, H. G. and Shi, J. L. (1995) *Arch. Biochem. Biophys.* **316**, 155–162
- Tsai, C. S., Shi, J. L. and Ye, H. G. (1995) *Arch. Biochem. Biophys.* **316**, 163–168
- Collinson, L. P. and Dawes, I. (1995) *Gene* **156**, 123–127
- Izawa, S., Inoue, Y. and Kimura, A. (1996) *Biochem. J.* **320**, 61–67

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- 30 Miki, T., Tsujimoto, Y., Miyabe, S., Sugiyama, K., Izawa, S., Inoue, Y. and Kimura, A. (1996) *Biosci. Biotech. Biochem.* **60**, 1207–1209
- 31 Kuby, S. A. and Noltman, E. A. (1966) *Methods Enzymol.* **9**, 116–125
- 32 Rippa, M. and Signorini, M. (1975) *Methods Enzymol.* **41**, 237–240
- 33 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **265**, 5267–5272
- 34 Tietze, F. (1969) *Anal. Biochem.* **27**, 502–522
- 35 Anderson, M. E. (1985) *Methods Enzymol.* **113**, 548–555
- 36 Schnell, N., Krems, B. and Entian, K.-D. (1992) *Curr. Genet.* **21**, 269–273
- 37 Sinha, A. and Maitra, P. (1992) *J. Gen. Microbiol.* **138**, 1865–1873
- 38 Miller, E. G. D. (1996) *Mol. Biol. Cell* **7**, 1805–1813
- 39 Thomas, D., Cherest, H. and Surdin-Kerjan, Y. (1991) *EMBO. J.* **10**, 547–553
- 40 Kirkman, H. N., Galiano, S. and Gaetani, G. F. (1987) *J. Biol. Chem.* **262**, 660–666
- 41 Hillar, A. and Nicholls, P. (1992) *FEBS Lett.* **314**, 179–182
- 42 Hillar, A., Nicholls, P., Switala, J. and Loewen, P. C. (1994) *Biochem. J.* **300**, 531–539
- 43 Kuge, S. and Jones, N. (1994) *EMBO J.* **13**, 655–664
- 44 Chae, H. Z., Chung, S. J. and Rhee, S. G. (1994) *J. Biol. Chem.* **269**, 27670–27678
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