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ORIGINAL PAPER

Decarbonylated cyclophilin A Cpr1 protein protects *Saccharomyces cerevisiae* KNU5377Y when exposed to stress induced by menadione

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Abstract Cyclophilins are conserved *cis–trans* peptidyl-prolyl isomerase that are implicated in protein folding and function as molecular chaperones. The accumulation of Cpr1 protein to menadione in *Saccharomyces cerevisiae* KNU5377Y suggests a possibility that this protein may participate in the mechanism of stress tolerance. Stress response of *S. cerevisiae* KNU5377Y *cpr1Δ* mutant strain was investigated in the presence of menadione (MD). The growth ability of the strain was confirmed in an oxidant-supplemented medium, and a relationship was established between diminishing levels of cell rescue enzymes and MD sensitivity. The results demonstrate the significant effect of *CPR1* disruption in the cellular growth rate, cell viability and morphology, and redox state in the presence of MD and suggest the possible role of Cpr1p in acquiring sensitivity to MD and its physiological role in cellular stress tolerance. The in vivo importance of Cpr1p for antioxidant-mediated reactive oxygen species (ROS) neutralization and chaperone-mediated protein folding was confirmed by analyzing the expression changes of a variety of cell rescue proteins in a *CPR1*-disrupted strain. The *cpr1Δ* to the exogenous MD showed reduced expression level of antioxidant enzymes, molecular chaperones, and metabolic enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH)- or adenosine triphosphate (ATP)-generating systems. More importantly, it was shown that *cpr1Δ* mutant caused imbalance in the cellular redox homeostasis and increased ROS levels in the cytosol as well as mitochondria and elevated iron concentrations. As a result of excess ROS production, the *cpr1Δ* mutant provoked an increase in oxidative damage and a reduction in antioxidant activity and free radical scavenger ability. However, there was no difference in the stress responses between the wild-type and the *cpr1Δ* mutant strains derived from *S. cerevisiae* BY4741 as a control strain under the same stress. Unlike BY4741, KNU5377Y Cpr1 protein was decarbonylated during MD stress. Decarbonylation of Cpr1 protein in KNU5377Y strain seems to be caused by a rapid and efficient gene expression program via stress response factors Hsf1, Yap1, and Msn2. Hence, the decarbonylated Cpr1 protein may be critical in cellular redox homeostasis and may be a potential chaperone to menadione.

Keywords Cyclophilin A Cpr1 · Oxidative stress · Antioxidant enzymes · Molecular chaperones · *Saccharomyces cerevisiae* KNU5377Y

Introduction

Cyclophilin A belongs to a large family of conserved and ubiquitous proteins ranging from bacteria to mammals and functions as peptidyl-prolyl *cis–trans* isomerases (PPIase), prolyl isomerases, or foldases (Arevalo-Rodriguez et al. 2000, 2004; Chen et al. 2007). The genomes of *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae* encode different numbers of the cyclophilin paralogs (Galat 2004). In *S. cerevisiae* and human T cells,

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the cyclophilin (Cyp)/cyclosporine (CsA) complex was found to inhibit the calcium/calmodulin-dependent serine/threonine phosphatase activity of the calcineurin protein (Arevalo-Rodriguez et al. 2000; Wang and Heitman 2005). Additionally, cyclophilins have been reported to be involved in a wide range of physiological roles, including cell division, transcriptional regulation, protein trafficking, cell signaling, pre-messenger RNA (mRNA) splicing (Wang et al. 2001; Arevalo-Rodriguez et al. 2004; Coaker et al. 2005; Chen et al. 2007; Massignan et al. 2007), molecular chaperoning, stress tolerance, and host–microbial interactions. Although mutants lacking single or multiple cyclophilins have a few number of phenotypes in the yeast *S. cerevisiae* under stress conditions (Arevalo-Rodriguez et al. 2000, 2004), the ubiquitous and conserved nature of cyclophilins suggests that they have fundamental cellular roles.

Among cyclophilins, the yeast Cpr1 (P14832), a single-domain cyclophilin peptidyl-prolyl *cis–trans* isomerase, is highly conserved and localized in the cytosol and nucleus (Wang and Heitman 2005). Only recently the endogenous functions of the protein have begun to be elucidated. Cpr1 is required for the glucose-stimulated transport of fructose-1,6-biphosphatase into the vacuole responsible for the import and degradation (Vid; Vid22p) of vesicles (Arevalo-Rodriguez et al. 2000). Cpr1 could promote the proper subcellular localization of an essential zinc finger protein (Zpr1) (Arevalo-Rodriguez and Heitman 2005). *CPR1* transcription has been known to be mediated by high temperature, NaCl, or sorbic acid (Arevalo-Rodriguez et al. 2004). In plants, the expression of *CYP* genes derived from different origins and CYP20-3 (ROC4; At5g62030) in *A. thaliana* has been upregulated in response to various external stimuli, including wounding, light, heat, salt, cold, salicylic acid, ethylene, jasmonate, viral infection, and fungal infection (Romano et al. 2004), which catalyze serine acetyltransferase (SAT1), a rate-limiting enzyme, in cysteine biosynthesis (Dominguez-Solis et al. 2008).

Menadione (MD; 2-methyl-1,4-naphtoquinone; vitamin K3) has been used as a model for studies coupled with oxidative stress (Castro et al. 2007). Oxidative stress is the result of an imbalance between oxidant and antioxidant systems (Jamieson 1998). In order to overcome transient alteration or continuous reactive oxygen species (ROS) challenges, cells have evolved a variety of enzymatic and nonenzymatic antioxidant defense systems that are capable of removing free radicals and their by-products to protect cell integrity as well as molecular chaperone systems (Jamieson 1998; Moradas-Ferreira and Costa 2000). In these systems, oxidative stress enhances the accumulation of chaperones, such as cyclophilins. Cyclophilin expression in plant tissues increases in response to types of stress such as heat shock and infection of pathogens (Perez and Weis 2008). In vascular smooth muscle cells, oxidative stress leads to increased expression and secretion of cyclophilin A (CypA), and it is believed that the PPIase activity of CypA is necessary for inhibiting nitric oxide-induced apoptosis and for activating extracellular signal-regulated kinase (ERK1/2) (Perez and Weis 2008). The role of Cpr1p as a component of cellular protection in yeast during oxidative stress conditions has not been studied because there was no noticeable difference in phenotypes to H_2O_2 stress in the wild-type and *cpr1*Δ cells in BY4741 (Costa et al. 2002).

We compare the expression changes between the wild-type strain and its isogenic *cpr1Δ* mutant from KNU5377Y and BY4741, in order to further investigate the origin of the sensitivity of the *cpr1Δ* mutant toward MD stress. Thus, we hope that the outcome of this study will provide new insights into the mechanism of action and will add a new dimension in cell signaling.

Materials and methods

Growth conditions

Pre-cultures aerobically grown at 30°C overnight in a YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) were transferred to a fresh YPD medium. Having reached mid-log phase (OD_{600}=1.0), cells were exposed to 0.4 mM MD (ethanol-soluble form; Sigma M5625) for 1 h at 30°C, harvested by centrifugation, washed twice with cold phosphate-buffered saline (PBS) buffer, and then used for subsequent experiments. In addition, their morphology was evaluated microscopically. Growth kinetics was measured by spectrophotometer analysis. Pre-cultures were inoculated into a fresh YPD broth in the presence of 50 μM MD and cultured at 30°C with shaking at 160 rpm. One-milliliter aliquot of the culture was used to monitor the growth of the cells by measuring the optical density at 600 nm (OD_{600}) for the indicated time. Initial optical density was adjusted to 0.2. For spotting assay, cells reaching the mid-log phase were challenged directly with 0.4 mM MD for 1 h at 30°C, serially diluted with cold distilled water, spotted onto YPD agar plates, and then incubated for 2 days at 30°C. Viable cell numbers were determined by counting the colonies that formed on YPD agar plates without or with 0.4 mM MD for 1 h at 30°C. Viability was determined as survival percentage values, which were calculated by dividing the viable cell numbers after stress application by the viable cell numbers determined under the same growth conditions but without stress exposure.

Cellular redox status and damage

The intracellular hydroperoxide level was determined by ferrous ion oxidation in the presence of a ferric ion indicator,

xylenol orange (Gay et al. 1999). To measure the levels of cytosolic and mitochondrial ROS, exponential cells were incubated for 20 min at 30°C with 100 μM dichlorodihydrofluorescein diacetate (DCFHDA) or 100 μM dihydrorhodamine 123, exposed to 0.4 mM for 1 h, washed twice with PBS buffer, and then resuspended in the same buffer. Cells loaded with the fluorescent probes were imaged with a fluorescence microscopy (excitation, 488 nm; emission, 525 nm) (Lee et al. 2008). Carbonyl content was visualized via immunoblot analysis with anti-dinitrophenyl (DNP) antibody (Sigma) after two-dimensional gel electrophoresis (Kim et al. 2006a). Carbonyl content was also measured by the spectrometric method reported previously (Kim et al. 2006a).

Colorimetric assay for iron quantitation

After growing under nonstressful and stressful conditions, cells were washed twice with ultra-pure water, suspended in 500 μl of 3% nitric acid, and then heated for 12 h at 94°C. Subsequently, the heated cells were centrifuged to collect a cleared supernatant, which was used for a colorimetric assay to quantify the total cellular iron. Nitric acid (400 μl, 3%) containing either standard or lysed yeast cells was mixed with 160 μl of 38 mg/ml sodium ascorbate, 320 μl of 1.7 mg/ml bathophenanthrolinedifulfonic acid (BPS; iron chelator) and 126 μl of 7.5 M ammonium acetate solution. After reaction for 5 min at room temperature, the absorbance of the iron chelator complex was observed at 535 nm as described previously (Tamarit et al. 2006).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis

Cellular total proteins were obtained from cells treated for 1 h at 30°C with 0.4 mM MD for KNU5377Y and BY4741 (Kim et al. 2006b). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by loading 25 μg of denatured proteins, which were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked, then incubated with primary antibodies Sod1, Sod2, Hsp104, and Hsp60 (Stressgen, Glanford Ave, VIC, Canada); thioredoxin 2, thioredoxin 3, and thioredoxin reductase 1 (kindly provided by Dr. Kang-Wha Kim in Korea); thioredoxin peroxidase I (kindly provided by Dr. Jeen-Woo Park in Korea); alcohol dehydrogenase and hexokinase (Rockland, Gilbertsville, PA, USA); glucose-6-phosphate dehydrogenase, Zpr1, glutathione reductase, and catalase (Sigma, Saint Louis, MI, USA); glutathione peroxidase (Agrisera, Vannas, Sweden); glyceraldehyde-3-phosphate dehydrogenase (Ab Frontier, Seoul, South Korea); porin (Invitrogen, Camarillo, CA, USA); and tubulin (SantaCruiz, Santa Cruz, CA, USA) as a control. Anti-Hsp82, Ssa, Sti, Grp, Hsp42, and Hsp26 antibodies were kindly provided by Dr. Johannes Buchner in Germany. Anti-Hsp31 and anti-ECS antibodies were synthesized with amino acid sequence in yeast and *Brassica rapa*, respectively. An appropriate secondary antibody was proved, developed by enhanced chemiluminescence (ECL kit; GE Healthcare), processed, and photographed.

Immunoprecipitation

Cell lysates were pre-cleared with 50 μl of pre-equilibrated protein-A agarose. The lysates were then immunoprecipitated by the addition of 10 μg of anti-DNP for 1 h at 4°C. After centrifugation and washing, the immunoprecipitates were boiled in a reducing sample buffer and separated by SDS-PAGE. The gel was electroblotted to PVDF membranes and blocked with 5% nonfat dry skim milk for 1 h at room temperature (RT). Anti-Cpr1p pAb (1:500 dilution) was incubated with the membrane in 5% nonfat dry skim milk at 4°C overnight. The membranes were washed three times for 30 min with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit pAb diluted to 1:1,000 for 1 h at RT. The membranes were then washed three times and developed with the enhanced chemiluminescence system (Amersham) and X-ray films.

Two-dimensional gel electrophoresis

Yeast cells grew to the mid-log phase and were exposed to menadione for 1 h at 30°C with shaking. Cells were harvested by centrifugation, washed twice with cold PBS buffer, resuspended in lysis buffer containing 50 mM Tris–HCl, pH 7.5, 5% glycerol, 1 mM ethylenediamine tetraacetic acid (EDTA), 1.5% mercaptoethanol plus 1 mM PMSF, and vortexed with MicroMixer. Protein crude extracts were obtained by high speed centrifugation, incubated with DNase/RNase/Mg mix on ice for 15 min, boiled for 5 min, and cooled on ice for 5 min. Sample preparation by TCA/acetone precipitation, isoelectric focusing, equilibration, and SDS-PAGE was followed as previously reported (Kim et al. 2006a).

Determination of intracellular NADPH level

The measurement of cellular NADPH was carried out as in previous studies (Fernandes et al. 2007; Tan et al. 2009). NADPH levels were expressed as an nanomole per milligram protein.

PPIase activity

PPIase assay mixture which include 20 mM HEPES buffer (pH 8.0) containing 0.1 M NaCl, 1 mM EDTA,

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
BY4741	*MATa*, *his3Δ1*, *leuΔ0*, *met15 Δ0*, *ura Δ0* (**bwt**)	EUROSCARF
	MATa, *his3Δ1*, *leuΔ0*, *met15 Δ0*, *ura Δ0*, *YDR155c::kanMX4* (*cpr1Δ*, **bc1**)	EUROSCARF
KNU5377Y	*MATa* (**kwt**)	Kim et al. (2006b)
	MATa, *YDR155c::kanMX4* (*cpr1Δ*, **kc1**)	Kim et al. (2006b)

EUROSCARF European *Saccharomyces cerevisiae* Archives for Functional Analysis

and 50 μM *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Sigma), was incubated at 10°C. Added to initiate the reaction was 20 μM α-chymotrypsin. Crude extract (100 μg/ml) or purified protein (10 g/ml) was added after 15 s. Absorbance was monitored every 2 s at 360 nm for 1 min by UV spectrophotometry. Control blanks without protein extract were included (Perez and Weis 2008).

Free radical scavenging ability on 1, 1-diphenyl-2-picrylhydrazyl

To assess the scavenging ability on 1,1-diphenyl-2-picryl-hydrazyl (DPPH), crude extract in ethanol was mixed with 1 ml of ethanolic solution containing 0.2 mM DPPH radicals. The mixture was shaken vigorously and left to stand for 30 min in the dark before measuring the absorbance at 517 nm (Chen et al. 2009).

Measurement of the antioxidant power by ferric reducing ability

The ferric reducing ability of yeasts was assessed following the method reported previously (Gillespie et al. 2007) Briefly, the ferric reducing/antioxidant power (FRAP) reagent contained 2.5 ml of 10 mM of 2,4,6-tris (2-pyridyl)-1,3,5-triazine solution in 40 mM HCl plus 2.5 ml of 20 mM $FeCl_3$ and 25 ml of 0.3 M acetate buffer, pH 3.6, which was freshly prepared and warmed at 37°C prior to analysis. Aliquots of samples were mixed with 0.2 ml distilled water and 1.8 ml of FRAP reagent was added. The final results were expressed as the concentration of antioxidants having ferric reducing ability equivalent to that of 1 mM $FeSO_4$, particularly expressed as mole Fe (II) equivalent/gram sample in wet weight.

Statistical analysis

All experiments were carried out in at least three independent repetitions. Results were expressed as mean ± standard deviation.

Results

The kc1 cells are hypersensitive to menadione stress

Cyclophilin A (Cpr1) expression was induced during fermentation at high temperature (40°C) in *S. cerevisiae* KNU5377 (Kim et al. 2006b). The fermentation process at high temperature as well as transient heat shock has been known to produce ROS. The observation and the result imply that KNU5377 Cpr1 may be involved in the oxidative stress response. As a source of ROS, menadione, solvent (ethanol or DMSO)-soluble form, used in this study, is well known to generate free radicals. To explore cyclophilin A for its physiological role in the less known Cpr1, wild-type cells (kwt) in haploid KNU5377Y were disrupted and named as kc1. Wild-type (bwt) and *cpr1Δ* mutant cells (bc1) from laboratory strain *S. cerevisiae* BY4741 were introduced as positive controls. A cell viability assay was performed to evaluate the effect of Cpr1 on stress response against MD. The kwt and kc1 cells showed a viability of 73% and 22%, respectively, as compared with the condition without MD treatment. Cell viability of bwt and bc1 was not significantly different when reduced by up to 25% and 20% under 0.4 mM MD, respectively (Fig. 1a). Figure 1b shows that the growth rate of the kc1 cells was lower than that of the kwt cells when the cells were cultured on liquid YPD medium in the presence of 50 μM MD. By contrast, the growth rate in the bwt and the bc1 cells did not show any effect in the indicated time under the same conditions. Cell growth kinetics in the presence of 50 μM MD was in the order of kwt, kc1, and BY4741 (bwt and bc1) cells. For the first 12 h, growth in the kwt cells occurred at almost the same rate as that in the other cells. A lag then ensued, with growth resuming, but more rapidly. The kwt cells entered the stationary phase stage at 20 h. The kc1, bwt, and bc1 cells had a longer lag phase period (22 and 30 h, respectively) as compared to a rapid restoration of growth kinetics in the kwt cells. The growth recovery of bwt and bc1 cells was created in the presence of MD, which shows no difference in growth kinetics. The appearance of the stationary phase was achieved at about 36 h after initial

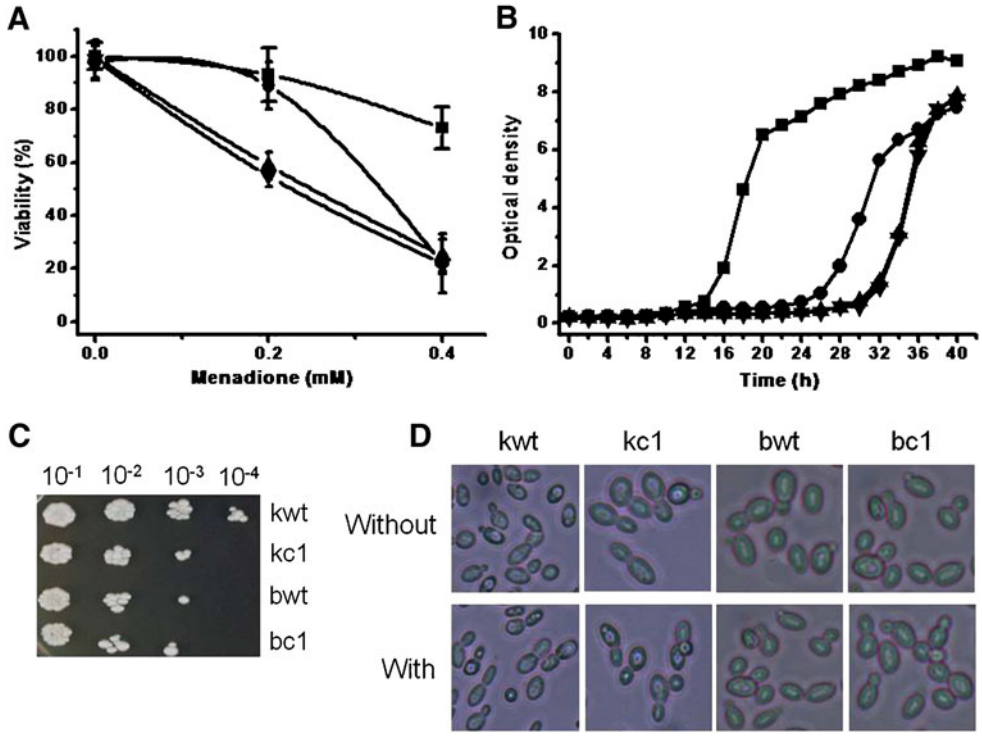

Fig. 1 Growth kinetics, stress sensitivity, cell viability, and morphology analysis in the presence of menadione in *S. cerevisiae*. **a** Mid-log phase yeast cells were exposed to 0.0, 0.2, and 0.4 mM MD for 1 h, diluted serially to 10^{-5}. One hundred microliters of the 10^{-5} diluted solutions was spread onto YPD agar plate. After incubation for 2 days, CFU was counted. Viability was calculated as 100% of the colony counting number in the nonstressed cells. **b** Growth rate was measured every 2 h for 40 h in the presence of 50 μM MD in four strains. kwt (*filled square*), kc1 (*filled circle*), bwt (*filled triangle*), and bc1 (*filled inverted triangle*). **c** Spotting assay was performed via serial dilution (10^{0}–10^{-4}) from yeast cells exposed to 0.4 mM MD for 1 h. **d** Cell morphology was photographed with a light microscope before (without) and after 0.4 mM MD treatment (with) for 1 h

inoculation. Stress sensitivity was also detected by a spotting assay with serial dilution. Stress acquisition of the kwt cells exposed to 0.4 mM MD was higher than that of kc1, bwt, and bc1 cells. The stress response of the kc1 cells was similar to the bwt and bc1 cells (Fig. 1c). Cell morphology analysis was carried out to observe cell shape during MD stress. As shown in Fig. 1d, there was no difference in the cell morphology before and after MD treatment among the four strains. These results suggest that the Cpr1 protein of KNU5377Y cells is an important factor of stress defense without affecting cell morphology because the kc1 cells were hypersensitive when exposed to MD as compared to the kwt cells, but not BY4741 cells (bwt and bc1).

The kc1 cells diminish expression of antioxidant enzymes and NADPH levels

Oxidant-induced protective responses often result from the coordinated activation of proteins involved in oxidant detoxification. Because cellular antioxidants act in a concerted manner, we investigated whether the modulation in cellular Cpr1 expression caused alterations in the activity of other major antioxidant enzymes. The expression transition of the antioxidant enzymes was conducted in exponential phase cells imposed on MD by Western blot analysis. As seen in Fig. 2a, the expression of cytosolic superoxide dismutase (Sod1), mitochondrial superoxide dismutase (Sod2), gamma glutamylcysteine synthetase (ECS or Gsh1), glutathione reductase (GR), thioredoxin isoform 2 (Trx2), cytosolic thioredoxin peroxidase (Tsa1), and porin (Por) was downregulated in the kc1 cells under MD stress, as compared to the kwt cells, whereas the expression of catalase increased in the kc1 cells in the presence of MD. Expression of these enzymes did not change in the bwt cells and the bc1 cells under the same conditions. The blocking of Cpr1 protein in the kc1 cells also caused the depression of metabolic enzymes, which contains glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH), and alcohol dehydrogenase (ADH). Changes in the aforementioned enzymes were not observed in both types of BY4741 strains (bwt and bc1 cells). Expression of GAPDH, ADH, and G6PDH in the bc1 cells was higher than that in the bwt cells during MD stress (Fig. 2b). Western blot analyses were strongly confirmed by enzyme activity assay. Analysis of enzyme activity of GR, GPX, G6PDH, and IDH was similar to protein expression by immunoblotting (Fig. S1). To examine the effect of G6PDH

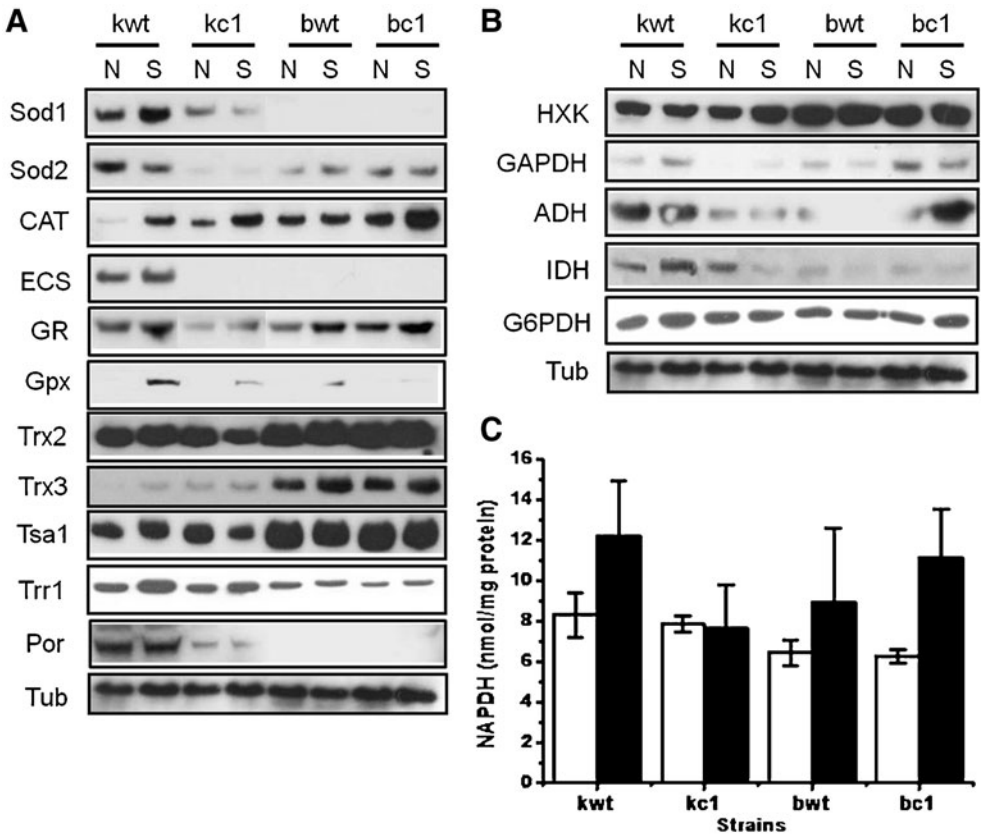

Fig. 2 The expression changes of antioxidant enzymes and metabolic enzymes and cellular NADPH assay against menadione stress. Mid-log phase yeast cells were cultured in the absence (*N*) and presence (*S*) of 0.4 mM MD for 1 h. Cells were collected and cellular proteins were used to detect antioxidant proteins (**a**) and metabolic enzymes (**b**) by Western blotting analysis following SDS-PAGE or to measure cellular NADPH level (**c**) *White bar*, nonstressed cells; *black bar*, MD-stressed cells. In immunoblot analysis, tubulin (*Tub*) was used for an internal standard

and IDH downregulation of the kc1 cells, the NADPH level was measured in the presence and absence of MD (Fig. 2c). During MD stress, the kwt, bwt, and bc1 cells led to a significant increase in the total pool of NADPH. MD treatment of the kc1 cells did not affect NADPH homeostasis. The level was highest in the kwt cells. Interestingly, the NADPH pool of the bc1 cells was higher than that of the bwt cells during MD exposure. This was probably due to increased accumulation of G6PDH. G6PDH (Zwf1p) produces NADPH in order to combat oxidative stress (Tan et al. 2009). According to previous reports, the transcriptional levels of genes encoding antioxidant proteins and metabolic enzymes were strongly induced to MD within the cells. The genes are as follows: superoxide dismutase (SOD1), catalase (CTT1), glutathione synthesis (GSH1), glutathione peroxidase (GPX1, GPX2, GPX3 or HYR1), glutathione reductase (GLR1), thioredoxin (TRX1 and TRX2), thioredoxin reductases (TRR1), thioredoxin peroxidase (TSA1, AHP1, and TSA2), pentose phosphate pathway (GND1 and GND2), and hexokinase (HXK1) (Gasch et al. 2000; Causton et al. 2001; Demasi et al. 2006). Taken together, these data indicate that there is an increased burden on antioxidant enzymes and on the metabolic enzymes containing NADPH-generating systems during MD stress. The enzyme level analyzed did not increase upon MD treatment in the kc1 cells except for CAT, Gpx, Trr1, and Hxk, which shows that Cpr1 has an effect on the expression of antioxidant proteins and metabolic enzymes, at least partially, to stress tolerance against MD stress in KNU5377Y.

Molecular chaperones require Cpr1 protein in stress response against menadione

One of the different characteristics of oxidative stress is the inducible expression of a highly conserved family of proteins known as the heat shock proteins (Hsps) or molecular chaperones, which confer a protective mechanism to ensure survival of the stressed cell against normally lethal forms of stress. We analyzed whether the lack of Cpr1 protein has an effect on the expression of chaperone proteins or its cofactors using immunoblotting analysis. As shown in Fig. 3a and b, Hsp104, Hsp90 (Hsp82), Hsp42, Hsp31, endoplasmic reticulum (ER) chaperone Grp, and Hsp90 cochaperone Sti1 were reduced in the Cpr1-deficient kc1 cells in the absence and presence of MD, as compared to the kwt cells. Ssb and Hsp60 were more strongly induced in the kc1 cells than in the kwt cells. Ssa, Hsp26, and a zinc finger protein Zpr1 in both cells were unchanged in the presence of MD. In the control strain, there was no difference in the expression levels of Hsp104, Ssb, Hsp60, Hsp31, Zpr1, and Grp in both bwt and bc1 cells, whereas the expression of Hsp90, Ssa, Hsp42, Hsp26, and Sti1 was higher in the bc1 cells than in

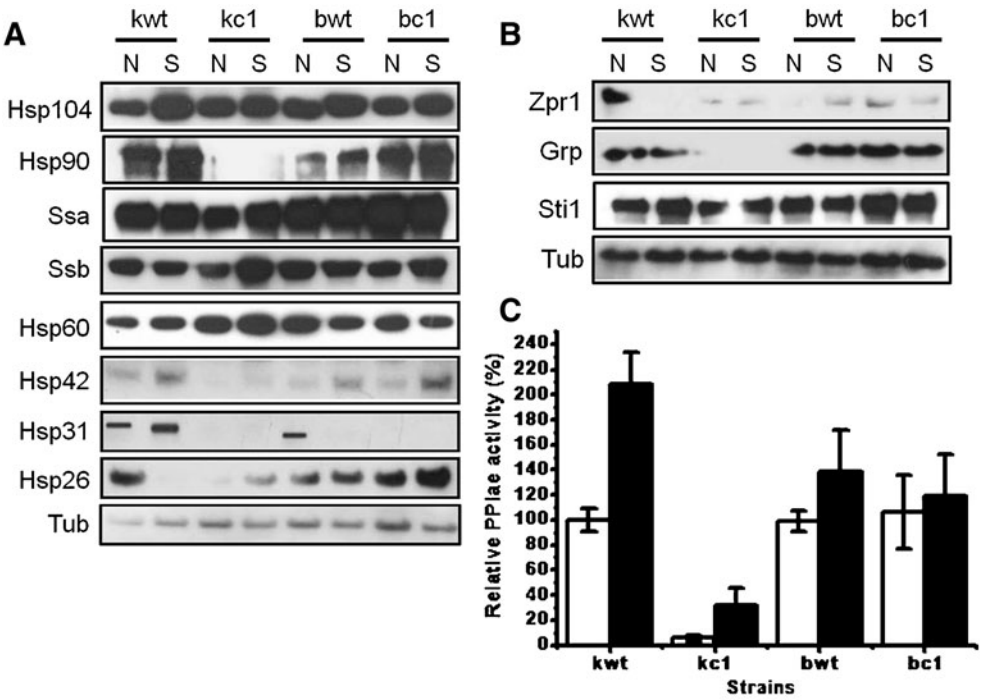

Fig. 3 Expression analyses of heat shock proteins, molecular chaperones, and its cofactors and PPIase activity assay under menadione stress. Western blot analyses were carried out properly with primary antibody diluted. Expression of Hsps and molecular chaperones (**a**) and its cofactor or substrates (**b**) was detected before MD treatment (*N*) and after MD treatment (*S*). Tubulin (*Tub*) was used as a housekeeping control. **c** PPIase activity with each crude extract was calculated under basis of comparison being 100% for the bwt cells untreated MD. *White bar*, nonstressed cells; *black bar*, MD-stressed cells

the bwt cells. In the transcriptional analysis, the transcripts of Hsp104, Hsp42, and Hsp78 were activated during MD stress, whereas the mRNA levels of Hsp26, Ssa3, Ssa4, and Sse were repressed under the same condition (Gasch et al. 2000). Hsp104 (Fig. S2) (Cashikar et al. 2005), Hsp90 (Imai and Yahara 2000), Hsp42 (Wotton et al. 1996), and Hsp31 (Skoneczna et al. 2007) are crucial for yeast stress tolerance. The chaperones have a PPIase activity that catalyzes the rate-limiting proline isomerization in the protein-folding process. Based on these facts, we measured PPIase activity with crude extract to determine whether the loss of molecular chaperone proteins due to Cpr1 protein deficiency affects the PPIase activity. The kc1 cells did not increase PPIase activity in the presence and absence of MD as compared to the kwt cells, where this activity was lower. There were no differences in the PPIase activity in both bwt and bc1 cells although the enzyme activity showed a moderate increase during MD stress (Fig. 3c). Hence, the Cpr1 protein plays an important role in molecular chaperone systems that protect cells against MD stress in the kwt cells since chaperone expression and PPIase activity were reduced in the kc1 cells. As a detrimental consequence, Cpr1 protein deficiency can lead to abnormal protein homeostasis following aberrant protein conformations and aggregation under MD stress.

Loss of Cpr1 protein causes enhancement of cellular ROS levels and damaged proteins

The difficulty of maintaining the redox homeostasis in the kc1 cells could render it unprotected against oxidative stress caused by MD. The breakdown of cellular redox balance involves the modulation of cellular oxidative biomarkers such as hydroperoxide and protein oxidation by carbonyl content increase. As seen in Fig. 4a, hydroperoxide levels using FOX reagent in the kc1 cells were approximately 2.0-fold higher than that in the kwt cells during MD management.

The hydroperoxide levels in the bwt and bc1 cells were unchanged under conditions supplemented with MD although the concentration was higher than in the kc1 cells (Fig. 4a). To confirm these findings, the levels of intracellular hydroperoxides were evaluated by fluorescence microscopy with cytosolic oxidant-sensitive probe DCFHDA and mitochondrial ROS-sensitive probe rhodamine 123. An increase in DCF and rhodamine 123 fluorescence was observed in both kwt and kc1 cells when they were exposed to MD. The increase in fluorescence was more pronounced in the kc1 cells as compared to the kwt cells. No distinguishable differences were observed in either the bwt or bc1 cells although the fluorescence intensity was strongly induced in these cells. In addition, we observed a moderate release of probe dye outside yeast cells in the bwt and bc1 cells (Fig. 4b). The results indicate that MD stress in the kc1 cells leads to increased ROS levels in the cytosol and mitochondria. Mitochondrial oxidative stress has been implicated in cell death and aging-related pathologies including some neurological diseases (Herrero et al. 2008). Furthermore, increased ROS levels are in agreement with a reduction of antioxidant enzymes in the kc1 cells due to Cpr1 protein deficiency. The ROS production causes damage to cell components such as proteins, lipids, and DNA. Among these macro-

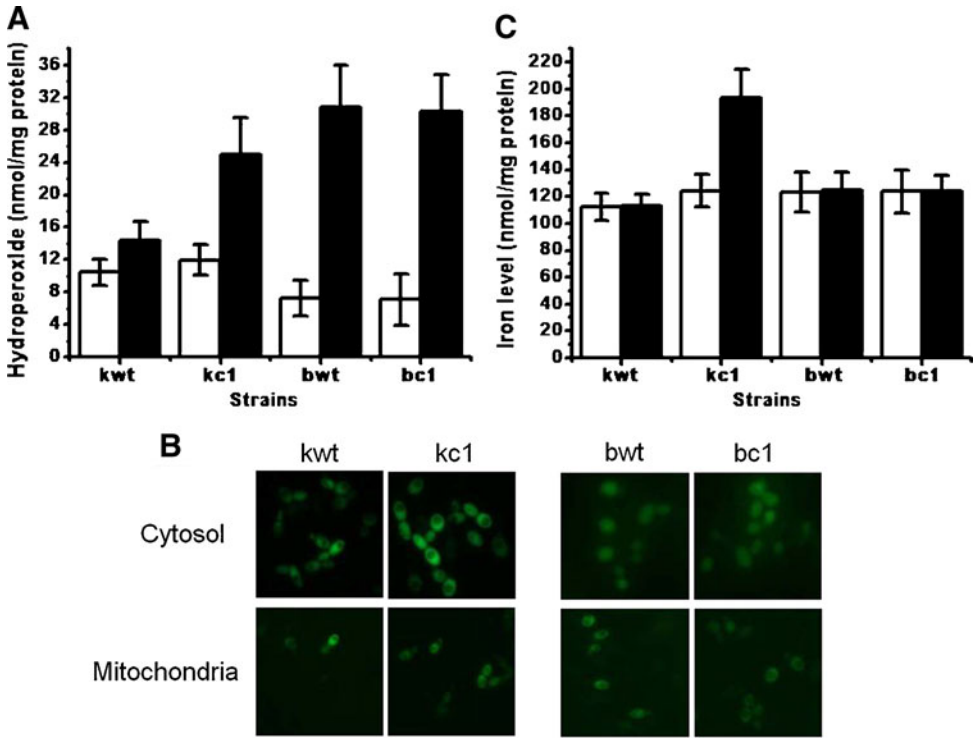

Fig. 4 Measurement of cellular ROS level and labile iron pool. **a** Cellular ROS level was measured with spectrophotometric method using FOX reagent. *White bar*, nonstressed cells; *black bar*, MD-stressed cells. **b** Measurement of in vivo molecular oxidation. DCF (cytosol) and DHAR 123 (mitochondria) fluorescences were measured in yeast cells exposed to MD. Fluorescence images were obtained under microscopy. **c** Intracellular pool of labile iron (micromole per milligram protein) was measured in four strains before and after menadione treatment. *White bar*, nonstressed cells; *black bar*, stressed cells

molecules, protein carbonylation is a unique and highly specific biomarker of MD-induced protein modification. Analysis of protein oxidation by carbonyl-deriving agents is usually used to measure oxidative damage at the molecular level. Levels of protein carbonylation were first measured spectrophotometrically using DNPH in the absence and presence of MD. Increased carbonylation was observed in the kc1 cells with average levels approximately 1.7-fold higher compared to the kwt cells (2.5 nmol/mg protein) during MD stress. On the other hand, carbonyl content of the bwt cells and bc1 cells increased and reached up to 4.5~4.7 nmol/mg protein although there were no differences in the levels under the same MD condition (Fig. 5a). The carbonyl content was similar to the level (5.2 nmol/mg protein) of frataxin mutant *yfh1* cells exposed to H_2O_2-induced oxidative stress (Kim et al. 2010b). Secondly, the pattern change of protein carbonylation was analyzed with Western blot using anti-DNP following two-dimensional analysis. In this experiment, the bwt and bc1 cells were excluded because any differences of protein carbonylation could not be observed. As shown in Fig. 5b, protein carbonylation clearly increased in the kc1 cells as compared to the kwt cells during MD stress. Carbonylation is an irreversible and irreparable modification although it has been noted that carbonylated proteins can be more sensitive to proteolytic degradation than nonoxidized proteins. According to a recent report, Adh1 (alcohol dehydrogenase), Tdh2 (glyceraldehyde-3-phosphate dehydrogenase), and Trx2 (thioredoxin) are major targets of protein damage under oxidative stress (Kim et al. 2010b). To sum up our results, increased protein damage is supported by the results from cell viability assays, ROS levels, and cell rescue proteins in the kc1 cells.

Yeast kc1 cells lacking Cpr1 protein increase cellular labile iron pool

Iron is a cofactor required for many cellular functions such as oxygen transport, electron transfer, and enzyme catalysis. Iron-containing enzymes are key components of many essential biochemical reactions, such as energy metabolism, DNA synthesis and repair, and neutralization of ROS (Kruszewski 2003). However, the same biochemical properties that make iron favorable in many cellular processes might cause unfavorable effects under specific conditions. Based on this fact, a measurement of total cellular iron level via a colorimetric method for nitric digestion of yeast cells was performed (Tamarit et al. 2006). As shown in Fig. 4c, a total iron pool (193.7±20.7 μM/mg protein) of the kc1 cells was about 1.5-fold higher than that (124.3±12.1 μM/mg protein) of the kwt cells after MD treatment, while there were no differences in the iron level in both bwt and bc1

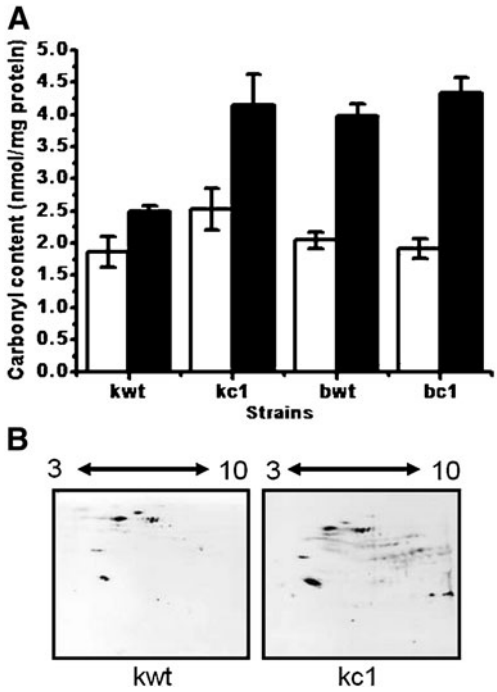

Fig. 5 Patterns of oxidatively damaged proteins against menadione-induced oxidative stress. **a** Carbonyl contents were measured by spectrophotometric assay. The levels were represented as nanomole per milligram protein. *White bar*, nonstressed cells; *black bar*, MD-stressed cells. **b** Pattern analysis of protein carbonylation by immunoblot analysis following two-dimensional analysis in the presence of MD in the kwt cells and the kc1 cells

cells, whose level was almost the same as the kwt cells without or with MD. In general, the cellular iron content of *S. cerevisiae* strain is from 90 to 120 μM, while that of the mutant strain of *YFH1* (*YDL120W*) regulating mitochondrial iron accumulation is approximately 250 μM (Babcock et al. 1997; Tamarit et al. 2006). The result indicates that the cellular labile iron pool in the kc1 cells is in excess as compared to the other cells in the presence of MD. The imbalance of iron homeostasis can catalyze one-electron reduction of oxygen that leads to oxidative stress generating very toxic hydroxyl radicals via the Fenton/Harber Weiss reaction cycle. Iron-driven production of free radicals is known to induce oxidation of proteins, lipids and lipoproteins, nucleic acids, carbohydrate, and other cellular molecules (Kruszewski 2003). The detrimental effect of labile iron pool has been related to cell phenotype changes, cell death, tissue necrosis, degenerative diseases such as Friedreich's ataxia (FRDA), sideroblastic anemia, aceruloplasminemiam, and cancer formation (Roy and Andrews 2001). Thus, free iron must be kept at the lowest physiologically relevant concentration to prevent the side effect of oxidative stress. This result suggests that the kc1 cells caused an imbalance of the cellular iron equilibration under MD stress, which could produce iron-derived oxidative stress.

Expression changes of Cpr1 and stress response factors, total antioxidant activity, and free radical scavenging ability

Certain biochemical and physiological properties were analyzed because the stress sensitivity of the kc1 cells was higher than that of the kwt cells against MD, unlike either bwt or bc1 cells. To begin with, whether Cpr1 is expressed or not under MD stress was considered. As seen in Fig. 6a (upper), the expression of Cpr1 protein in the kwt cells was strongly elevated during MD stress, as compared to the bwt cells. Several studies have shown that Cpr1 protein is carbonylated under hydrogen peroxide-induced oxidative stress (Costa et al. 2007). In our study, concern was focused on whether Cpr1p is modified under adverse conditions. To examine this possibility, protein crude extracts prepared from each of the cell types were immunoprecipitated with anti-DNP antibody. After the reaction, lysates were collected with protein A-Sepharose beads and subjected to Western blot analysis probing for Cpr1 protein. Unlike the bwt strain, the Cpr1 protein from the kwt strain was not carbonylated during MD stress (Fig. 6a, middle). The result from the bwt strain was consistent with the previous report that Cpr1p was carbonylated under oxidative stress (Costa et al. 2002). Yeast cells respond to oxidative stress by altering their transcriptional regulation in a complex way. Secondly, expression changes of general and specific stress response factors were analyzed between the kwt cells and the bwt cells during MD exposure. Among these factors, the expression patterns of Hsf1, Yap1, and Msn2 with each antibody were observed. The translational expression level of these factors in the kwt cells compared with the bwt cells was 1.5–3 times against MD stress although Yap1 and Msn2 in the bwt cells were induced. Hsf1p in the bwt cells was not detected during luminescence in the indicated time. Hsf1 (Fig. S3) as well as the two factors Yap1 and Msn2 is implicated in the stress response against oxidative stress such as hydrogen peroxide, superoxide such as MD, singlet oxygen and cadmium, which participate in part cooperatively (Herrero et al. 2008). After oxidative stress, Hsf1 has been shown to play a role in the induction of heat shock genes and some antioxidant genes (Raitt et al. 2000). Finally, depending on the increase of these proteins, the changes of antioxidant capacity and free radical scavenging ability were compared within the cells. The principle behind antioxidant activity is the availability of electrons to neutralize any so-called free radicals. In this work, the antioxidant activity of the ethanolic extract between yeast cells was evaluated using DPPH scavenging, FRAP. As shown in Fig. 6c, a significant difference was observed among the respective values obtained. The values varied, with the higher activity for the kwt cells under MD stress, as compared with the bwt cells. There were no differences

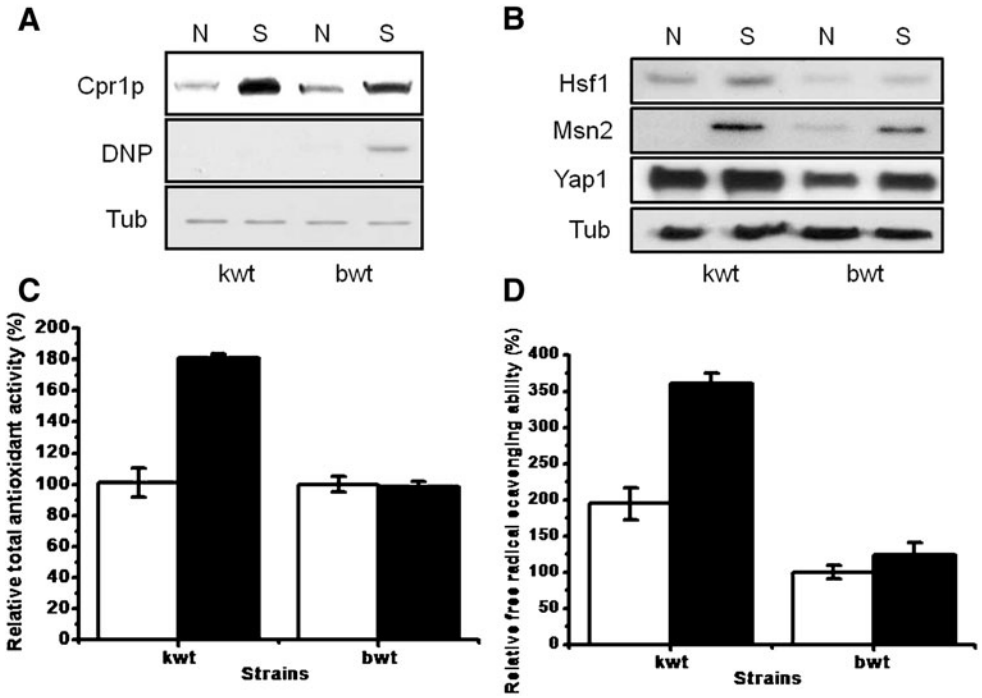

Fig. 6 Induction and carbonylation of Cpr1 protein, expression of transcription factors, and total antioxidant activity and free radical scavenger ability under MD stress. **a** Induction of Cpr1 protein and protein carbonylation by immunoprecipitation with total crude extract in the kwt and bwt cells. **b** Expression analysis of transcription factors Hsf1, Msn2, and Yap1 without MD (*N*) or with MD (*S*). Tubulin (*Tub*) was used as a housekeeping control. Relative total antioxidant activity (**c**) and relative free radical scavenger ability (**d**) were measured with spectrophometric assay in the kwt and bwt cells. Activity with each crude extract was calculated under basis of comparison being 100% for the bwt cells untreated MD. *White bar*, nonstressed cells; *black bar*, MD-stressed cells

in the levels in the bwt cells before and after MD stress. Free radical scavenging ability was strongly consistent with antioxidant capacity during MD stress (Fig. 6d).

Discussion

It was previously demonstrated that KNU5377Y Cpr1p is essential for oxidative defense against ROS (Kim et al. 2006b). Remarkably, we have shown here that Cpr1p is very specific in the protection of cells against MD. The protective action of Cpr1p was demonstrated by the growth kinetics and the viability in the kc1 cells (Fig. 1). Aerobic organisms have a range of mechanisms that cope with ROS derived from endogenous and exogenous sources. As an exogenous source, menadione can form ROS such as superoxide, hydrogen peroxide, and semiquinone radicals. ROS levels in the kc1 cells were higher than those in the kwt cells (Fig. 4a, b). ROS production can affect cellular components and increase oxidative biomarkers including lipid peroxidation, nucleic acid damage such as DNA fragmentation, and protein oxidation (protein carbonylation) (Fig. 5). To overcome or prevent overwhelming production of ROS, cells have many enzymatic and nonenzymatic mechanisms to detoxify ROS. Nonenzymatic components include small molecules such as glutathione (GSH) and thioredoxins (Trx1–3). GSH is synthesized in two sequential reactions catalyzed by gamma-glutamylcysteine synthetase (EC 6.3.2.2, Gsh1p or ECS) and glutathione synthetase (EC 6.3.2.3, Gsh2p or GS) in the presence of ATP. The *GSH1* and *GSH2* gene is regulated by Yap1p in the oxidative stress response, and the intracellular GSH content is increased with oxidative stress (Sugiyama et al. 2000). Enzymatic systems include superoxide dismutases (Sod1 and Sod2) that convert superoxide anion to hydrogen peroxide and oxygen, whereas the glutathione-dependent peroxidases (Gpx), thioredoxin-dependent peroxidases (Tsa1, etc.), and catalase (CAT) convert hydrogen peroxide to water and oxygen. In GSH and TRX systems, oxidized forms (GSSG) and thioredoxin (oTrx) are reduced by glutathione reductase (GR) and thioredoxin reductase (Trr), respectively. As seen in the MD-induced kc1 cells (Fig. 2), deletion or downregulation of these antioxidant metabolites leads to a number of oxygen-dependent phenotypes, including oxygen sensitivity, slow growth, hypersensitivity to superoxide generating agents such as MD or paraquat, which are believed to accelerate aging, and auxotrophy for methione and lysine. Lysine auxotrophy is attributed to oxidation of the iron sulfur center of homoaconitase, whereas methionine auxotrophy is thought to result from depletion of cellular NADPH by

superoxide anion (Tan et al. 2009) In this study, we analyzed results of this auxotrophy. Given the results showing increased iron levels (Fig. 4c) and the NADPH depletion (Fig. 2c), methionine and proline auxotrophy could be observed in the kc1 cells, but not lysine auxotrophy (data not shown). In addition, hexokinase (Hxk1), Sod1, Trx2, thioredoxin peroxidase (Tsa1, Tsa2, and Prx1), glutathione precursor synthesis (Ecm38), and glutathione peroxidase (Gpx1, Hyr1) are involved in the stress response to menadione (Gasch et al. 2000; Tucker and Fields 2004). Cellular NADPH that functions as a reducing power for GR and Trr is important for tolerance to ROS. Deleted or repressed (kc1 cells) genes involved in the nonoxidative branch of the pentose phosphate pathway that are important for NADPH production, such as G6PDH or IDH, are sensitive to oxidants and also defective in adaptation to oxidative stress (Tan et al. 2009). As the kc1 cells treated with MD rely on glycosylation to produce ATP, carbohydrate metabolism redirection after MD challenge would be affected and the generation of NADPH would be diminished. In cytosol, furthermore, the mitochondrial function requires maintaining redox homeostasis, which is mediated primarily by the voltage-dependent anion channel (VDAC; porin pore). The VDAC releases superoxide anion from mitochondria to cytosol (Han et al. 2003). These results were observed in this study. Porin expression was apparently reduced during MD stress, as compared to the kwt cells (Fig. 2a). The decrease of other antioxidant systems in the mitochondria such as Gpx and Sod2 and the porin protein (Fig. 2a) led to an increase in mitochondria ROS levels (Fig. 4b). The elevated ROS makes it difficult to modify neutralizing ROS or to repair oxidative damage, which is associated with sensitivity to oxidative stress.

The yeast cyclophilin A Cpr1 is a homolog of hCypA and AtCYP20-3, which shares 65% and 58% identity in the amino acid sequence, respectively, and is present in the cytoplasm. The Cpr1 protein contains two cysteine residues (Cys38 and Cys117) as well as the amino acid residues known to be involved in catalysis and binding to substrates (data not shown). Decarbonylated Cpr1 protein maintains the redox homeostasis by regulating a wide range of antioxidant enzymes, especially the thioredoxin system. The redox regulation of cyclophilins has been reported for hCypA from T cells, in which key cysteine residues are glutathionylated (Ghezzi et al. 2006). Another example is chloroplast CypA from *A. thaliana* (AtCypA), which is redox-regulated by thiol modification via thioredoxin (Trx). AtCypA is inactive in its oxidized state but activated when Trx reduces the two disulfide bonds involving four conserved cysteine residues (Motohashi et al. 2003). Moreover, CypA is implicated in oxidative stress in many ways. In mammalian cells, the CypA protein protects cells from oxidative stress (Doyle et al. 1999) by binding to thiol-specific antioxidants, Aop1 protein (Jaschke et al. 1998), peroxiredoxins (Lee et al. 2001), calreticulum—a calcium sequestering protein (Reddy and Atreya 1999), and glutathione-S-transferase (Jaschke et al. 1998; Piotukh et al. 2005). In plants, a cyclophilin from *Pisum sativum* enhanced the protective activity of 2-Cys Prx in a DNA protection assay and regenerated the peroxide-detoxifying activity (Bernier-Villamor et al. 2004). These results indicate that cyclophilin protein may directly cooperate with antioxidant proteins, especially thioredoxin systems.

Molecular chaperones are known as a group of highly interactive proteins that modulate the folding and unfolding of other proteins, or assembly and disassembly of protein–protein, protein–DNA, and protein–RNA complexes. Chaperones are known to be involved in many cellular processes and pathways such as protein translocation across membrane, ribosomal RNA processing, and ER-associated protein degradation (Gong et al. 2009). Many chaperones, through constitutively expression, are synthesized at increased levels under MD stress (Fig. 3a, b) and are named as stress proteins or Hsps. Chaperones that participate broadly in protein refolding, such as Hsp70 family (Ssa and Ssb), Hsp90, Hsp104, and sHsps, promote the folding process through cycles of substrate binding and by cofactor proteins (Hartl and Hayer-Hartl 2002). Hsp104 and Hsp70 act together with Hsp26 in protein refolding after stress-induced unfolding (Haslbeck et al. 2005). Also, Hsp90 depends on its association with a variety of cochaperones and cofactors. Cochaperones include Hsp70 and Hsp40 (Hsp42) (Zhao et al. 2005) and cyclophlins Cpr6 and Cpr7 (Mayr et al. 2000). The formation of a complex between Hsp70 and Hsp90 is mediated through the association of both chaperones to an adaptor protein termed an activator of the Ssa proteins Sti1 (Wegele et al. 2003) and an essential ER chaperone Grp (Grp94) that functions as an interaction domain of Hsp90 (Chu et al. 2006). For a growing number of proteins, chaperone function is combined with an additional function in certain proteins such as disulfide isomerases and peptidyl-prolyl isomerases, enzymes that catalyze rate-limiting steps in the folding of some proteins (Hartl and Hayer-Hartl 2002). According to a previous report by Gasch et al. (2000), transcriptional activation in Hsp104, Hsp42, and Hsp78 was induced by MD stress, whereas transcripts in Hsp26, Ssa4, Ssa3, and Sse3 were unchanged or repressed under the same conditions. As an example to support these results, *hsp104* mutant cells were introduced in this study. Stress sensitivity (Fig. S2b) and redox state (Fig. S2c) in *hsp104* mutant (*hsp104Δ*) from KNU5377Y (kwt) were increased when exposed to MD. In addition, Cpr1 protein expression in *hsp104* mutant cells was downregulated in the presence of MD (Fig. S2a). These results indicate that the Cpr1 protein is (in)directly involved in other molecular chaper-

ons in stress response to MD. Although the chaperones network of physiological and genetic interactions has been well known, a comprehensive knowledge of chaperone interaction with chaperones and their cofactors (or substrates) has not yet been publicized. Recently, systematic analysis of physical TAP-tag-based protein–protein interactions of all known 63 chaperones in *S. cerevisiae* was carried out. According to this result, Hsp70s have a large number of interactions with Hsp104, Hsp90, CCT, PFD, Hsp40, and sHsps. In general, chaperones in the cytoplasm and nucleus share many more interactions than those in the ER and mitochondria. However, both ScJ1 in the ER and Hsp78 in the mitochondria share a considerable number of interactors with cytoplasmic chaperones (Gong et al. 2009). This suggests that a coregulation of the chaperone systems could play a major role in mediating the translocation of proteins from the cytoplasm to the respective organelles. In this study, the kc1 cells caused the reduction of these chaperone proteins except Ssb, Hsp60, and Hsp26, whereas expression of these chaperones in the bwt cells was Cpr1 protein-independent because chaperone proteins were unchanged or increased in the bc1 cells during MD stress. Hence, the kwt Cpr1 protein is required for redox buffering or chaperone machinery systems (Fig. S2) in the presence of MD, but not in the bwt cells. An imbalance of chaperone machinery systems in the kc1 cells creates a difficulty in protein (re)folding under unstressed and stressed conditions that are accompanied by elevated protein oxidation (Fig. 5) and a decrease in PPIase activity under elevated ROS in the cytosol and mitochondria (Fig. 4c), whereas there is no noticeable difference between the bwt cells and the bc1 cells. As shown in Fig. 3a and b, Hsp104, Hsp90 (Hsp82), Hsp42, Hsp31, ER chaperone Grp, and Hsp90 cochaperone Sti1 are reduced in the Cpr1-deficient kc1 cells in the absence and presence of MD, as compared to the kwt cells. Under normal conditions, reduction of these proteins induces the slow growth rate in the kc1 cells rather than in the kwt cells (Fig. S4), which results in the increase in protein carbonyl content (Fig. 5), followed by a decrease in the PPIase activity (Fig. 3c). In yeast and fungi, the role of Cyp in growth has been reported. In particular, Cyp mutants induced growth defects in fungus. In addition, Cyp mutants in neuronal cells caused apoptosis and defects in the signaling response, respectively (Wang and Heitman 2005). Thus, reduction of these proteins in the kc1 cells under normal conditions suggests that Cpr1 plays a fundamental and cellular redox role under stressed and unstressed conditions. In addition, Zpr1 is a zinc finger protein that translocates to the nucleus in response to growth stimuli, which is a lethal partner with Cpr1p for PPIase activity (Ansari et al. 2002). The Zpr1 is essential for cell viability in diverse eukaryotic organisms containing yeast and mice. Reduction of Zpr1 expression in mammalian cells by antisense or small interfering RNA knockdown causes defects in transcription, prevents DNA synthesis, and results in an accumulation of cells in the G_1 and G_2 phases of the cell cycle (Mishra et al. 2007). However, Zpr1p in stress response of the kwt cells decreased during MD exposure, which had no effect on cell viability. Our result does not show a potential interaction between Cpr1p and Zpr1p.

Cyclophilins are housekeeping proteins with many roles, including PPIase and protein folding, interaction, and trafficking. They are evolutionarily highly conserved proteins found in prokaryotes to eukaryotes. The amino acid sequence of Cpr1p from KNU5377Y was the same as BY4741 (data not shown). Unlike the kwt cells, MD stress induced carbonylation of Cpr1 protein in the bwt cells, as observed in H_2O_2 stress (Costa et al. 2002). Protein carbonylation is irreversible and correlated with oxidative stress-induced cell death. Protein carbonylation of the Cpr1 protein in the bwt cells did not increase stress tolerance as compared with the mutant cells lacking CPR1. We recently identified that the homologous Cpr1 overexpression improves stress tolerance to oxidative stress and heavy metals in the bwt cells (Kim et al. 2010a). At this point, we need to consider whether protein carbonylation of the Cpr1p does not occur in the kwt strain. One of the reasons seems to be the superior antioxidant ability of the kwt strain. Expression levels of the cell rescue proteins including superoxide dismutase (Sod1 and Sod2), NADPH generation proteins (G6PDH and IDH), glutathione system (ECS and Gpx) and thioredoxin system (Trr), porin (Por), heat shock proteins or its cofactors (Hsp104, Hsp90, Hsp42, Hsp31, Cpr1, and Sti1), and metabolic enzymes (ADH and GAPDH) of the kwt cells were higher than those of the bwt cells during MD stress (Figs. 2 and 3). Upregulation of these proteins increases antioxidant capacity (Fig. 6c) and chaperone activity (Fig. 3c), which is directly related to the ROS scavenger ability (Fig. 6d). Why does a higher stress tolerance via elevated induction of these proteins occur under MD stress in the kwt cells rather than the bwt cells? It seems to be caused by the stress response factor. As shown in Fig. 6b, expression levels of the general and specific stress response factors Hsf1 (Fig. S3), Yap1, and Msn2 in the kwt cells were higher than those in the bwt cells, which enable rapid and efficient remodeling of yeast genome expression in response to environmental change induced by MD. It is well known that transcription factors and antioxidant enzymes play very important roles in cellular defense against oxidative stress in yeast (Jamieson 1998; Moradas-Ferreira and Costa 2000; Fernandes et al. 2007; Herrero et al. 2008), which affects redox balance within the cells.

In conclusion, we found a phenotype associated with deletion of CPR1 in *S. cerevisiae* KNU5377Y that was suggestive of Cpr1's natural function. We measured the

activity of Cpr1 in KNU5377Y cells, which was involved in critical functions that shift the expression of cell rescue proteins and metabolic enzymes under MD stress. The elevation or overexpression of these proteins could help elucidate the function of Cpr1 as a potential molecular chaperone.

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