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Molecules and Cells

A Cyclophilin A CPR1 Overexpression Enhances Stress Acquisition in *Saccharomyces cerevisiae*

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Cyclophilins are conserved *cis-trans* peptidyl-prolyl isomerase that are implicated in protein folding and function as molecular chaperones. We found the expression of cyclophilin A, Cpr1, changes in response to exposure to yeast *Saccharomyces cerevisiae* to abiotic stress conditions. The effect of Cpr1 overexpression in stress responses was therefore examined. The CPR1 gene was cloned to the yeast expression vector pVTU260 under regulation of an endogenous alcohol dehydrogenase (ADH) promoter. The overexpression of Cpr1 drastically increased cell viability of yeast in the presence of stress inducers, such as cadmium, cobalt, copper, hydrogen peroxide, *tert*-butyl hydroperoxide (*t*-BOOH), and sodium dodecyl sulfate (SDS). The Cpr1 expression also enhanced the cell rescue program resulting in a variety of antioxidanr enzymes including thioredoxin system (particularly, thioredoxin peroxidase), metabolic enzymes (glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase), and molecular chaperones (Hsp104, Hsp90, Hsp60 and Hsp42). Thus, our study illustrates the importance of Cpr1 as a molecular chaperone that improves cellular stress responses through collaborative relationships with other proteins when yeast cells are exposed to adverse conditions, and it also premises the improvement of yeast strains.

INTRODUCTION

Cyclophilins were originally identified as the intracellular receptors of the immunorepressive compound cyclosporine A (CsA). Cyclophilin and CsA form a complex that binds to and inhibits the protein phosphatase calcineurin, preventing the activation of human T-cells (Arevalo-Rodriguez and Heitman, 2005; Wang and Heitman, 2005). Cyclophilins found in mammals, plants, insects, fungi, and bacteria are all have peptidyl-prolyl *cis-trans* isomerase, or prolyl isomerase (PPIase), which catalyze *cis-trans* isomerization of the peptide bonds preceding the proline residues (Arevalo-Rodriguez et al., 2000; 2004; Galat, 2004; Wang and Heitman, 2005). There are seven major cyclophilins (hCypA, hCypB, hCypC, hCypD, hCypE, hCyp40, ans hCypNK) and a total of 16 unique cyclophilin proteins in humans (Arevalo-Rodriguez and Heitman, 2005; Wang and Heitman, 2005). The fly *Drosophila* has at least nine cyclophilins and the plant *Arabidopsis thaliana* has 29 putative cyclophilins (Wang and Heitman, 2005), and *Saccharomyces cerevisiae* has a total of eight cyclophilin proteins, Cpr1-Cpr8 (Arevalo-Rodriguez and Heitman, 2005; Wang and Heitman, 2005). Previous extensive studies of various model organisms have suggested that cyclophilins are involved in a wide range of cellular processes, including cell division, transcriptional regulation, protein trafficking, signaling, pre-mRNA splicing, molecular chaperone, stress tolerance, *etc*. (Chen et al., 2007; Coaker et al., 2005; Massignan et al., 2007; Wang et al., 2001). Nevertheless, the complete biological functions and conserved groups of proteins remain to be explored.

As the predominant member of the cyclophilins, the yeast CypA, Cpr1 (P14832), contains a single-domain cyclophilin peptidyl-prolyl *cis-trans* isomerase motif and is localized in the cytosol and nucleus (Arevalo-Rodriguez and Heitman, 2005; Wang and Heitman, 2005). Cpr1 functions as a cyclosporin A receptor, a component of the Set3 histone deacetylase complex, and is involved in the regulation of meiosis (Arevalo-Rodriguez et al., 2004). In addition, Cpr1 is required for the glucose-stimulated transport of fructose-1,6-biphoaphatase (FBPase) into the vacuoles (Vid; Vid22p) and its degradation (Brown et al., 2001). Cpr1 could also promote the proper subcellular localization of an essential zinc finger protein (Zpr1) (Arevalo-Rodriguez and Heitman, 2005). Moreover, Cpr1 transcription is induced by high temperatures, NaCl, or sorbic acid, but not by ethanol (Moskvina et al., 1998), and Cpr1 expression is activated also by stress conditions, indicating that Cpr1 may participate in protecting the cell against cellular stresses (Arevalo-Rodriguez et al., 2004).

We previously found that Cpr1 expression in *S. cerevisiae* is strongly induced during the fermentation process at elevated temperatures (Kim et al., 2006), which allows us to hypothesize that overexpression of Cpr1 might enhances stress tolerance. In this study, we examined the effect of Cpr1-overexpression of Cpr1 and found that is significantly enhanced yeast stress tolerance. Our study suggests, for the first time, that the accumulation of a single domain Cpr1 could increase an acquired stress tolerance in eukaryotic microorganism.

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MATERIALS AND METHODS

Growth conditions

S. cerevisiae BY4741 wild-type (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0;* EUROSCARF) and *tsa1Δ* (*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YML028w::kanMX4;* EUROSCARF) mutant cells grown overnight at 30°C were inoculated into a fresh YPD medium containing 1% yeast extract, 2% peptone, and 2% glucose. Once reaching the mid-log phase (OD_{600} = 1.0), yeast cells were used for subsequent experiments. In the case of the stress response of CPR1, cells grown to the mid-log phase were challenged to a variety of stressors (20 mM H_2O_2, 20 mM *t*-BOOH, 0.1 mM $CdCl_2$, 20 mM $CoCl_2$, 20 mM $CuSO_4$, and 0.2% SDS) for 1 h at 30°C with shaking, and used for future experiments such. For heat shock, cells were exposed for 5 min at 50°C and then prepared for protein isolation.

Construction of the Cpr1-expressing recombinant plasmid

Total RNA was isolated from *S. cerevisiae* S288C (ATCC No. 204508; *MATα/MATa SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*). The cDNA probe was prepared by reverse transcription-polymerase chain reaction (RT-PCR). The *CPR1* coding region containing start- and stop codons was amplified from cDNA by PCR using *Taq* and *Pfu* polymerase (Roche). PCR reaction conditions are as follows: initial denaturation cycle at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final extension of 7 min at 72°C.

The primers utilized for PCR cloning of *CPR1* gene were 5′-CCACTCCCCGCTAGCATGTCCCAAGTCTATTTTGATGTCG-3′ (*Nhe*I site underlined) and 5′-GCACTGGGATCCTTATAA-TTCACCGGACTTGGCAACA-3′ (*BamH*I site underlined) as sense and antisense primers, respectively. The PCR product was inserted into the TOPO TA cloning vector (Invirogen). The cloned plasmid was sequenced to confirm that no PCR-induced mutations were introduced (GenoTech Inc.), and ligated into the yeast expression vector pVTU260 (EUROSCARF). To transform, the cells of yeast grown overnight in YPD medium were cultured until reaching the mid-log phase (A_{600} = 1.0), and were then transformed with pVTU260-CPR1 via the PEG/LiCl method (Chen et al., 2001; Gietz and Schiestl, 2007). Transformants were selected in a minimal medium containing 2% glucose but lacking uracil (amino acid used as auxotrophic marker) in yeast. Colonies were then restreaked and incubated under the same conditions.

Semi-quantitative RT-PCR analysis

Total RNA from mid-log phase yeast cells was obtained using a RNeasy Mini Kit (Qiagen). Semi-quantitative RT-PCR of the *CPR1* gene was amplified with One-Step RT-PCR PreMix (Intron) using a template RNA (0.1 μg) and a gene-specific primer. The oligonucleotides used were CPR1-F and CPR1-R, the sequences of which were: 5′-TTTTGATGTCGAAGCTGATGG-3′ and 5′-TAATTCACCGGACTTGGCAA-3′, respectively. PCR conditions are as follows: one cycle of 30 min at 45°C and 5 min at 94°C for cDNA synthesis, and initial denaturation cycle at 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final extension of 7 min at 72°C for the *CPR1* gene amplication. The PCR amplicon was separated onto a 1.2% agarose gel in 0.5× TBE buffer, stained with ethium bromide, visualized and photographed. The PCR amplicon of *PDA1* was used as a reference control. Sense and antisense primers used are as follows: 5′-CTTCATTCAAACGC-CAACCA-3′ and 5′-GAGGCAAAACCTTGCTTTTTG-3′ (Wenzel et al., 1995).

Protein extraction, SDS-PAGE, and Western-blot analysis

A crude protein extract from yeast was prepared using a lysis buffer (50 mM HEPES, 5% glycerol, 1 mM DTT, 1 mM PMSF, 2 μM pepstatin A and EDTA-free protease inhibitor cocktails), with an equal volume of glass beads (425-600 μm; Sigma). After vigorously vortexing, the cleared supernatant was collected by centrifugation (Kim et al., 2006). Protein concentration was determined via the Protein Dye Reagent (Bio-Rad). The protein extract (30 μg) was loaded on 12% SDS-PAGE, run at 70 V, stained with Coomassie Brilliant Blue R-250, and destained (Laemmli, 1970). For immunoblot analysis, the gel was electrophoretically transferred to a PVDF membrane (Bio-Rad) at 4°C at 180 mA for 3 h after SDS-PAGE as described above. The membranes were blocked with 5% skim milk in a TBST buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.5% Tween-20 for 1 h at room temperature, and incubated overnight at 4°C with anti-His tag diluted to 1:500 (Millipore), anti-Cpr1p diluted to 1:1000 (provided friendly by Dr. Joseph Heitman, Duke University, USA), anti-Tsa1p diluted to 1:2000 (provided friendly by Dr. Jeen-Woo Park, Kyungppok National University, Korea), anti-Trx2, -Trx3 and -Trr1 diluted to 1:4000, respectively (provided friendly by Dr. Kang-Wha Kim, Chonnam National University, Korea), anti-glucose-6-phosphate dehydrogenase diluted to 1:4000 (G6PDH; Sigma), glyceraldehyde-3-phosphate dehydrogenase diluted to 1:2000 (GAPDH; Ab frontiers), anti-alcohol dehydrogenase (Adh) and aldehyde dehydrogenase diluted to 1:5000 (Ald; Rockland), anti-Hsp104 and anti-Hsp60 diluted to 1:1000 (Stressgen), anti-Hsp90 diluted to 1:5000, anti-Ssa and anti-Ssb diluted to 1:8000 (a gift from E. A. Craig, Universtiy of Wisconsin-Madison, USA), and anti-Hsp42 and -Hsp26 diluted to 1:2000 (provided friendly by Dr. Jahannes Buchner, Technische Universität München, Germany). Anti-Hsp30 antibody diluted to 1:1000 was synthesized. Anti-yeast tubulin (Tub; SantaCruiz) diluted to 1:500 was used as a housekeeping control. Blots were washed with TBST, and incubated with a horseradish peroxidase coupled to anti-rabbit (Promega) or anti-mouse (Millipore) secondary antibodies diluted to 1:1000-1:10,000 for 1.5 h at room temperature (Wu et al., 2007). After washing with TBST, the signal was visualized using the ECL Western blotting detection reagent (GE Healthcare).

Growth recovery curves and stress response assay

Fresh overnight cultures of the recombinant cells with Cpr1 and yeast cells with vector alone grown in a YPD medium and were resuspended in a fresh YPD media containing a variety of concentrations of cadmium chloride (Cd_2Cl_2) and cobalt chloride (Co_2Cl_2) at A_{600} = 0.1. After culture for 12 h at 30°C with properly shaking, aliquots of each sample were taken and the absorbance was measured. When A_{600} > 1.0, aliquots were serially diluted and new measurements were taken. To examine the effect of stress response by the overexpression of CPR1, experiments were divided into three parts containing the growth rate (I), spotting assay (II), and streaking assay (III). I. Fresh cells were resuspended at approximately A_{600} = 0.2 in a fresh YPD media supplemented with each stressor (0.1 mM $CdCl_2$, 2.0 mM $CoCl_2$, 5.0 mM H_2O_2, 1.0 mM *tert*-butyl hydroperoxide (*t*-BOOH), 10.0 mM $CuSO_4$, and 0.3% SDS) and incubated at 30°C with shaking. Aliquots were taken at regular intervals and the absorbance was measured. II. Once reaching the mid-log phase (A_{600} = 1.5), cells were exposed to 10 mM $CdCl_2$ and 20 mM $CoCl_2$ for 1 h at 30°C with shaking, 10-fold serially diluted and spotted on a YPD medium. III. Cells grown to A_{600} = 1.0 were plated on a YPD medium supplemented with 0.1 mM $CdCl_2$ and 2.0 mM $CoCl_2$, and incubated for 2-3 days at 30°C.

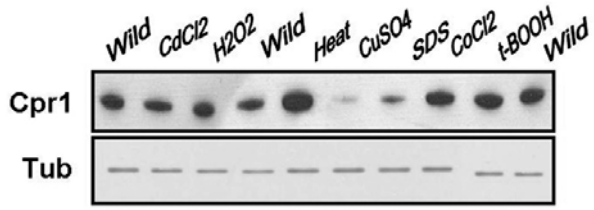

Fig. 1. Expression changes of Cpr1 protein are changed under a variety of stresses. Non-transformed yeast cells (BY4741) grown to mid-log phase were exposed to 20 mM H_2O_2, 20 mM *t*-BOOH, 0.1 mM $CdCl_2$, 20 mM $CoCl_2$, 20 mM $CuSO_4$, and 0.2% SDS for 1 h at 30°C with shaking, and harvested by centrifugation. For heat shock, cells were treated for 5 min at 50°C. After cell lysis, protein extracts were used for Western blot following SDS-PAGE (12%). Anti-tubulin (Tub) antibody was used for a standard control. Wild, yeast cells without stressor.

The plate was photographed.

RESULTS

Expression of the Cpr1 protein is dependent on the stress type in yeast

In order to understand the involvement of Cpr1 in the stress response, we first examined whether the Cpr1 expression was altered by external stresses. When compared to a favorable condition, the expression of Cpr1 was induced under heat shock, unchanged to H_2O_2, $CdCl_2$, *t*-BOOH, and $CoCl_2$, and reduced upon $CuSO_4$ and SDS stresses (Fig. 1). To date, *CPR1* transcription was reported to be induced by high temperature, NaCl, and sorbic acid in yeast (Arevalo-Rodriguez et al., 2004). The results may suggest that overexpression of the Cpr1 protein could affect the tolerance level of yeast cells to certain stresses, and we determined to investigate that.

Recombinant yeast cells store homologous Cpr1 protein

The identification of a cyclophilin A CPR1 from *S. cerevisiae* was described previously (Kim et al., 2006). A cDNA containing the open reading frame (ORF; 489 bp) of the *CPR1* gene from the *S. cerevisiae* was subcloned into a yeast expression vector, pVTU260, at the *Nhe*I-*BamH*I site, which was regulated under an ADH (alcohol dehydrogenase) promoter in the presence glucose (Fig. 2A). This plasmid was used to transform *S. cerevisiae* BY4741 to characterize the expression virtue of the CPR1. To explore whether the CPR1 gene is effectively expressed in yeast, semi-quantitative RT-PCR, SDS-PAGE, and Immunoblotting analysis were performed. PCR products generated from the house-keeping gene *PDA1*, showed that a single DNA fragment of 472 bp corresponding to the region within *CPR1* ORF was amplified in the Cpr1-expressing cells (recombinant cells). Amplification signal was also detected in the control cells with vector alone (control cells) because of the presence of endogenous CPR1. However, signal intensity was stronger in the recombinant cells compared to the control cells (Fig. 2B). SDS-PAGE and Western blotting showed that the Cpr1 protein was normally expressed in yeast. As shown in semi RT-PCR, the Cpr1 protein in the control cells was detected in Western-blot analysis using an anti-Cpr1p antibody (Fig. 2C). It may be noted that both the control cells and recombinant cells had endogenous CPR1 genes in their chromosomes. To exclude any endogenous CPR1 protein in yeast, Western blotting using an anti-His tag antibody was also examined, and revealed that the endogenous protein in the control cells was non-reactive to His-tag (Fig. 2C). Judging from the intensity, the results suggest that Cpr1 expression in the transformed cells contributes approximately 50% of the total Cpr1 protein expression, while half of the total Cpr1 expression might be due to the endogenous Cpr1 protein. The pVTU260-CPR1 with the six histidine residues at the N-terminal showed approximately 18 kDa of molecular weight (MW) on SDS-PAGE (Fig. 2D). The band represented with an arrow was identified by MALDI-TOF analysis, the MW and isoelectric point (pI) of which were 17.37 kDa and 6.9, respectively. The Mowse score and coverage were 2.11 and 40%, respectively (data not shown). These results evidently show that the positive signal came from recombinant Cpr1, and not intrinsic yeast Cpr1.

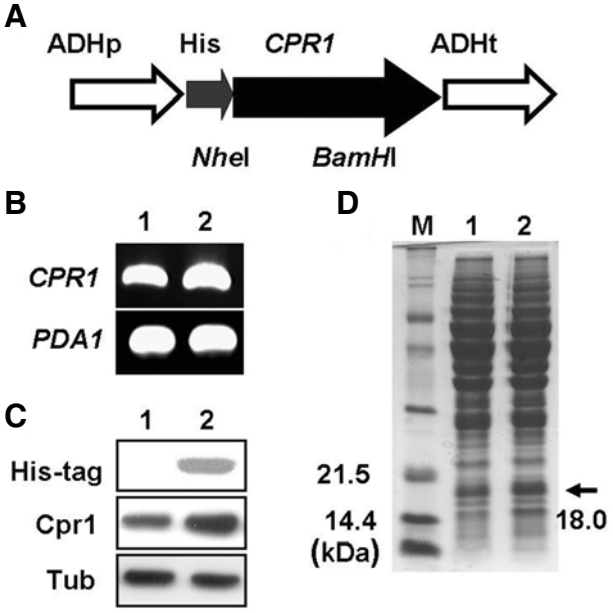

Fig. 2. Over-expression of Cpr1 in *S. cerevisiae* BY4741 cells. (A) Schematic diagram of the pVTU260-CPR1 construct. Restriction sites used to fuse *CPR1* genes to the promoter were displayed under the gene as *Nhe*I and *BamH*I. ADH p, ADH promoter; His, 6x His-tag; ADHt, ADH terminator. Arrows indicate the direction of transcription (5′-3′). (B) The mRNA expression of the transformed yeast cells was detected by semi-quantitative RT-PCR analysis using housekeeping *PDA1* gene. Lane 1, yeast cells with vector alone; lane 2, transformed cells with pVTU260-CPR1. Protein expression of Cpr1 was confirmed by Western blotting analysis using housekeeping anti-Tubulin (Tub) antibody (C) and SDS-PAGE (D). M, protein marker; lane 1, yeast cells with vector alone; lane 2, transformed cells with pVTU260-CPR1.

Cpr1-overexpression has a marked effect on stress tolerance under diverse stresses

Stress containing oxidative stress is caused during respiratory process for energy production or by exogenous stimuli, which can alter the intracellular environment, allowing the cellular redox state to become more oxidized. Aerobic organisms have developed a set of cell rescue systems to minimize or prevent the detrimental effects to stress. Among these defense systems, it has been proposed elsewhere that cyclophilin A protein participates in cell protection against stress conditions, such as oxidative stress by maintaining the adequate redox balance in the intracellular environment and, thus, regulating various cellu-

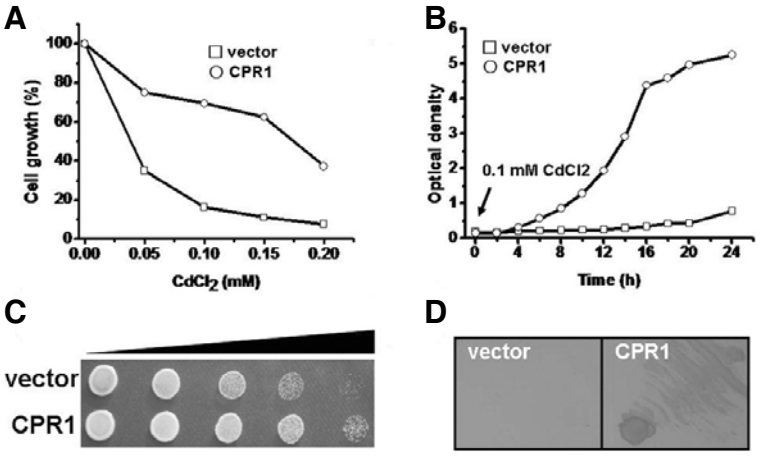

Fig. 3. Stress tolerance assay to cadmium in yeast cells expressing recombinant Cpr1. (A) Yeast cells precultured in YPD were treated with different concentrations of $CdCl_2$ for 12 h at 30°C. Cell growth was represented as a relative optical density. (B) To monitor growth rate, cells were diluted in the presence of 0.1 mM $CdCl_2$ and measured at 600 nm. Tansformant expressing vector alone (□); transformant expressing recombinant Cpr1 (○). Cell survival was performed by spotting assay using serial dilution (C) and streaking assay in 0.1 mM $CdCl_2$-amended YPD agar medium (D) as described in *Materials and methods*. Vector, transformant with only vector; CPR1, transformant with recombinant Cpr1. Data shown are a representative of at least three independent experiments.

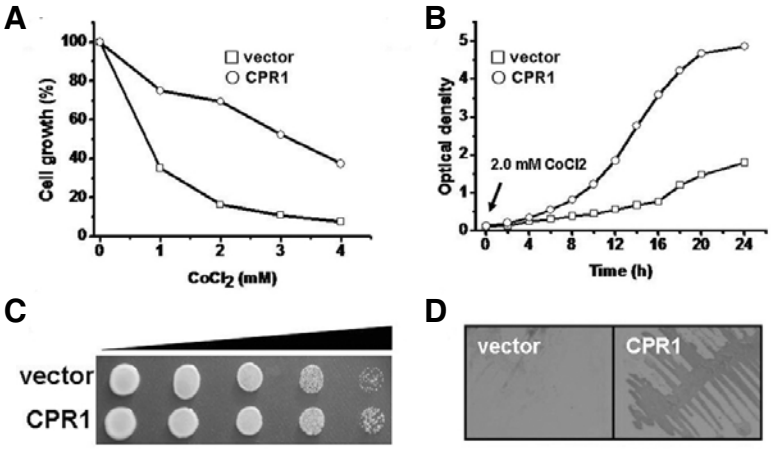

Fig. 4. Stress tolerance assay to cobalt in yeast cells expressing recombinant Cpr1. Tolerance test to cobalt chloride was obtained via cell survival assay by monitoring cell growth (A), growth kinetics (B), spotting assay (C), and streaking assay (D). Vector (□), transformant with only vector; CPR1 (○), transformant with recombinant Cpr1.

lar activities in animals and plants, but not yeast (Arevalo-Rodriguez et al., 2004; Wang and Heitman, 2005). Based on previous reports, this experiment was carried out whether Cpr1 expression could involve stress tolerance to different kinds of stressor such as oxidants (hydrogen peroxide and *t*-BOOH), heavy metals (cadmium, cobalt, and copper), and a detergent sodium dodecyl sulfate (SDS) in yeast cells. A comparative analysis of stress tolerance was produced using yeast cells transformed with either pVTU260-CPR1 or an empty vector. Cell survival of the recombinant cells that constitutively expresses Cpr1 protein under ADH (alcohol dehydrogenase) promoter was observed under elevated concentration of $CdCl_2$ and $CoCl_2$, respectively, as compared to the control cells with the vector alone (Figs. 3A and 4A). In $CdCl_2$- and $CoCl_2$-amended YPD medium, the recombinant yeast cells exhibited a significant difference of growth kinetics, as measured by changes in A_{600}. For the first 4 h, growth in the Cpr1-expressing cells occurred at almost the same rate as that in the control cells. A temporary lag then ensued, with growth resuming, but more rapidly. The final A_{600} in the transformed cells with Cpr1 was considerably higher than that in the control cells with the vector alone (Figs. 3B and 4B). Expression of Cpr1 dramatically created rapid restoration of growth kinetics. In addition to growth kinetics, an attenuation of stress sensitivity by Cpr1 expression was seen in the streaking and spotting assay. The cell viability of the transformed cells with Cpr1 was higher than that of the control cells with vector alone under 10 mM $CdCl_2$ and 20 mM $CoCl_2$ (Figs. 3C and 4C). Also, the survival of the Cpr1-expressing cells rapidly recovered in the YPD medium supplemented with 0.1 mM $CdCl_2$ and 2.0 mM $CoCl_2$, as compared to that of the vector cells (Figs. 3D and 4D). The Cpr1-expressing cells also showed an improved resistance against copper stress ($CuSO_4$), and detergent SDS exposure, with the growth kinetics (Figs. 5A and 5B). As a final sample, overexpression of the Cpr1 protein dramatically increased the growth rate and cell survival as well when cells were grown in the presence of pro-oxidant hydrogen peroxide (H_2O_2) and *t*-BOOH (Figs. 5C and 5D). Thus, these results suggest that the Cpr1 over-expression in yeast could enhance an acquisition stress tolerance to heavy metals, a detergent SDS, or pro-oxidant, as compared to the control cells.

Accumulation of Cpr1 protein leads to an enhanced expression of cell rescue proteins

This study shows that the Cpr1-expressing cells undergo physiological changes that enhance their capacity to withstand stresses exposed. Thus, Cpr1 expression sets into motion remedial measures, and if these are concerned with countering the stress tolerance effect of Cpr1, as suspected, then Cpr1-expressing cells and the vector cells should exhibit different levels of stress. To test this notion, we made an effort to address the likely mechanism involved in increased stress resis-

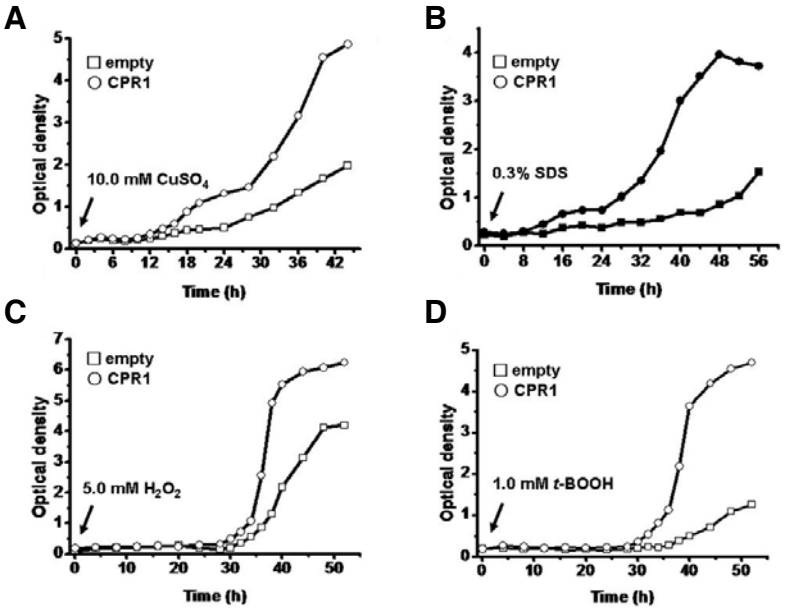

Fig. 5. Stress tolerance assay in the presence of copper, SDS, H_2O_2 and *t*-BOOH from homologous Cpr1-expressing yeast cells. Yeast strains were inoculated on liquid YPD containing 10 mM $CuSO_4$ (A), 0.3% SDS (B), 5.0 mM H_2O_2 (C) and 1.0 mM *t*-BOOH (D), and then grown for different time periods. Growth kinetics of cells was determined by sampling at every 4 h interval. Empty (□), transformant with only vector; CPR1 (○), transformant with recombinant Cpr1.

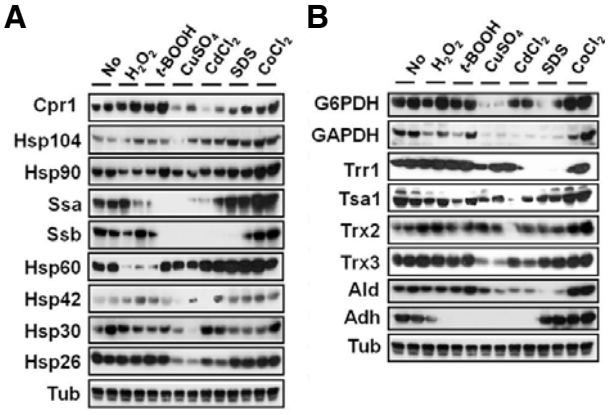

Fig. 6. Expression changes of a variety of cell rescue proteins by Western blot. Yeast cells grown to mid-log phase were challenged to 20 mM H_2O_2, 20 mM t-BOOH, 0.1 mM $CdCl_2$, 20 mM $CoCl_2$, 20 mM $CuSO_4$ and 0.2% SDS for 1 h at 30°C with shaking, harvested by centrifugation, and washed twice cold PBS buffer. Protein was extracted by physiological method using glass beads and used for Immunoblot analysis. Tubulin (Tub) was used as a housekeeping standard. No, yeast cells without stressor treatment; left, yeast cells with vector alone; right, transformed cells with recombinant Cpr1.

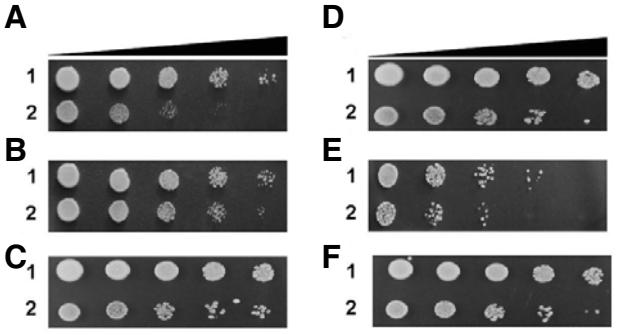

Fig. 7. Stress sensitivity of *tsa1Δ* mutant cells against multiple stressors. Yeast cells reached to mid-log phase were exposed to 0.1 mM $CdCl_2$ (A), 20 mM $CoCl_2$ (B), 10 mM $CuSO_2$ (C), 0.2% SDS (D), 20 mM H_2O_2 (E), and 20 mM *t*-BOOH (F) for 1 h at 30°C with shaking, and properly diluted from 10^0 to 10^{-4} with distilled deionized water. Five μl of diluted sample were spotted onto a YPD agar plate. 1, wild-type cells; 2, *tsa1Δ* mutant cells.

tance. As a method, we executed Western-blot analysis. As shown in Fig. 6, a distinguishable difference of expression levels for antioxidant proteins, metabolic proteins, and heat shock proteins (Hsps) or molecular chaperones was observed. Also, protein expression pattern varied between stress types or between transformants. First, Cpr1 protein level in Cpr1-expressing cells was higher than in transformant cells with vector alone under normal condition and a variety of stresses although much of the difference (Fig. 6A). Second, the expression level of Hsp104, Hsp90, Hsp60, and Hsp42 was distinctly elevated in the Cpr1-expressing cells under stress conditions analyzed, compared to wild-type cells. There were no differences of Ssa, Ssb, Hsp30, Hsp26 expression in both cells under the same conditions, except cobalt stress (Fig. 6A). Finally, thioredoxin 2 (Trx2) and 3 (Trx3), thioredoxin peroxidase 1 (Tsa1), and thioredoxin reductase 1 (Trr1) involved in thiothioredoxin system, glucose-6-phosphate dehydrogenase (G6PDH) producing reducing power NADPH as a cofactor of thioredoxin system, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a metabolic enzyme were induced under environmental challenges, compared to wild-type cells with vector alone (Fig. 6B). GAPDH and Trr1 expressions in both cells did not detect under copper, cadmium, and SDS, respectively. Particularly, repression of Tsa1 protein in the wild-type cells was strongly observed. We introduced the *tsa1* mutant strain to elucidate whether down-regulation of the Tsa1 protein increase stress sensitivity. As seen in Fig. 7, *tsa1Δ* mutant cells were hypersensitive to hydrogen peroxide, *t*-BOOH, cadmium, copper, cobalt, and SDS stress. These results suggest that Cpr1-overexpression enhances expression of a variety of cell rescue proteins, indicating up-regulated proteins by exogenous Cpr1 induction are important factors.

DISCUSSION

S. cerevisiae has been applied as a useful model system for studying cell stress response because its genetic systems have been studied extensively. Oxidative stress is a condition associated with an increased rate of cellular damage induced by reactive oxygen species (ROS) in organisms. The ROS is commonly generated by intra- and extracellular environments (Sikka, 1996). Hydrogen peroxide (H_2O_2) and *t*-BOOH as well as temperature shift are a major source of oxidative stress. These oxidants have been widely used and many effects on cell metabolism have been reported. For example, oxidant treatment causes changes in calcium homeostasis, increase of lipid peroxidation or the decrease of mitochondrial membrane potential modification of SH groups, and oxidative modification of iron-sulfur in various proteins complexes (Drahota et al., 2005; Izawa et al., 2007; Medina-Silva et al., 2006). Redox-active metals (copper, cobalt) are known to be capable of inducing various reactive hydroxyl radical production and promoting oxidative stress through Fenton reaction. Non-redox-active metals, such as cadmium, can indirectly enhance oxidative stress by depleting free-radical scavengers such as glutathione and binding to protein sulphydryl groups (Howlett and Avery, 1997; Lewinska and Bartosz, 2007). Most effects of heavy metals are related to their interaction with carbonyl and thiol groups of proteins, to their ionophoretic properties, and to their ability to direct or indirectly generate free radicals and, hence, induce oxidative stress (Lewinska and Bartosz, 2007; Mendoza-Cozatl et al., 2005). SDS also cause damage to membrane and alterations in carbon metabolism, and induce the oxidative stress (Sirisattha et al., 2004). Taken together, the major effect of heavy metals and SDS on a living cell is oxidative stress. This stress arises when cellular defenses and/or repair systems against oxidative damage are either compromised or inhibited by ROS. In response to the detrimental nature of ROS, organisms containing yeast have evolved a wide range of cellular defense mechanisms containing cyclophilin, which include enzymes or molecules that eliminate and restore the products of oxidatively damaged molecules (Adamis et al., 2004).

Cyclophilins are housekeeping proteins with many roles, including PPIase and protein folding. Cyclophilin A (CypA or Cyp18) has been involved in oxidative stress response in mammals and plants, however is not yet in yeast (Cyp17). Although a yeast mutant lacking cyclophilin has been shown to be viable, it is more likely that this have a requirement in specific environmental signaling cues or under natural stress (Barik, 2006). The yeast cyclophilin A CPR1 is a homolog of *Homo sapiens* CypA (Cyp18), *Arabidopsis thaliana* CYP20-3 and *E. coli* CypA. It shares a 65%, 58% and 23% identity in the amino-acid sequence, respectively, and is well conserved in PPIase consensus domain (data not shown). In rat tissues, CyP18 transcript levels are about 0.4% of total cellular mRNA (Santos et al., 2000). Ubiquitous enzyme CPR1 is difficult to explain why higher levels are required during stress response. According to previous reports, yeast cells increase the oxidation state of a specific group of oxidized proteins containing molecular chaperone cyclophilin A Cpr1 and Hsp60 when exposed to H_2O_2 (Cabiscol et al., 2000; Costa et al., 2002; 2007). The inactivation of molecular chaperones such as Cpr1 affects cell metabolism since these proteins display multiple functions such as control of protein folding and membrane translocation (Costa et al., 2002). For example, Hsp60 deficient cells are not viable and Hsp60 over-expression increases oxidative stress resistance (Cabiscol et al., 2002). Thus, increased level of Cpr1 might be necessary to assist in rapid protein control quality, to accelerate folding and maturation of proteins with ROS protective functions (Hsp104, Hsp90, Hsp60 and Hsp42) (Fig. 6A), or to protect cells by antioxidant proteins or molecules (thioredoxin system) (Fig. 6B) via rapid and effective gene operating program from ROS. An over-expression of Cpr1 may be a part of a general cellular response to oxidative stress induced by various stimuli.

Our results indicate that the basal expression of the Cpr1 in yeast (Fig. 2) is not sufficient to overcome unfavorable conditions containing oxidants, and heavy metals in the control cells. In addition, Cpr1-overexpression assisted induction of a variety of antioxidant proteins such as thioredoxin peroxidase. This function would be consistent with the role of human Cyp18 and *Arabidopsis* CYP20-3 in the tracking and folding of proteins under stress conditions. In mammalian cells, cyclophilin A (CypA) is implicated in oxidative stress or hypoxia treatment in many ways (Santos et al., 2000). The CypA protein could protect 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), and could form mixed disulfide even under oxidative stress (Fratelli et al., 2002). Overexpression of CypA in mammalian *SOD1* mutant cells led to an increase in viability (Lee et al., 1999; Massignan et al., 2007). Additionally, overexpression of cyclophilin B performs a crucial function in protecting cells against ROS by ER stress via PPIase activity, and maintaining Ca^{2+} homeostasis (Kim et al., 2008). In plants, the expression of *CYP* genes derived from different origins has been up-regulated 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). Overexpression of the *CYP1* gene (ThCYP1) derived from salt-tolerant *Thellungiella halophila* improved the salt tolerance of yeasts and plants (Chen et al., 2007). A cyclophilin, CYP20-3 (ROC4; At3g62030) in *Arabidopsis thaliana* was shown to be involved in defense against cellular damages from oxidative condition caused by high light, high salt concentration, with osmotic shock, via catalyzing SAT, a rate-limiting enzyme, in cysteine biosynthesis (Dominguez-Solis et al., 2008), and homologous serine *O*-acetyltransferase (SATase) (EC 2.3.1. 30) (Laxa et al., 2007). A cyclophilin from *Pisum sativum*, the CYP20-3 homologue, was able to enhance the protective activity of 2-Cys Prx and regenerated the peroxide-detoxifying activity (Bernier-Villamor et al., 2004). Overexpression of rice FKBP20 (FK506-binding protein) in yeast endowed capacity of high temperature tolerance to yeast cells (Nigam et al., 2008). In bacteria such as *Escherichia coli* (FkpA, PpiA, PpiD and SurA), *Bacillus subtilis* (PpiB and TF), *Legionella pneumophila* (Icyp), and *Streptomyces chrysomallus* (CypA and CypB), a large array of cyclophilin-like proteins is also produced (Wong et al., 1999). Cyclophilins from gram-negative bacteria such as *E. coli* have no or little affinity to CsA. Disruption of the cyclophilin gene in the bacteria mentioned above did not affect the viability of the cells, but the mutation affected growth in specific conditions such as nutrient starvation (Berger et al., 1999) as seen in yeast (Dolinski et al., 1997). Therefore, the over-expression of Cpr1 in yeast is in fact responsible for stress response, and is involved in participating the mechanism of the acquisition tolerance when the cells are exposed to stresses.

In conclusion, cyclophilins are ubiquitously distributed peptidyl prolyl isomerases that are implicated in many cellular processes, including stress responses, which have been well documented in many model systems. Here, we carefully examined the role of cyclophilin A Cpr1 overexpression in the stress re-

sponse of the yeast *S. cerevisiae* and found that Cpr1 has a conserved role in protecting cells against oxidative stress inducers, heavy metals and SDS, agent that perturb cell walls. The increased stress response resulted from enhanced cell rescue proteins including thioredoxin peroxidase 1 (Tsa1) in yeast.

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