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Redox regulation of the tumor suppressor PTEN by glutaredoxin 5 and Ycp4

Yujeong Kim [a,1], Kee-Oh Chay [a,1], Inyoung Kim [a,1], Yong Bhum Song [b], Tae-Youl Kim [b], Seong-Jeong Han [c], Younghee Ahn [d], Seung-Hyun Cho [e], Kwang-Lae Hoe [f], Bong Whan Ahn [a], Won-Ki Huh [b,*], Seung-Rock Lee [a,*]

[a] Department of Biochemistry, Research Center for Aging and Geriatrics, Research Institute of Medical Sciences, Chonnam National University Medical School, Gwangju 501-190, Republic of Korea
[b] School of Biological Sciences and Research Center for Functional Cellulomics, Institute of Microbiology, Seoul National University, Seoul 151-747, Republic of Korea
[c] School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea
[d] Department of Pediatrics, University of Calgary and Alberta Health Services-Calgary Zone, 2888 Shaganappi Trail, NW Calgary, Alberta, Canada T3B 6A8
[e] de Duve Institute, Université catholique de Louvain, B-1200 Brussels, Belgium
[f] Integrative Omics Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yusong, Daejeon, Republic of Korea

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ABSTRACT

Human PTEN (phosphatase and tensin homolog deleted on chromosome 10; a phosphatidylinositol 3-phosphatase) expressed in *Saccharomyces cerevisiae* was oxidized in a time- and H_2O_2-concentration-dependent manner. Oxidized hPTEN was reduced by cellular reductants as in human cells. The reduction rate of oxidized hPTEN was monitored in *S. cerevisiae* mutants in which the genes involved in redox homeostasis had been disrupted. Reduction of hPTEN was delayed in each of *S. cerevisiae grx5Δ* and *ycp4Δ* mutants. Expression of Grx5 and Ycp4 in each of the mutants rescued the reduction rate of oxidized hPTEN. Furthermore, an *in vitro* assay revealed that the human Grx5/GSH system efficiently catalyzed the reduction of oxidized hPTEN. These results suggest that the reduction of oxidized hPTEN is regulated by Grx5 and Ycp4.

1. Introduction

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a phosphatidylinositol (3,4,5)-trisphosphate (PIP3) 3-phosphatase that catalyzes the removal of the phosphate attached to the 3′-hydroxyl group of the phosphoinositide inositol ring. PTEN plays an essential role in regulating signaling pathways involved in cell proliferation and apoptosis. PTEN functions as an important tumor suppressor by negatively modulating the phosphoinositide 3-kinase (PI3K)-Akt mediated signaling pathway.

Mutations and deletion in the *PTEN* gene cause tumorigenesis in a number of human tissues, including brain, breast, prostate, and other advanced malignant tissues. In addition, germline *PTEN* mutations have been detected in different diseases including Cowden disease, Bannayan–Zonana syndrome and Lhermitte–Duclos disease (LDD) [1]. To study the role of PTEN *in vivo*, many mice strains have been generated as mammalian models, including PTEN-knockout, -conditional knockout and -transgenic mice. PTEN has also been studied in other vertebrates, including amphibians and fish [2].

The budding yeast *Saccharomyces cerevisiae* has been widely used as a eukaryotic model fungal organism. *S. cerevisiae* encodes an apparent PTEN ortholog (Tep1) as well as orthologs to classic mammalian PIP3 targets such as the AGC kinases, PDK1 and PKB/Akt. But, there is no evidence that the PTEN ortholog Tep1 acts as a phosphoinositide phosphatase in *S. cerevisiae* [2].

Oxidation of human PTEN with endogenous H_2O_2 *in vitro* or by exposure of cells with H_2O_2 leads to the formation of a disulfide bond between the active site Cys^{124} residue and a nearby Cys^{71} residue [3]. Phorbol ester (PMA) or lipopolysaccharide oxidizes a fraction of the endogenous PTEN to increase from 5% to 16% in macrophages, generating high levels of endogenous reactive oxygen species (ROS) [4]. Significantly, peptide growth factors such as insulin, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) have been shown to induce the production of H_2O_2 in various nonphagocytic cells and the oxidation of PTEN has been demonstrated in cells stimulated with these peptide growth factors at greatly lower levels than that found in macrophages. PTEN oxidation has also been observed in HEK293 cells stimulated with insulin, HeLa cells stimulated with EGF, and NIH3T3 fibroblasts stimulated with PDGF [5,6]. Similarly, the oxidation of PTEN has been observed in neuroblastoma cells

Abbreviations: Cys, cysteine; Grx, glutaredoxin; H_2O_2, hydrogen peroxide; NEM, *n*-ethylmaleimide; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Trx, thioredoxin.

* Corresponding authors. Fax: +82 2 873 4740 (W.-K. Huh), +82 62 223 8321 (S.-R. Lee).
E-mail addresses: wkh@snu.ac.kr (W.-K. Huh), leesr@chonnam.ac.kr (S.-R. Lee).

[1] These authors contributed equally to this work.

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stimulated with insulin, although the activation of PI 3-kinase was unaffected by insulin-stimulated ROS production [6]. PTEN oxidation has been identified in cells increasing the production of mitochondrial H_2O_2 by overexpressing manganese superoxide dismutase (MnSOD), leading to downstream regulation of vascular endothelial growth factor (VEGF) production and angiogenesis [7]. PTEN is oxidized in response to insulin in cell culture systems *in vitro* to promote PI3K signaling [6] and the oxidation of PTEN by *S*-nitrosothiols has also been shown *in vitro* [8]. More recently, oxidized inactivation of PTEN has been shown in mitochondrial respiration-deficient cells because of the accumulation of NADH, which competitively blocks NADPH-dependent thioredoxin-reductive PTEN activation [9].

The reduction of H_2O_2-oxidized PTEN in cells might be dependent on thioredoxin (Trx) [3]. It has been proposed that peroxiredoxin1 (Prdx1) promotes the tumor suppressive function by binding PTEN and protecting its lipid phosphatase activity from H_2O_2-induced inactivation [10]. Also, we have recently demonstrated that reduction of human PTEN (hPTEN) was delayed in each of *S. cerevisiae gsh1Δ* and *gsh2Δ* mutants which were deficient in glutathione (GSH) [11]. In the same study, oxidized hPTEN was reduced by glutathione in a concentration- and time-dependent manner *in vitro*. Incubation of 293T cells with buthionine sulfoximine (BSO) and knockdown expression of glutamate-cysteine ligase catalytic subunit (GCLc) in HeLa cells by siRNA resulted in the delay of reduction of oxidized PTEN. In HeLa cells transfected with GCLc siRNA, stimulation with epidermal growth factor resulted in the increase of oxidized PTEN and downstream PI3K-dependent Akt activation. Although the reduction of oxidized hPTEN was markedly delayed in the *S. cerevisiae gsh1Δ*, it was completely reduced within 200 min, suggesting existence of other reductants besides GSH.

Intracellular concentrations of GSH in hepatic cells are reported to be about 6.6 mM [12]. However, oxidized hPTEN was reduced in an environment of relatively high GSH concentrations (>7 mM) [11]. In this study, we have shown whether glutaredoxin catalyzes the reduction of oxidized hPTEN and identified other reductants of hPTEN by monitoring the redox state of hPTEN after exposure to H_2O_2 in *S. cerevisiae* mutants in which were disrupted the genes involved in redox homeostasis.

2. Materials and methods

2.1. Yeast strains, medium and culture conditions

S. cerevisiae strains were derived from BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). All mutants were generated by a one-step PCR-based method using the *URA3* or *KanMX4* gene as a selectable marker [13]. Revertant strains were generated using the disintegrator vector system as described previously [14]. All strains with p426GAL-PTEN were selected by growth to mid-logarithmic phase at 30 °C in synthetic complete medium (0.67% yeast nitrogen base without amino acids, 2% glucose; SC) lacking the appropriate amino acids for selection. For induction of p426GAL-PTEN, strains were grown in a nutrient-rich medium (1% yeast extract, 2% peptone, 2% galactose; YPG) for 2 h.

2.2. Construction of plasmids

For construction of p416GAL-hPTEN, the coding sequence of hPTEN was amplified by PCR using pCGN-hPTEN as a template using forward 5′-GTGGATCCATGACAGCCATCATCAAAGAG-3′ and reverse 5′-GTAAGCTTCTAGACTTTTGTAATTTGTGTATG-3′ primers. The obtained PCR products were cut with *Bam*HI and *Hind*III and inserted into the *Bam*HI–*Hind*III site of the p416GAL vector. For construction of the *GRX5* integrating vector, the coding region of *GRX5* containing the promoter and terminator sequences was amplified from *S. cerevisiae* genomic DNA as a template using 5′-TCAGTCTAGAGTCCATTATGACGACTGTAG-3′ forward and 5′-AG TCCTCGAGGTCAATTCATTTGGCAAAGC-3′ reverse primers. The *YCP4* gene was also obtained by 5′-TCAGTCTAGAACGTTACTATTTCCTTC CTC-3′ forward and 5′-AGTCCTCGAGTGGCTTAACAAAATGAGGTC-3′ reverse primers. The amplified DNA was digested with *Xba*I and *Xho*I and cloned into the *Xba*I–*Xho*I site of the pIS373 disintegrator plasmid [14].

2.3. Validation of GRX5 and YCP4 revertant strains

To confirm the reversion from each deletion mutants, mRNA expression was monitored by reverse transcription PCR (RT-PCR). Total RNA was isolated using the RNeasy MiniKit (Qiagen). cDNA was generated using the M-MLV Reverse Transcriptase Kit (Mbiotech) according to the manufacturer's instruction. The expression of *GRX5* were analyzed using 5′-TTAGAAGACCCAGAGCTACG-3′ forward and 5′-CAACGATCTTTGGTTTCTTC-3′ reverse primers and the expression of *YCP4* were analyzed using 5′-AAGAACTGCGTCTC-CACTTG-3′ forward and 5′-TTACATGACAGTACAGCAGG-3′ reverse primers. The *ACT1* gene was used as internal control and its mRNA level was also measured using 5′-TGACTGACTACTTGATGAAG-3′ forward and 5′-ACAGAAGGATGGAACAAAGC-3′ reverse primers.

2.4. Immunoblot analysis

Cultured *S. cerevisiae* strains (wild-type and all mutants with p426GAL-PTEN) were harvested and transferred to a 2 ml screwcap tube with 0.5 mm glass beads and lysis buffer containing 10 mM *n*-ethylmaleimide (NEM). The cells were lysed using a bead-beater and the lysate was clarified by centrifugation. Protein concentrations

Table 1
List of the genes having an effect on reduction of oxidized hPTEN in *S. cerevisiae*.

Gene ontology terms	Associated gene(s)	Effective genes on reduction of hPTEN
Electron carrier activity	YCP4, YPR004C, OPT1	YCP4
Oxidoreductase activity	CYC2, DfT2, FMP46, GRE2, YPR1, YGL039W, YGL157W, YOR246C, HSP82, FMP40, FMP46, SFA1	None
Age-dependent response to reactive oxygen species during chronological cell aging	SOD1, SOD2	None
Response to reactive oxygen species	CTT1, HSP150, URE2, RAS2	None
Peroxiredoxin activity	TSA1, TSA2, AHP1, GPX2, OT01, GPX1, GLR1, PRX1, GPX3	None
Thiol-disulfide exchange intermediate activity	GRX1, GRX2, GFQC3, GRX4, GRXS, TRX1, TRX2, TRX3, QTT1, GTT2	GRXS
Thioredoxin peroxidase activity	COX17, DOTS, HYR1, PRX1	None
Thioredoxin-disulfide reductase activity	TRR2	None
Glutathione synthetase activity	GSH1, GSH2	GSH1, GSH2

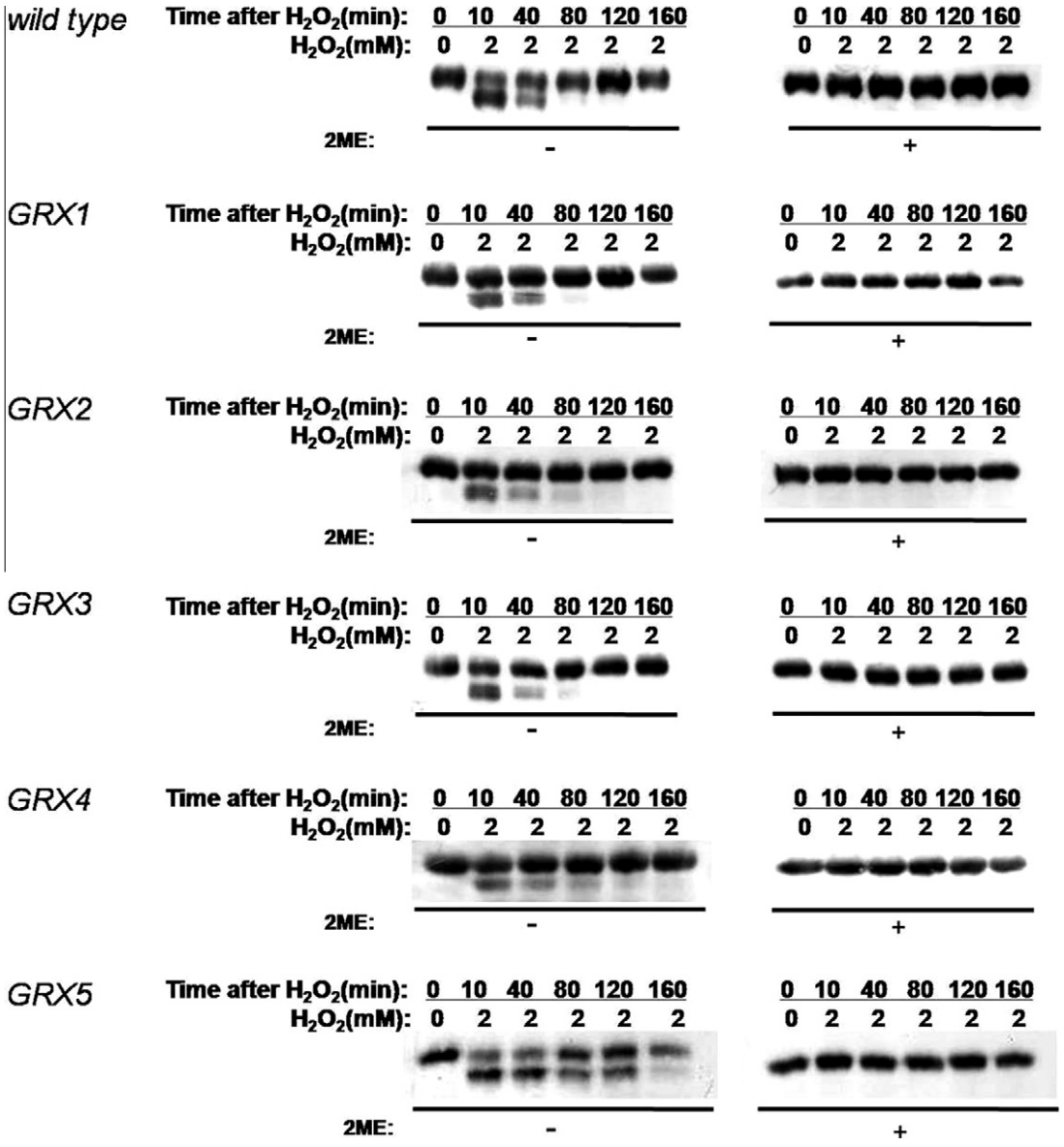

Fig. 1. The effect of glutaredoxin knockout on reduction of oxidized hPTEN in *S. cerevisiae*. Wild-type, *grx1Δ*, *grx2Δ*, *grx3Δ*, *grx4Δ* and *grx5Δ* mutants were transformed with p426GAL-hPTEN. Cells were treated with 2 mM H_2O_2 for 10 min and incubated for the indicated times after the removal of H_2O_2 by catalase. Cellular protein extracts were then alkylated with 10 mM NEM and subjected to nonreducing (left) and reducing (right) SDS–PAGE followed by immunoblotting with antibodies to hPTEN. The data are representative of three separate experiments.

were determined using a BCA assay kit (Pierce). Protein samples were electrophoresed on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing and reducing conditions and transferred onto a nitrocellulose membrane (Whatman). The membranes were blocked in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) nonfat dry milk and then reacted with primary antibodies to hPTEN overnight at 4 °C. After several washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG antibodies. Proteins in the membranes were then visualized using the enhanced chemiluminescence (ECL) detection kit (Pierce) according to the manufacturer's instructions.

2.5. Protein purification

Human Grx5 cDNA cloned in the pQE30 vector for expression of the protein with a histidine tag at the NH_2 terminus. The histidine-tagged hGrx5 protein was expressed in *Escherichia coli* according to standard protocols and purified with an immobilized nickel affinity resin (Qiagen) as described previously [11]. The purified proteins were eluted by 20 mM Tris–HCl buffer (pH 7.5) containing 150 mM sodium chloride (NaCl), 5% glycerol, 0.1% Nonidet P40 (NP-40) and 1 μM phenylmethanesulfonyl fluoride (PMSF) and then stored at −80 °C.

3. Results

To identify the genes involved in the redox regulation of hPTEN, 47 genes involved in redox homeostasis in *S. cerevisiae* were selected from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) for screening (Table 1). Then, we obtained deletion mutants by a one-step PCR-based method using the *URA3* gene as a selectable marker for each of the genes [13]. The p426 GAL-hPTEN plasmid was transformed into all single-gene knockout mutants for expression of human PTEN protein. Also, we initially

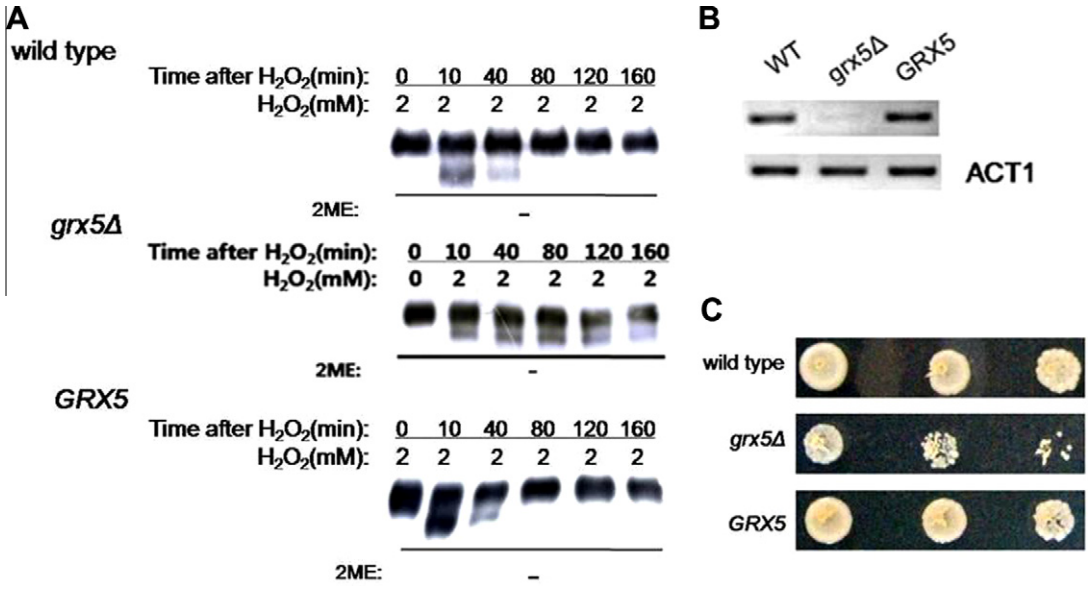

Fig. 2. Reduction of oxidized hPTEN in *GRX5* revertant strain. The *GRX5* revertant strain was obtained using the disintegrator vector system. (A) Time-dependent reduction of oxidized hPTEN in wild-type, *grx5*Δ and *GRX5* revertant strains. (B) Validation of *GRX5* reversion by RT-PCR. (C) Growth assay. Wild-type, *grx5*Δ and *GRX5* revertant strains were grown to stationary phase, and the A_{600} was adjusted to 1, 0.1, or 0.01 before strains were spotted onto plates. Growth was monitored after 3 days of incubation at 30 °C. Preparation of the cellular extracts and analysis was performed as described in Fig 1. The data are representative of three separate experiments.

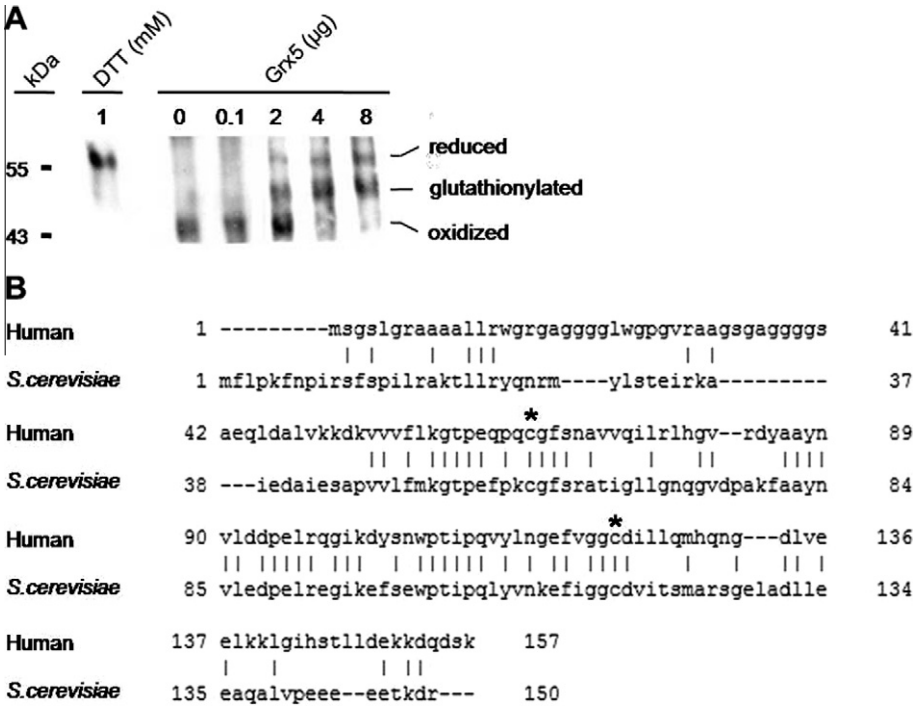

Fig. 3. Redox regulation of oxidized hPTEN by the Grx5/GSH system *in vitro*. (A) Recombinant hPTEN was completely reduced by incubation with 1 mM DTT at room temperature for 2 h (far left lane). Oxidized hPTEN (0.5 μg) was incubated with human Grx5 (0, 0.1, 2, 4 and 8 μg) in the presence of 4 mM GSH at room temperature for 2 h. All samples were modified with NEM and subjected to nonreducing SDS–PAGE followed by immunoblot analysis with antibodies to PTEN. (B) Sequence alignment of *S. cerevisiae* Grx5 and human ortholog showing conservation of the active cysteine residues (asterisks).

demonstrated that hPTEN expressed in *S. cerevisiae* can be reversibly oxidized by H_2O_2 as in human cells on the basis of the mobility shift in nonreducing gels [11]. Exposure of *S. cerevisiae* cells to H_2O_2 resulted in the appearance of the higher mobility (oxidized) form of hPTEN, and the intensity of this band increased as the concentration of H_2O_2 increased as in HeLa cells [11]. As well, oxidized hPTEN in *S. cerevisiae* was reduced by cellular reductants in a time-dependent manner as in HeLa cells [11]. The reduction of H_2O_2-oxidized hPTEN was delayed in *S. cerevisiae gsh1Δ* and *gsh2Δ* mutants as shown previously [11]. Two new genes, *GRX5* and *YCP4*, were identified as hPTEN redox regulators from among 47 genes that are involved in redox homeostasis in *S. cerevisiae* (Table 1).

Glutaredoxins (Grxs) are thiol-disulfide oxidoreductases that are conserved in both prokaryotes and eukaryotes [15]. Grxs

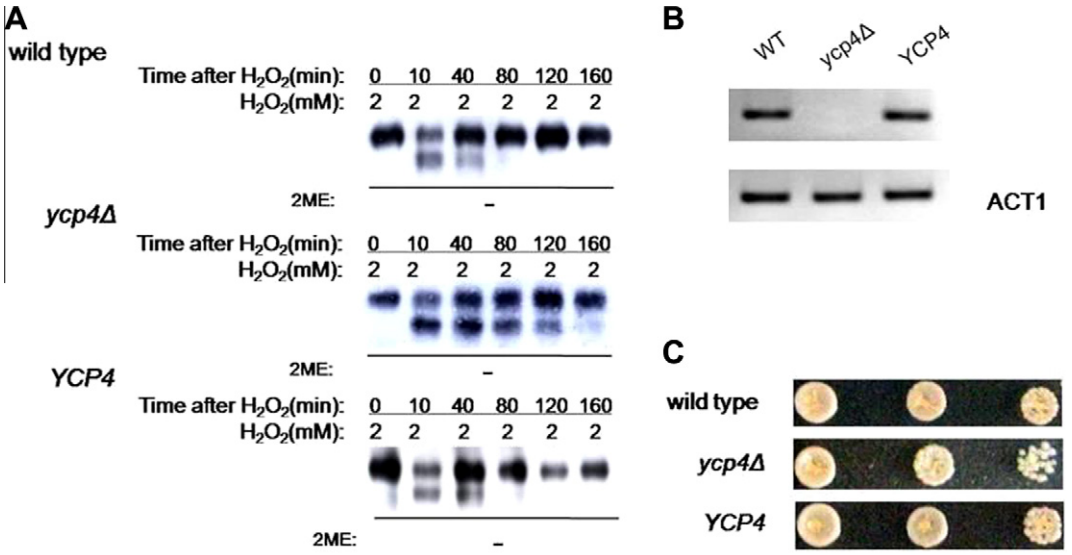

Fig. 4. Reduction of oxidized hPTEN in the *YCP4* revertant strain. The *YCP4* revertant strain was obtained using the disintegrator vector system. (A) Time-dependent reduction of oxidized hPTEN in wild-type, *ycp4Δ* and *YCP4* revertant strain. (B) Validation of *YCP4* reversion by RT-PCR. (C) Growth assay. The cellular extracts were prepared and analyzed as described in Fig 1. The data are representative of three separate experiments.

can catalyze the reduction of protein disulfides and of protein-S-S-glutathione mixed disulfide via a dithiol or monothiol mechanism. Dithiol Grxs can reduce protein disulfides using both the active site cysteines, and monothiol Grxs use only the N-terminal cysteine for the reduction of glutathione mixed disulfides. Oxidized Grx is recycled to the reduced form using glutathione as a hydrogen donor. These characteristic interactions of Grx with GSH distinguish it from Trx using NADPH as a hydrogen donor via TrxR. *S. cerevisiae* contains two cytoplasmic dithiol Grxs (Grx1 and Grx2) and three monothiol Grxs (a cytosolic Grx3 and Grx4, and a mitochondrial Grx5). Mammalian Grx is known to have two dithiol Grxs (a cytosolic Grx1 and a mitochondrial Grx2) and two monothiol Grxs (a multidomain Grx3 and a mitochondrial Grx5).

The reduction of oxidized hPTEN was markedly delayed in the *S. cerevisiae grx5Δ* mutant (Fig. 1). However, the reduction of oxidized hPTEN was slightly delayed in the *grx4Δ* mutant and was not nearly delayed in the *grx1Δ, grx2Δ* and *grx3Δ* mutants (Fig. 1). To confirm that the *GRX5* gene affected the reduction of oxidized hPTEN, we engineered a strain that reverted to *grx5Δ* mutant. Generation of *GRX5* revertant strain was carried out using the disintegrator vector system as described previously [14]. The *grx5Δ* mutant and revertant strain were confirmed by RT-PCR, which revealed the presence of the expected product in the wild-type strain and the *GRX5* revertant strain, but not in the *grx5Δ* mutant (Fig. 2B). Strains were grown to stationary phase and spotted onto SC-Ura plates. This analysis confirmed that growth defects shown in the *grx5Δ* mutant were rescued in the *GRX5* revertant strain (Fig. 2C). In contrast to the *grx5Δ* mutant, reduction of oxidized hPTEN in the *GRX5* revertant strain completely recovered within 80 min, as was observed in the wild-type strain (Fig. 2A), suggesting that disruption of the *GRX5* gene affects the reduction of oxidized hPTEN.

S. cerevisiae grx5 is 150 amino acid residues long and is conserved in human (Fig. 3B). To further clarify the molecular mechanism by which GSH/Grx5 system regulates the redox state of hPTEN, we examined whether human Grx5 (hGrx5) could accelerate reduction of oxidized hPTEN mediated by GSH *in vitro*. First we purified hGrx5 using the His-tag fusion protein expression system as described previously [11]. Then we examined the effect of purified hGrx5 on the reduction of oxidized recombinant hPTEN on the basis of the mobility shift in non-reducing gels. hPTEN was rapidly reduced by treatment of 1 mM DTT (Fig. 3A, far left lane). hGrx5 accelerated the reduction of hPTEN in a dose-dependent manner in the presence of 4 mM GSH, a concentration that did not affect the reduction of oxidized hPTEN (Fig. 3A) [11]. These results indicate that hGrx5 regulates the redox state of hPTEN in concert with GSH to regenerate oxidized hPTEN.

We also found that the *ycp4Δ* mutant lead to a delay in the reduction of oxidized hPTEN (Fig. 4A, middle). Ycp4 is a protein of unknown function, which has sequence and structural similarity to flavodoxins. To confirm that the reduction of oxidized hPTEN was affected by the *YCP4* gene, we engineered a *YCP4* revertant strain and performed RT-PCR as was done in the *GRX5* revertant strain (Fig. 4B). The *ycp4Δ* mutant exhibited slight growth defects that were rescued in the *YCP4* revertant strain (Fig. 4C). The reduction of oxidized hPTEN in the *YCP4* revertant strain also completely recovered within 80 min, as was observed in the wild-type strain (Fig. 4A).

4. Discussion

Mammalian Grx1 and *S*-glutathionylation have been shown to regulate the redox status of various cellular proteins including Akt [16], human immunodeficiency virus-1 (HIV-1) protease [17], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [18], nuclear factor I (NF1) [19], ASK1 [20], tubulin [21], actin [22], Ras [23] and protein-tyrosine phosphatase 1B (PTP1B) [24]. The other form of Grx, Grx2, has also been shown to regulate the redox status of mitochondrial proteins such as complex I protein [25]. Many proteins maintain a redox balance by glutathione or glutaredoxin system that protect against oxidative damage. However, we demonstrated that oxidized PTEN was reduced by both glutathione and glutaredoxin system. Furthermore, we had previously reported that the reduction of oxidized PTEN might be dependent on the thioredoxin system [3]. These data show the important role of tumor suppressor PTEN in cells. Further studies are required to determine which system are activated during various oxidative stress conditions to understand the redox regulation of PTEN.

The yeast *GRX5* gene has been shown to participate mainly in the synthesis and assembly of iron–sulfur clusters [26]. The *grx5Δ* mutant in yeast leads to the oxidative damage generated by iron accumulation [27]. Moreover, cells lacking *GRX5* gene exhibit high sensitivity to hydrogen peroxide and menadione, severe growth defects on minimal medium, and no growth on glycerol respiratory medium [27]. The human Grx5 isoform is highly homologous to the yeast Grx5 and it has been recently shown that the function of Grx5 is conserved in humans [28].

S. cerevisiae Ycp4 has not been shown to have human homolog. However, Ycp4 does have a Flavodoxin_1 (PFAM Accession No. PF00258) domain. We analyzed the domain architecture by using the SMART tool (http://smart.emblheidelberg.de/) and found eight human proteins including nitric oxide synthase 1, nitric oxide synthase 2, nitric oxide synthase 3, methionine synthase reductase, NADPH dependent diflavin oxidoreductase 1, tRNA wybutosine-synthesizing protein 1 homolog A, NADPH-cytochrome P450 reductase, and annotation of which was not available. We are not certain whether or not Ycp4 ortholog exists among these proteins. Hence, further studies are required to determine whether Ycp4 ortholog exists in humans and if so, whether it is really involved in redox regulation of PTEN.

Grx5 is a mitochondrial glutaredoxin (Kim and Lee, unpublished result) and Ycp4 is also detected in highly purified mitochondria in high-throughput studies. But, PTEN has a punctate cytoplasmic distribution and a substantial presence in the nucleus. This raises an interesting question. If oxidized PTEN is converted to reduced PTEN by Grx5 or Ycp4, then at which place is PTEN reduced? Grx5 is essential for iron–sulfur cluster biosynthesis and for the maintenance not only of mitochondrial but also of cytosolic iron homeostasis in human cells [29]. Based on these results, further studies on the function and interaction of PTEN redox-regulating proteins in mammalian system are needed to better understand the redox mechanisms of PTEN.

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