Antioxidant activity is required for the protective effects of cyclophilin A against oxidative stress

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Received May 3, 2014; Accepted January 7, 2015

DOI: 10.3892/mmr.2015.3392

Abstract. Cyclophilin (Cyp) belongs to a group of proteins that have peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. CypA is the major cellular target for the immunosuppressive drug cyclosporin A and mediates its actions. Previous studies have demonstrated that CypA has diverse cellular functions and have suggested that CypA may function as an antioxidant. The present study investigated the antioxidant activity of CypA and its association with PPIase activity. The purified CypA/wild-type (WT) and CypA/P16S mutant proteins were active in PPIase assays. A total antioxidant capacity assay revealed that the purified CypA/WT protein had significantly higher antioxidant activity, whereas the CypA/P16S mutant was defective in its antioxidant activity. To confirm the importance of CypA antioxidant activity, CypA/P16S was overexpressed in Chang human liver cells and the rate of cell death was measured following treatment with cisplatin or H_2O_2. Overexpression of CypA/WT protected the cells against cisplatin or H_2O_2-induced oxidative damage, however, the CypA/P16S mutant had no effect. These findings suggested that CypA exhibits a protective antioxidant effect.

Introduction

Cyclophilin (Cyp) is present in prokaryotes and eukaryotes and is an immunophilin and a cytosolic receptor for the immunosuppressive drug, cyclosporin A (1). In addition, Cyp possesses enzymatic peptidyl-prolyl *cis-trans* isomerase (PPIase) activity, which is essential for protein folding *in vivo* (2). PPIase activity has been suggested to facilitate protein folding, intracellular trafficking and the maintenance of multiprotein complex stability (3). There are several isoforms of Cyp, including CypA (4), CypB (5), CypC (6) and CypD (7). CypA is predominantly localized in the cytoplasm and is a highly conserved protein in mammalian cells (4,8). In our previous study (9), CypA protected cells against cellular stresses, including hypoxia and cisplatin-induced effects, and it was hypothesized that the protective effects of CypA were, at least in part due, to its antioxidant activity (9). However, the antioxidant activity of CypA remains to be fully elucidated.

Several mutations in CypA have been identified. The CypA mutant, which carries a serine (Ser/S) instead of proline (Pro/P) at amino acid 16, has been reported to alter the folding/assembly pathway and its refolding intermediates have been observed to fall into a kinetic trap in the refolding process under the observed time course, resulting in a small fraction of multimerized CypA (10). It has been demonstrated that another CypA mutant, which carries alanine (Ala/A) instead of arginine (Arg/R) at amino acid 55, retains <1% PPIase catalytic activity compared with the CypA/wild-type (WT) (11,12).

The present study aimed to demonstrate the antioxidant activity of CypA by measuring the total antioxidant capacity of the purified CypA proteins. The association between the antioxidant activity of CypA and its PPIase catalytic activity was also investigated.

Materials and methods

Construction of glutatione S-transferase (GST)-CypA fusion proteins. The present study was approved by the Ethics Committee of Kyunghee University (Seoul, South Korea). Plasmids encoding GST fusion proteins were constructed using pGEX-KG vectors (American Type Culture Collection, Manassas, MA, USA). All the plasmid DNA was prepared using a modified alkaline lysis method (10). Briefly, the mutagens were created through site-direct mutagenesis as previously described (13) CypA/WT, CypA/R55A, the PPIase-defective mutate gene, and CypA/P16S, which is a mutant gene with a more flexible structure, were digested using *Bam*HI and

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Key words: cyclophilin A, oxidative stress, peptidyl-prolyl *cis-trans* isomerase activity, antioxidant

*Eco*RI [New England Biolab (NEB), Inc., Ipswich, MA, USA] and ligated into the pGEX-KG expression vector. The ligation mixture (NEB, Inc.) was used to transform an *Escherichia coli* DH5α strain (Life Technologies, Grand Island, NY, USA). All the constructs were verified by DNA sequencing.

Site-directed mutagenesis of CypA. Mutations in CypA were produced by site-directed mutagenesis using modified rapid polymerase chain reaction (PCR) (14). Substitution of Ser16 into Pro and Ala55 into Arg were performed by PCR in two steps with a MyCycler (Bio-Rad, Hercules, CA, USA). Firstly, the two fragments, containing the sequences upstream and downstream of the Ser16 and Ala55 residues, were amplified using primers containing the desired mutations. A PCR mixture, total volume 50 μl [2 μg CypA/WT template DNA; 1 μl each forward and reverse primers (100 pmol; Macrogen, Inc., Seoul, Republic of Korea); 5 μl 10X buffer; 3 μl deoxyribonucleotide; 1 μl taq polymerase (Takara Bio, Inc., Otsu, Japan); and 37 μl distilled water], was used. The cycling conditions were as follows: 94˚C for 5 min, followed by 25 cycles of 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 1 min, the reaction was terminated by 72˚C for 10 min. The primer sequences used were as follows: R55A forward, AAATTTGGATCCATGGTCAACCCCACCG and reverse, GGCGGAATTCTTAGAGTTGTCCACAGTC; and P16S forward, ACTGTAAGCTTATGGTCAACCCCACCG and reverse, CCCGGGGATATCTTAGAGTTGTCCACAG. Secondly, the two amplified fragments were used as templates for a second PCR reaction at the same conditions, in which only a primer for the 5' end of the first fragment and a primer for the 3' end of the second fragment were used, resulting in a full length Pro16 and Arg55 mutated CypA.

Expression and purification of the recombinant fusion proteins. The pGEX-KG, pGEX-KG/CypA/WT, pGEX-KG/CypA/P16S and pGEX-KG/CypA/R55A plasmids were transformed into the DH5α *E. coli* strain. The bacterial cells were grown at 37˚C in 200 ml Lysogeny broth (Life Technologies) containing ampicillin (100 μg/ml; Duchefa Biochemie, Haarlem, Netherlands) from an overnight culture. At at absorbance$_{600}$ of ~0.6 (NovaspecII; Biotek Instruments, Inc., Winooski, VT, USA), the bacterial cells were induced using 0.1 mM isopropyl β-d-thiogalactopy ranoside (IPTG) for 3 h at 30˚C. The cells were harvested by centrifugation at 890 x g for 15 min and resuspended in 2 ml 1X phosphate-buffered saline (PBS; Bioworld, Bundang, South Korea) containing 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained on ice and lyzed by sonication (Sonicator W-375 Cell disruptor; Heat Systems Ultrasonics Inc., Wehnrath, Germany). Following centrifugation at 890 x g for 15 min at 4˚C, the cell debris was removed and the supernatant was incubated overnight with washed glutathione (GSH)-agarose (Sigma-Aldrich) at 4˚C with gentle rotation (Rotamix SLRM I; Seoulin Bioscience Co., Seoul, South Korea). The samples were centrifuged at 100 x g for 1 min at 4˚C and the GST-only or the GST-CypA fusions bound to the GSH-agarose beads (Sigma-Aldrich) were washed five times with ice-cold PBS containing 1% Triton X-100. The target fusion protein was eluted from the resin using an elution buffer, containing 20 mM reduced GSH in 100 mM Tris-HCl (pH 9.0; Duchefa Biochemie) overnight at 4˚C. Following centrifugation at 100 x g for 1 min at 4˚C, the supernatant containing the fusion proteins was mixed with 6X SDS sample buffer (Sigma-Aldrich), containing 0.35 M Tris-HCl (pH 6.8), 10.3% SDS, 36% glycerol, 0.6% bromophenol blue and 0.6 M dithiothreitol. The samples were analyzed by SDS-PAGE, native-PAGE and immunoblot analysis.

Removal of the GST by selective cleavage using thrombin. The GST tag was cleaved from the GST-CypA using a Thrombin CleanCleave™ kit (Sigma-Aldrich) according to the manufacturer's instructions. The fusion protein (1 mg/ml) in cleavage buffer containing 500 mM Tris-HCl (pH 8.0) and 100 mM $CaCl_2$ (Duchefa Biochemie), was incubated at 4˚C with gentle rotation overnight in the presence of 100 μl bovine thrombin agarose beads (Sigma-Aldrich). Following the reaction, the thrombin agarose was removed from the mixture by centrifugation at 100 x g for 1 min. The supernatant was assessed by SDS-PAGE, native-PAGE and immunoblot analysis.

SDS-PAGE, immunoblot analysis and native-PAGE. The total cell lysate, purified fusion proteins and cleaved fusion proteins were separated by 12 % SDS-PAGE (Life Technologies) and the proteins were transferred onto nitrocellulose membranes (Pall Corp., Pensacola, FL, USA). Transfer of the proteins was assessed by Ponceau red staining (Sigma-Aldrich) and the membranes were subsequently blocked for 1 h at room temperature in 3% bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline containing 10 mM Tris-HCl (pH 8.0; Life Technologies) and 150 mM NaCl (Duchefa Biochemie), supplemented with 0.05% Tween-20 (TBST). The nitrocellulose membrane was washed with 1X TBST three times for 15 min and incubated with the following primary antibodies: Mouse monoclonal anti-GST (1:1,000; sc-138; Santa Cruz Biotechnology, Inc. Dallas, TX, USA), rabbit polyclonal anti-CypA (1:1,000; 07-313; Millipore, Billerica, MA, USA), mouse monoclonal anti-HA (1:1,000; sc-7392; Santa Cruz Biotechnology, Inc.) and mouse polyclonal anti-GAPDH (1:1,000; csa-335; Enzo Life Sciences, Farmingdale, NY, USA), for 1 h at room temperature. Following washing with TBST three times for 15 min, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 45 min at room temperature.

Native-PAGE analysis was performed using a 12% gel. The stacking gel, separating gel and the running buffer were prepared as for SDS-PAGE, however, no SDS was added and the sample was not heated.

PPIase activity assay. The PPIase activity assay was performed, as described previously (15-17), with the suggested substrate solvent application (18). This assay determines the rate of conversion of *cis*-to-*trans* in proline-containing peptides, based on the principle that α-chymotrypsin cleaves the peptide only when it is in the *trans* conformation (18). The N-succinyl-Ala-Ala-Pro-phenylalanine-*p*-nitroanilide peptide substrate (Sigma-Aldrich) was dissolved in >99% trifluoroethanol (Sigma-Aldrich) with 470 mM LiCl (Sigma-Aldrich) to prepare a 100 mM stock solution, which was further diluted to 4 mM prior to use. α-chymotrypsin was dissolved

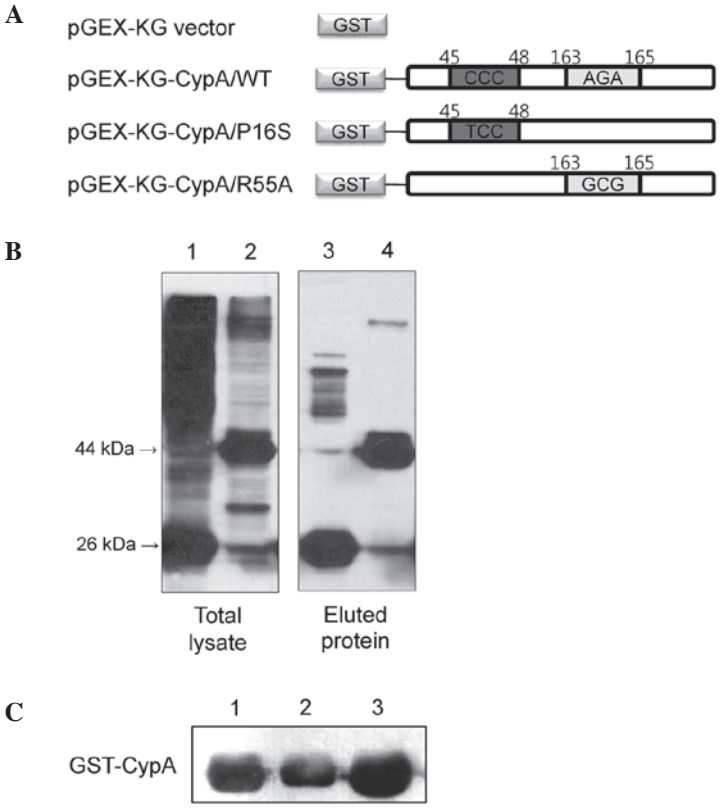

Figure 1. (A) Schematic of the GST-CypA constructs. (B) Western blot analysis of the purified GST-CypA fusion protein. The total GST protein lysate and the eluted GST protein lysate were separated by SDS-PAGE. The proteins were detected using an anti-GST antibody. Lane 1, total GST protein lysate; lane 2, total GST-CypA fusion protein lysate; lane 3, eluted GST protein lysate; Lane 4, eluted GST-CypA fusion protein lysate. (C) Western blot analysis of the purified GST-CypA/WT, GST-CypA/P16S, and GST-CypA/R55A fusion proteins. The proteins were detected using an anti-CypA antibody. Lane 1, eluted GST-CypA/WT; lane 2, eluted GST-CypA/P16S; lane 3, eluted GST-CypA/R55A. GST, glutatione S-transferase; CypA, cyclophilin A.

in 1 mM HCl (Junsei Chemical Co., Ltd, Tokyo, Japan) with 2 mM $CaCl_2$ to prpare a 1 M stock solution. GST-CypA was diluted into 50 mM HEPES (Sigma-Aldrich) and 86 mM NaCl (pH 8.0), to prepare the PPIase buffer. In a 1 ml cuvette (Sigma-Aldrich), 100 μl protein (10 μM final concentration) was added to 890 μl PPIase buffer. The reaction was initiated by the addition of 10 μl (40 μM) peptide substrate and α-chymotrypsin (Sigma-Aldrich) of 0.5 μl (500 μM), followed by rapid mixing using a pipette. The change in absorbance at 390 nm, following the cleavage of the *trans* form of the peptide and release of *p*-nitroaniline, was monitored using a biosciences spectrophotometer (VICTOR; PerkinElmer, Waltham, MA, USA).

Antioxidant activity assay. The antioxidant activity was determined using a Total Antioxidant Capacity Assay kit (BioVision Research Products, Mountain View, CA, USA) and measured by monitoring the reduction of Cu^{++} reagent by the increase of absorbance at 570 nm. The degree of quenching of radical generation in individual samples, indicative of the presence of antioxidant activity, was quantified by comparison with a traditional standard trolox (Sigma-Aldrich) and the assay results were expressed in terms of μmol/trolox.

Cell culture and reagent. Chang human liver cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (GE Healthcare, Logan, WV, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; GE Healthcare) and antibiotics [100 U/ml penicillin (Duchefa Biochemie) and 100 μg/ml streptomycin sulfate (Sigma-Aldrich)] in a 5% CO_2 incubator. To induce oxidative stress, the cells were treated with cisplatin (0, 15 or 20 μmol/l) or H_2O_2 (0, 400 or 500 μmol/l) for 24 h.

Transfection. CypA/WT and CypA/R55A were tagged (hemagglutinin tag, 5'-TAC CCA TAC GAC GTC CCA GAC TAC GCT-3') at the 5' end. The cells were cultured in a 12-well plate at 37˚C in a 5% CO_2 incubator for a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were transfected using Lipofectamine 2000™ reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Following transfection, cells were also incubated in the same conditions for 18-24 h.

MTT assay. Following 24 h treatment with cisplatin or H_2O_2, the cell viability was evaluated using an MTT conversion assay in a 12-well plate. The culture medium (DMEM supplementd with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate) was replaced with 1 ml medium containing 0.5 mg/ml MTT (Sigma-Aldrich; dissolved in filtration water) and incubated for 60 min at 37˚C. The medium was then carefully aspirated from the plates and the blue-colored tetrazolium crystals, resulting from mitochondrial enzymatic activity, on the MTT substrate were solubilized in 150 μl 100% dimethylsulfoxide (Junsei, Tokyo, Japan). The absorbance was measured at 595 nm in a Model 680 Microplate

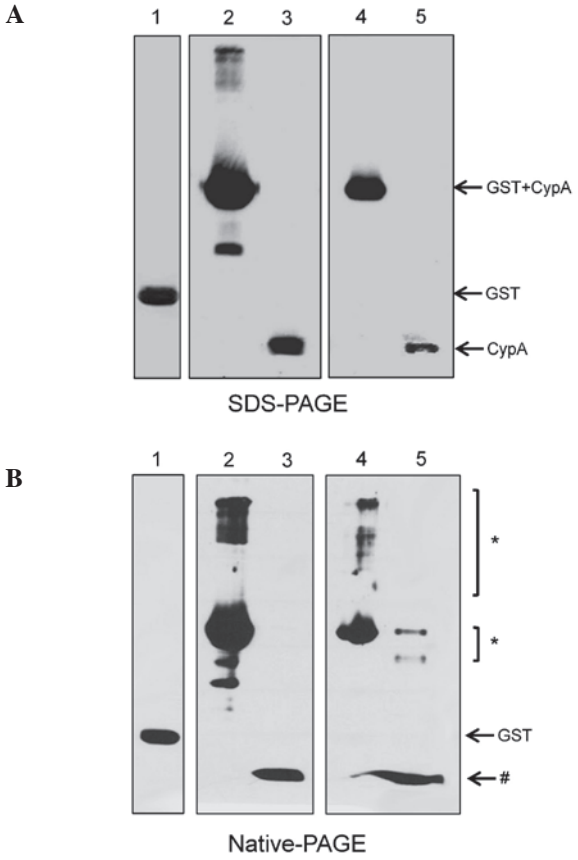

Figure 2. (A) Western blot analysis of the recombinant CypA proteins. CypA recombinant fusion proteins were separated by SDS-PAGE following GST cleavage. The proteins were detected using an anti-GST antibody (lane 1) and an anti-CypA antibody (lanes 2-5). Lane 1, eluted GST protein; lane 2, eluted GST-CypA/WT protein; lane 3, CypA/WT protein following cleavage; lane 4, eluted GST-CypA/P16S protein; lane 5, recombinant CypA/P16S protein following GST cleavage. (B) Recombinant CypA fusion proteins were separated by Native-PAGE gel. The proteins were detected using anti-GST (lane 1) and anti-CypA (lane 2-5). Lane 1, eluted GST protein alone; lane 2, eluted GST-CypA/WT protein; lane 3, CypA/WT protein following cleavage; lane 4, eluted GST-CypA/P16S protein; lane 5, CypA/P16S protein following cleavage. *Multimers of CypA protein; #monomer of CypA protein. CypA, cyclophilin; GST, glutatione S-transferase.

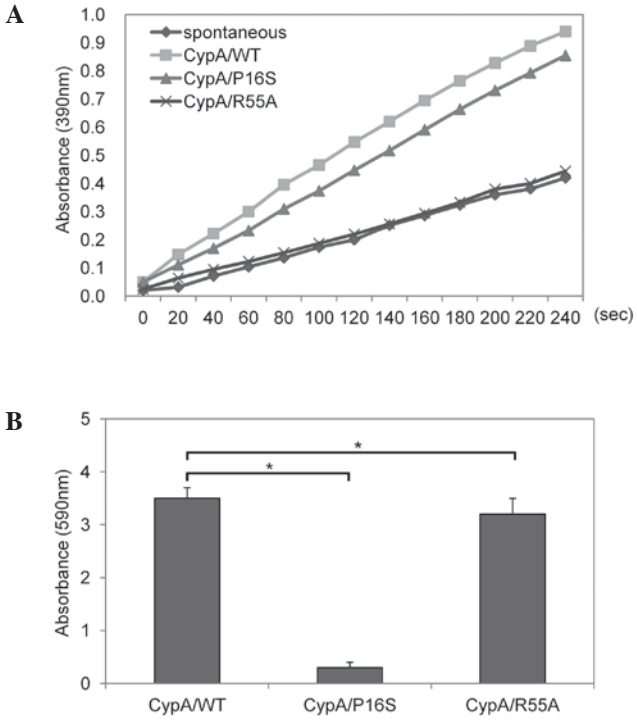

Figure 3. (A) PPIase activity of 10 μM GST-CypA WT, GST-CypA/P16S, GST-CypA/R55A and without enzymes. (B) Antioxidant catalytic activity of 1 μM CypA WT, CypA/P16S and CypA/R55A protein. The data are expressed as the mean ± standard deviation of three independent experiments. *P<0.05 vs. Cyp/WT-transfected cells. CypA, cyclophilin A; GST, glutatione S-transferase; WT, wild-type; PPIase, peptidyl-prolyl *cis-trans* isomerase.

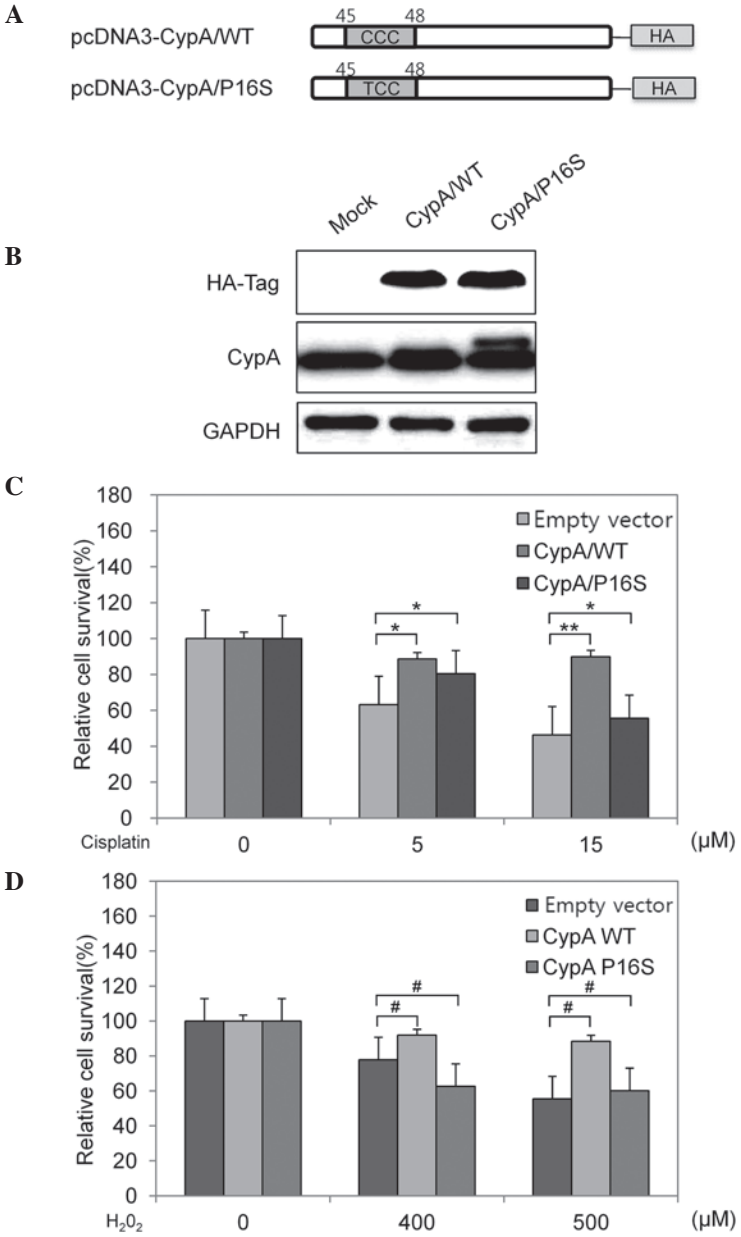

Figure 4. Effects of CypA on cell death following treatment with cisplatin or H_2O_2. (A) Schematic of the CypA constructs in mammalian expression vectors. (B) Protein expression level of CypA. The expression of CypA was determined by western blotting using anti-CypA or anti-HA antibodies. Chang cells were transfected with the empty vector, CypA/WT and CypA/P16S and were treated with (C) 0, 5 or 15 μM cisplatin or (D) 0, 400 or 500 μM H_2O_2 for 24 h. Cell viability was measured using an MTT assay and survival rate was calculated relative to that of 0 μM H_2O_2. The data are expressed as the mean ± standard deviation of three independent experiments. *P< 0.05 and **P<0.01 vs. empty vector-transfected cells treated with cisplatin, $^{\#}$P< 0.05 vs. empty vector-transfected cells treated with H_2O_2. WT, wild-type; HA, hemagglutinin; Cyp, cyclophilin.

Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell survival was expressed as the percentage of absorbance relative to that of the untreated cells.

Results

Purification of the expressed GST-CypA fusion proteins. In order to investigate the biochemical activity of CypA, the GST-CypA/WT, GST-CypA/P16S and GST-CypA/R55A plasmids were expressed in *E. coli*. A schematic of the constructed expression plasmids is shown in Fig. 1A. The recombinant GST-CypA fusion proteins were successfully overexpressed by the addition of 0.1 mM IPTG in *E. coli* (Fig. 1B). The GST-only and GST-CypA fusion proteins were eluted using GSH-beads (Fig. 1B). The expression levels of GST-CypA/WT, GST-CypA/P16S and GST-CypA/R55A were analyzed by western blotting (Fig. 1C). The 18-kDa recombinant CypA proteins were purified following GST cleavage with thrombin, and the purified proteins were confirmed by western blotting using an anti-CypA antibody (Fig. 2A). A small fraction of the CypA/P16S mutant protein has been reported to exhibit a multimerized structure due to its low yield of refolding (10). Therefore, in order to confirm the multimerized CypA/P16S, native-PAGE in non-denaturing conditions was used (Fig. 2B). The results demonstrated that a significant level of multimerized complexes of CypA/P16S remains, consistent with

the previous report (10). However, CypA/WT remained as a monomer (Fig. 2A).

PPIase and antioxidant activity assays of purified CypA protein. The PPIase activity of the purified CypA protein was measured. The PPIase activity assay is based on the observation that α-chymotrypsin cleaves the C-terminal amide bond only in the *trans* X-pro conformer of the chromogenic substrate, X-Pro-Phe-pNA. The rapid hydrolysis perturbs the *cis-trans* conformational equilibrium, which enables the PPIase-catalyzed *cis*-to-trans isomeriztion to be monitored. The PPIase activity was assayed in an α-chymotrysin coupled assay. As shown in Fig. 3A, recombinant CypA/WT and CypA/P16S exhibited PPIase activity, although CypA/P16S demonstrated marginally less efficient activity. Consistent with a previous report, the CypA/R55A was defective in its PPIase activity, as shown in Fig. 3A (13). The total antioxidant activity of the purified CypA protein was also measured. CypA/WT and CypA/R55A demonstrated significantly higher antioxidant activities, however, CypA/P16S exhibited no antioxidant activity (Fig. 3B). GST-only was used as a negative control.

Overexpression of CypA reduces cell death induced by cisplatin or H_2O_2. To confirm the importance of the antioxidant activity of CypA during oxidative stress, HA-tagged CypA was expressed in Chang human liver cells (Fig. 4A). The overexpression of CypA/WT or CypA/P16S was analyzed by western blotting (Fig. 4B). The effects of overexpressed CypA on reactive oxygen species-mediated cell death was determined using an MTT assay with various concentrations of H_2O_2 (400 and 500 μM) for 24 h. The CypA/WT-transfected cells demonstrated a higher survival rate compared with the empty vector-transfected cells. By contrast, the CypA/P16S-transfected cells exhibited a higher sensitivity to H_2O_2-mediated cell death compared with the CypA/WT-transfected cells (Fig. 4C). The chemoresistance of the CypA/WT- and CypA/P16S-transfected cells following treatment with cisplatin were monitored using an MTT assay. Cisplatin is known to induce apoptosis, partly through the generation of oxidative stress (19-21). The cells transfected with CypA/WT had a reduced level of cell death following treatment with cisplatin, compared with the empty vector-transfected cells. By contrast, the CypA/P16S-transfected cells exhibited a lower survival rate following treatment with cisplatin, compared with the CypA/WT-transfected cells (Fig. 4D). These findings suggested that antioxidant activity is required for the protective effects of CypA against oxidative stress.

Discussion

CypA protects cells from several types of cellular stress, including oxidative stress (14) and endoplasmic reticulum stress (22). PPIase activity is reported to be associated with several cellular functions, in addition to its biochemical activity (23). Several reports have demonstrated that the PPIase activity of CypA is important (24) and our previous study revealed that overexpression of CypA protects several cell lines from oxidative stress in a PPIase activity-dependent manner (9). However, the antioxidant activity of CypA and its importance remain to be fully elucidated. Therefore, the present study focused on the antioxidant activity of CypA and identified for the first time, to the best of our knowledge, its importance in the cellular response to oxidative stress.

The antioxidant activity of recombinant CypA, purified from bacterial extracts, was measured using a Total Antioxidant Capacity Assay kit. The results demonstrated that a significant level of antioxidant activity was associated with CypA/WT and CypA/R55A, while CypA/P16S was defective in antioxidant activity. The PPIase activity of each protein was also measured and its association with antioxidant activity was investigated. As shown in Fig. 2A, CypA/WT and CypA/P16S exhibited PPIase activity, although CypA/P16S had marginally less efficient activity. This indicated that Pro16 in the structure of CypA does not affect the catalytic PPIase activity since Pro16 is located at a region distant from the catalytic center of the PPIase. In addition, these results suggested that the antioxidant activity of CypA is independent of PPIase activity, since CypA/R55A, which is defective in PPIase activity, exhibits antioxidant activity. Notably, CypA/P16S formed a multimerized complex, while CypA/WT existed as a monomer, as shown in Fig. 2B. Multimerization may inhibit the antioxidant activity of CypA/P16S. However, further studies are required to elucidate the association between the multimerization of CypA and its antioxidant activity.

Our previous study demonstrated that the overexpression of CypA/WT may be important in tumorigenesis by reducing apoptosis under hypoxic conditions and by treatment with cisplatin (9). Therefore, the present study hypothesized that the overexpression of CypA/WT, however, not CypA/P16S, may reveal a protective effect on oxidative stress-induced cell death. As hypothesized, the CypA/WT protected the cells from H_2O_2 or cisplatin-mediated cell death, while the CypA/P16S mutant was unable to protect the cells (Fig. 4).

In conclusion, the present study demonstrated that antioxidant activity was associated with CypA and was independent of PPIase activity. In addition, the antioxidant activity of CypA was required for the protective effects of CypA against H_2O_2 or cisplatin-mediated cell death. These findings may be useful for identifying a novel chemotherapeutic target in tumor cells, since cancer cells are usually resistant to oxidative stress, which is induced by anti-cancer drugs, such as Cisplatin (25).

Acknowledgements

This study was supported by grants from Kyung Hee University (Seoul, Republic of Korea) in 2012 (no. KHU-20121733) and the Basic Science Research Program through the National Research Foundation of Korea, funded by the Ministry of Education (no. NRF-2013R1A1A2060694).

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