Overexpressed Cyclophilin A in Cancer Cells Renders Resistance to Hypoxia- and Cisplatin-Induced Cell Death

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Abstract

Cyclophilin A (CypA) has been reported to be overexpressed in cancer cells, especially in solid tumors. To determine the role of CypA in tumorigenesis, we investigated the induction of CypA as well as the role it plays in cancer cells. Here, we have shown that induction of CypA is associated with hypoxia in a variety of cells, including DU145 human prostate cancer cell line. Our analysis of the CypA promoter clearly showed that CypA up-regulation is mediated by hypoxia-inducible factor-1α (HIF-1α) transcription factor. Interestingly, overexpression of CypA prevented hypoxia- and cisplatin-induced apoptosis, and this was associated with the suppression of reactive oxygen species generation and depolarization of mitochondrial membrane potential, whereas small interfering RNA-based CypA knockdown aggravated these factors. These results suggest that CypA is important in tumorigenesis, especially in tumor apoptosis. [Cancer Res 2007;67(8):3654–62]

Introduction

Cyclophilin A (CypA), the prototypical member of the cyclophilin family, is a highly conserved protein in mammalian cells (1). Traditionally, the cyclophilins are composed of four isoforms (cyclophilins A, B, C, and D). Sequence homology analysis of human CypA shows that it is highly homologous to human cyclophilin B (CypB), C (CypC), and D (CypD; ref. 2). Although CypB and CypC have the NH2-terminal sequences that target them to endoplasmic reticulum, CypA, an 18-kDa protein, is predominantly localized in the cytoplasm (2). It is known as an immunophilin and a cytosolic receptor for the immunosuppressive drug cyclosporin A (1). In addition, it possesses enzymatic peptidyl-prolyl cis-trans-isomerase (PPIase) activity, which is essential for protein folding in vivo. In addition, the PPIase activity of cyclophilins has recently been shown to be involved in diverse cellular processes, including intracellular protein trafficking (3, 4), mitochondrial function (5, 6), pre-mRNA splicing (7), and maintenance of multiprotein complex stability (3).

Although little is known about the function of CypA in cancer cells, it has been recently reported that CypA is overexpressed in many cancer cells, including human pancreatic cancer cells (8, 9), oral squamous cancer cells (10, 11) and non–small cell lung cancer (12, 13). Recent reports have shown that CypA interacts with the retinoblastoma susceptibility gene product (Rb) in Jurkat T cells (14). Although the role of CypA in apoptosis is not clear at this point, several reports show that CypA is released from cardiac myocytes in response to hypoxia/reoxygenation and may protect cardiac myocytes from oxidative stress–induced apoptosis (15), and that CypA also protects other cells from oxidative stress–induced apoptosis (16, 17). These reports suggest that CypA might be important for tumorigenesis in solid tumors.

To elucidate the role of overexpressed CypA in cancer cells, we examined CypA induction under hypoxic conditions and investigated the effect of CypA on hypoxia- or cisplatin-induced cell death. In this study, we show, for the first time, that CypA is transcriptionally induced by hypoxia-inducible factor-1α (HIF-1α) transcription factor. Furthermore, we show that overexpressed CypA in cancer cells protects cells against cellular stresses, including hypoxia and cisplatin treatment, at least in part as a result of its antioxidant function.

Materials and Methods

Cell culture and hypoxia. Human prostate carcinoma DU145, human cervical carcinoma HeLa, human colorectal carcinoma HCT116 p53+/− and p53−/− cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Mouse embryonic fibroblast NIH3T3, human hepatoblastoma HepG2 and human embryonic kidney HEK 293 cells were propagated in DMEM supplemented as above. Hypoxic conditions were generated by placing the cells in the hypoxia chamber (0.5% O2) for indicated periods of time.

Reverse transcription-PCR. For the analysis of CypA mRNA, total RNA was prepared from cells using the TRizol reagent (Invitrogen, San Diego, CA). Total RNA (2 μg) was amplified by a two-step protocol using the avian myeloblastosis virus reverse-transcriptase (Promega, Madison, WI) and Taq polymerase. Primers for amplifying CypA transcripts were as follows: forward, 5’-CGAGCTTACCATGGTCAACCCCACC-3’ and reverse, 5’-CCGGTACCGAGGTGTGTTCCACAGTGGCA-3’. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for reverse transcription-PCR (RT-PCR). Amplified products were separated on 1% agarose gel.

Construction of plasmids for CypA promoter analysis. The CypA promoter sequence was analyzed using MatInspector of Genomatix. Four hypoxia-responsive element (HRE) candidates were identified in the CypA promoter sequence (14). Although the role of CypA in apoptosis is not clear at this point, several reports show that CypA is released from cardiac myocytes in response to hypoxia/reoxygenation and may protect cardiac myocytes from oxidative stress–induced apoptosis (15), and that CypA also protects other cells from oxidative stress–induced apoptosis (16, 17). These reports suggest that CypA might be important for tumorigenesis in solid tumors.

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pGL3-CypA-4TM forward, 5'-GGGAGCCGCCGTTATACGTTTGCAAC-3'
pGL3-CypA-4TM reverse, 5'-GTCCGCAAAACCGTATAGCTGGCTTCCC-3'
pGL3-CypA-4TM forward, 5'-GCCCCCTTTATACGTCCCCGCCGCC-3'
pGL3-CypA-4TM reverse, 5'-GGGGGCCGCGCATGTCATCG-3'
pGL3-CypA-309M forward, 5'-AATCGACCCCTTATATACCGGCCGTCCTCG-3'
pGL3-CypA-309M reverse, 5'-ACCGGACGCGCGTGATAGGGAGGCTGATC-3'
pGL3-CypA-589M forward, 5'-GGGAGGCCGAGGCTAGTTTGCTCCG-3'
pGL3-CypA-589M reverse, 5'-GGGGCGGAACAACTATACCGGTCTCTANCA-3'

Luciferase assay. NIH3T3, DU145, and HeLa cells were transfected with 0.5 μg amounts of the pGL3 basic-derived plasmids, together with internal control plasmid pSV-luc (Promega). The cells were exposed to hypoxia for 12 h and harvested in 150 ml of lysis buffer [1% Triton X-100, 25 mM L-Gly-Gly, 15 mM MgSO4, and 2 mM L-EGTA (pH 8.0)]. Luciferase and β-gal activities (data not shown) were measured using 20 μl of each cell lysate using a microplate reader (Bio-Rad, Richmond, CA), and luciferase activity was normalized based on β-gal activity, as reported previously (18).

Preparation of nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared according to previous reports (19–21). Electrophoretic mobility shift assay was done with oligonucleotides ([CypA-WT, 5'-CCCTTTTATACACAGTGCCGGCCG-3'; CypA-M, 5'-GCCCTTTTATACACAGTGCCGGCCGCGC-3'] derived from the CypA promoter sequences. Sense and antisense strands of oligonucleotides were annealed into double-stranded oligonucleotides and were labeled with [γ-32P]-ATP (Amersham Biosciences, Buckinghamshire, United Kingdom). Nuclear extracts were obtained from DU145 and NIH3T3 cells after hypoxic incubation for 12 h. The nuclear extracts (10 μg) were incubated in the presence of 5 μl binding buffer [25% glycerol, 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM DTT, 1 mg/ml poly(deoxyinosinic-deoxyguanylic acid), 5 mM L-EDTA] for 30 min at room temperature. For a competition study, a 100-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture for 30 min before the addition of the radiolabeled probe. The samples were run on a 5% nondenaturing polyacrylamide gel. The gels were then dried and exposed to X-ray film with an intensifying screen at −80°C.

Hoechst staining. Cells were incubated for 30 min with Hoechst 33342 (Molecular Probes) loading dye and fixed for 20 min in 4% formaldehyde. After washing with ice-cold PBS thrice, stained cells were monitored using the confocal laser microscope (META 510, Zeiss, Thornwood, NY). Apoptotic cells were identified by nuclear condensation and fragmentation.

Stable transfection. pcDNA3-CypA/WT (3 μg) or pcDNA3 (3 μg) alone was transfected into DU145 cells using GenePORTER (Genlantis, San Diego, CA). Cells used for stable transfection were selected in the culture medium with 600 μg/ml G418 for a month. Then, drug-resistant individual colonies were isolated and transferred to a six-well plate for further amplification in the presence of selective medium.

Small interfering RNA. Small interfering RNAs (siRNA) specific to either CypA (CypA-siRNA) or control sequence (control-siRNA) were prepared by Eurogene tech (Intron Biotechnology, Kyungki, Korea). siRNA (0.5 μg) was transfected into cells using META FECTENE transfection reagent (Biontex, Munich, Germany). siRNA target sequences were as follows: CypA-siRNA (sense, 5'-UGACUUCACACGCCAUAAUdTdT-3'); antisense, 5'-AUUAUGGCGGCCGAAATAUGTT-3') and control-siRNA (universal negative control). The efficiency of siRNA-based interference of CypA was monitored by immunoblot.

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay in a 12-well plate. The culture medium was replaced with 1 ml medium containing 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO) and incubated for 60 min at 37°C. The blue-colored tetrazolium crystals resulting from mitochondrial enzymatic activity on the MTT substrate were solubilized with 150 μl DMSO. The absorbance was read at 595 nm in a microplate reader (Bio-Rad). Cell survival was expressed as the percentage of absorbance relative to that of untreated cells.

Cell cycle analysis. Cells were grown to 60% confluence and treated with 2 mM/L thymidine for up to 24 h to arrest the cells in the G1 phase. The cells were washed in PBS and released in fresh medium for specified periods of time. For cell cycle analysis, cells were harvested, rinsed with PBS, and fixed in 70% ethanol overnight at 4°C. The fixed cells were centrifuged and resuspended in 1 ml of PBS containing 0.05 mg/ml propidium iodide and 0.2 mg/ml RNase A and then incubated at 4°C for 1 h. DNA content was determined by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) to analyze the cell cycle.

Analysis of ROS and mitochondrial membrane potential (ΔΨm) measurement. Reactive oxygen species (ROS) were measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probe) dye. Human prostate carcinoma DU145 cells were grown to 80% confluence in complete media and treated with and without cisplatin (30 μmol/L). Then, the cells were loaded with 10 μmol/L DCF-DA at 37°C for 30 min and resuspended in 1 ml of PBS. Fluorescence was measured by a flow cytometer (FACSCalibur, Becton Dickinson). The mean DCF fluorescence intensity was measured with excitation at 488 nm and emission at 525 nm. Untreated cells were used as a reference for ROS levels. The mitochondrial membrane potential was measured using 3, 3-diethoxyxacarbocyanine iodide dye (DiOC6; Molecular Probes, Eugene, OR). After treatment with cisplatin (30 μmol/L), cells were loaded with 40 μmol/L DiOC6 for 40 min at 37°C and resuspended in 1 ml of PBS before the flow-cytometric analysis. DiOC6 was excited at 488 nm, and fluorescence was analyzed at 525 nm.

Subcellular fractionation. Cells were suspended in lysis buffer [250 mM/L sucrose, 0.1 mM/L EDTA, 2 mM/L HEPES (pH 7.4)], homogenized, and centrifuged at 900 × g for 10 min. Then, the supernatants were recentrifuged at 10,000 × g for 20 min. The resulting supernatants (cytosol) were subjected to 15% SDS-PAGE and analyzed by immunoblotting with anti–cytochrome c oxidase subunit IV (COX4) antibody (Clontech, Palo Alto, CA) to confirm that the cytosolic fraction was successfully separated from the mitochondrial fraction (data not shown).

Figure 1. CypA is transcriptionally up-regulated by hypoxia. A to C, RT-PCR analysis. Total RNA was extracted from NIH3T3 cells exposed to hypoxia (A) or treated with 100 μmol/L CoCl2 (B) for the indicated periods of time and subjected to RT-PCR analysis of CypA and GAPDH mRNA. C, cells were exposed to hypoxic conditions for 9 h and then incubated with actinomycin D (5 μg/ml) for 0, 1, 3, 6, 9, and 12 h under normoxic or hypoxic conditions. GAPDH was used as a loading control, D, immunoblotting. Total cell lysates (10 μg) extracted after exposure to hypoxia for the indicated periods of time were subjected to immunoblotting analysis.
Recombinant adenovirus. CypA/WT cDNA was cloned into pCA14. For homologous recombination, pCA14-CypA was transformed into Escherichia coli BJ S183 along with vmRL-H5dl 324Bst. Recombinant adenovirus was propagated in HEK 293 cells. The viral DNA constructs were confirmed by HindIII restriction enzyme digestion (data not shown). Adenovirus stocks were purified through a cesium gradient for 4 h (444,000 × g, 1°C) and titrated using a standard plaque assay. Cells were infected with recombinant adenovirus at a multiplicity of infection (MOI) of 300 for 24 h to express exogeneous CypA. Uninfected or infected cells with adenovirus-GFP (Ad) were used as a negative control for CypA expression.

Immunoblotting. Cell extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with the indicated primary antibody, followed by incubation with a secondary antibody. Samples were detected with enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Santa Cruz, CA). Unless specified, actin protein was immunoblotted to standardize the amount of sample proteins for the immunoblot analysis. Antibodies against CypA, PARP, cytochrome c, or actin were obtained from Santa Cruz Biotechnology and Upstate Biotechnology (Lake Placid, NY).

Statistical analysis. Results were expressed as means ± SE from at least three independent experiments. Statistical analysis was done by using Student’s t test. Unless otherwise indicated, P < 0.05 was deemed significant.

Results

CypA is transcriptionally up-regulated in response to hypoxia. To investigate whether the CypA gene can be induced
An increase in CypA mRNA was also observed after 12 h of CoCl2 treatment of NIH3T3 fibroblasts exposed to hypoxia for indicated periods of time. CypA transcripts increased after 3 h incubation and kept increasing up to 12 h under hypoxic conditions, whereas GAPDH mRNA used as a control was unchanged (Fig. 1A). To confirm this result, we repeated the RT-PCR analysis using a drug that mimics hypoxia, CoCl2, instead of incubation under hypoxic conditions. An increase in CypA mRNA was also observed after 12 h of CoCl2 treatment (Fig. 1B). We assumed that the increase in gene expression of CypA occurred at the transcriptional level by increasing the transcriptional rate or at the post-transcriptional level by stabilizing mRNA. To rule out the possibility of stabilizing mRNA, the level of CypA mRNA in hypoxia was compared with that in normoxia by induction of CypA mRNA in hypoxia for 9 h and subsequent treatment with actinomycin D (5 μg/mL) up to 12 h under hypoxic or normoxic conditions. Actinomycin D was used to inhibit the de novo synthesis of the CypA transcript. As shown in Fig. 1C, the half-life of CypA mRNA turned out to be similar in both conditions. These results showed that transcriptional activation, not mRNA stabilization, causes induction of CypA under hypoxic conditions. To determine whether accumulation of CypA mRNA would lead to an elevated level of CypA protein, immunoblotting analysis was done using total cell lysate from NIH3T3 fibroblasts subjected to hypoxic conditions for the specified periods of time. As shown in Fig. 1D, a rapid increase in the CypA protein level was detected after 3 h exposure to hypoxia and kept continuously increasing up to 12 h exposure, whereas the actin protein level, used as a control, was constant.

**CypA promoter is activated by the HIF-1α transcription factor.** Up-regulation of CypA by hypoxia led us to explore whether the CypA promoter has a HRE, the HIF-1α binding site. First, bioinformatic analysis of the promoter of the human CypA gene was done to search for a consensus HRE sequence 5'-CGTG-3'. We identified four putative HRE sequences located at -47 bp (HRE1), -77 bp (HRE2), -309 bp (HRE3), and -589 bp (HRE4) upstream to the CypA start codon (Fig. 2A).

To identify which HRE was responsive to hypoxia, several luciferase reporter constructs, containing various regions of the 1,012-bp CypA promoter, were constructed (Fig. 2A). The activity of the CypA promoter was monitored by luciferase assay after 12 h incubation under hypoxic conditions. Neither under normoxic nor hypoxic conditions was any luciferase activity observed for the empty pGL3-Basic plasmid. However, for pGL3-CypA-200 and pGL3-CypA-1012, there was an almost 2-fold increase in luciferase activity under hypoxic conditions compared with normoxic conditions (Fig. 2B). Therefore, this suggested that pGL3-CypA-200 and pGL3-CypA-1012 showed hypoxia-induced luciferase activity because they contain the HRE1 and HRE2 sites. Further deletion analysis of the CypA promoter showed that any luciferase construct without the HRE2 site did not respond to hypoxic conditions (data not shown). To confirm that the HRE2 site is responsible for the induction of hypoxia, we mutated each HRE site (5'-CGTG-3') to 5'-TATA-3' on the CypA promoter. pGL3-CypA-47M, pGL3-CypA-309M, and pGL3-CypA-589M showed about a 2-fold increase in luciferase activity in response to hypoxia compared with normoxic conditions. By contrast, the luciferase activity of pGL3-CypA-77M containing the mutated HRE2 was not induced by hypoxia, indicating that the HRE2 site is responsible for the induction of CypA by hypoxia. HIF-1α is known to be a major transcription factor in hypoxia (22). Thus, we overexpressed HIF-1α by transfection of pcDNA3-HIF-1α and compared the luciferase activity of the transfectants in normoxia with that of controls to determine whether HIF-1α induces the transcription of CypA. As in the hypoxia experiments, all luciferase constructs containing a functional HRE2 showed about a 2-fold increase in luciferase activity when pcDNA3-HIF-1α was cotransfected (Fig. 2B). Interestingly, pGL3-CypA-200 displayed a low luciferase activity when pcDNA3-HIF-1α was cotransfected (Fig. 2B). Figure 3. Effects of CypA on cell death induced by hypoxia, cisplatin, or H2O2 treatment. A, expression level of CypA. CypA expression level was monitored by immunoblotting in DU145 cells transfected with mock, pcDNA3-CypA/WT, and CypA-siRNA. B, MTT assay. Each transfectant was exposed to hypoxia for 4 d, treated with 120 μmol/L cisplatin for 48 h or 400 μmol/L H2O2 for 24 h. Columns, means of results from three independent experiments; bars, SE. *P < 0.05 compared with Con-siRNA. C, ROS measurement. Each transfectant was treated with (+) or without (-) 30 μmol/L cisplatin for 24 h. Then, ROS was measured by flow cytometry after loading with DCF-DA. D, measurement of mitochondrial membrane potential. Each transfectant was treated with (+) or without (-) 30 μmol/L cisplatin for 48 h. Then, the cells were incubated with DiOC6 and subjected to flow-cytometric analysis. Arrows, mitochondrial membrane potential depolarized cells.
under normoxic conditions, compared with other CypA luciferase constructs (Fig. 2B). In addition, pGL3-CypA-200 showed a weaker basal luciferase activity, compared with pGL3-CypA-1012 in the HIF-1α transfection experiment, indicating that other cis-acting elements required for the general expression of CypA are located upstream of HRE2. We finally located a putative GC box at \(-317\) bp from the starting codon of CypA (data not shown).

Next, because CypA has been reported to be expressed more in tumor than in normal cells (8–13), the luciferase activity of pGL3-CypA-1012 was measured in cancer cells, such as DU145 and HeLa cells. As shown in Fig. 2C, the luciferase activity was increased by 3.5- and 3-fold in these cancer cells by hypoxia and HIF-1α transfection, respectively. Taken together, these data suggest that HIF-1α regulates CypA expression in response to hypoxia.

**Binding of HIF-1α to the HRE sequence of CypA promoter.** To determine if HIF-1α binds to the HRE2 sequence of the CypA promoter, EMSA experiments were done using nuclear extracts from DU145 cells incubated for 12 h under hypoxic conditions. Oligonucleotides containing wild-type (WT) or mutated version of HRE2 (see Materials and Methods) were used in the experiments. First, we tested whether HIF-1α binds to the HRE2 sequences of the CypA promoter under normoxic conditions. \(^{32}\)P-labeled oligonucleotides containing WT HRE2 were complexed with nuclear protein extracts from DU145 cells cultured under normoxic conditions, whereas the \(^{32}\)P-labeled oligonucleotides containing mutated HRE2 were not (Fig. 2D, lanes 1 and 2). The complex was more easily observed when cultured under conditions of hypoxia, whereas oligonucleotides containing mutated HRE2 did not form complexes (Fig. 2D, lanes 4 and 5). The specificity of this complex was verified by competitive inhibition with an excess of the same unlabeled oligonucleotide (Fig. 2D, lanes 3 and 6). We verified the results by reproducing consistent EMSA data with nuclear extracts from NIH3T3 (data not shown). Therefore, we concluded that up-regulation of CypA is indeed mediated by HIF-1α in response to hypoxia.

**CypA desensitizes cells to hypoxia- and cisplatin-induced cell death by suppression of ROS increase and loss of mitochondrial membrane potential.** HIF-1α has been known to play an important role in growth and survival of solid tumor cells (23, 24). We have shown that CypA is induced under hypoxic conditions via the HIF-1α transcription factor. Therefore, we hypothesized that CypA might play an important role in growth and survival of solid tumor cells, especially in response to cellular stress including hypoxia, cisplatin, and oxidative stress such as H\(_2\)O\(_2\) treatment.

First, to assess the role of CypA in hypoxia-induced cell death, we did a MTT assay after DU145 cells were stably transfected with mock vector, pcDNA3-CypA/WT or transiently transfected with CypA-siRNA and were exposed to hypoxia for up to 4 days. Before the hypoxic experiments, the expression level of exogenous CypA was evaluated in the transfected cells by immunoblotting (Fig. 3A). Compared with endogenous CypA, exogenous CypA showed an almost 2-fold increase in expression. In addition, the expression of CypA was almost 95% suppressed by specific siRNA interference (Fig. 3A). As presented in Fig. 3B, CypA transfectants exposed to hypoxia for 4 days showed significantly higher cell survival rates than mock transfectants; CypA knockdown showed the highest cell death rate among the transfectants, indicating that CypA protects cells from hypoxia-induced cell death.

Next, we monitored the chemoresistance of CypA transfectants and CypA knockdown to cisplatin by MTT assay. Cisplatin gave rise to cellular death, but CypA transfectants showed a higher cell survival rate than mock transfectants. By contrast, CypA knockdown cells showed significantly lower cell survival rates than control siRNA transfectants (Fig. 3B). Cisplatin is known to induce apoptosis partly through the generation of ROS (25–27). To determine if overexpression of CypA attenuates apoptosis by suppression of increased ROS generation, we tested cellular resistance to H\(_2\)O\(_2\) treatment in both CypA transfectants and CypA knockdown samples. As shown in Fig. 3B, H\(_2\)O\(_2\) caused cellular death, but CypA transfectants showed a higher cell survival rate than mock transfectants. By contrast, CypA knockdown samples showed...
a significantly lower cell survival rate than control siRNA transfectants. Therefore, we assessed for antioxidant activity of CypA in each transfected cell line. As clearly shown in Fig. 3C, treatment with cisplatin gave rise to the generation of ROS. Interestingly, CypA overexpression did not increase ROS significantly, whereas CypA knockdown aggravated ROS generation. In addition, mitochondrial membrane potentials were also analyzed with or without cisplatin treatment. As presented in Fig. 3D, CypA knockdown showed the most significant cisplatin-induced mitochondrial membrane depolarization, followed by mock-transfected samples. CypA-overexpressed cells led to a negligible cisplatin-induced depolarization of mitochondrial membrane potential. These data show that CypA overexpression suppresses cisplatin-induced ROS increase and the loss of mitochondrial membrane potential. Next, we monitored cellular ROS and mitochondrial membrane potential in CypA transfectants and CypA knockdown samples under hypoxic and H2O2 treatment conditions. As in cisplatin-induced apoptosis, hypoxia and H2O2 treatment induced a similar pattern of changes in ROS generation and depolarization of mitochondrial membrane in these transfected cells (data not shown). Therefore, our findings show that CypA protects cells from stress-induced cell death at least in part as a result of its antioxidant role.

**Cytoprotective roles of CypA against apoptosis.** The effects of hypoxia, cisplatin, and H2O2 treatment on the processing of apoptosis-related protein PARP and cytochrome c release from mitochondria to cytoplasm in CypA transfectants and CypA knockdown were investigated using immunoblotting. Incubation of cells under hypoxic conditions or treatment with cisplatin or H2O2 showed cleavage of the caspase-3 substrate, PARP, as indicated by the appearance of the 85-kDa cleaved PARP product (Fig. 4A and B). Accordingly, the extent of cytochrome c release was also increased with cellular stress. As expected, overexpression of CypA reduced the processing of PARP and cytochrome c release (Fig. 4A). By contrast, CypA knockdown increased the processing of PARP and cytosolic release of cytochrome c significantly, compared with control-siRNA (Fig. 4B). In addition, suppression of CypA in cisplatin-induced apoptosis was verified by Hoechst staining (Fig. 4C). These data show that CypA overexpression desensitizes responses of apoptosis-related proteins to hypoxia, cisplatin, and H2O2, suggesting that overexpressed CypA in cancer cells protects cells by attenuating stress-induced apoptosis.

![Figure 5](https://www.aacrjournals.org/3659) Cytoprotective role of CypA against cell death is not limited to a cell type. A, expression level of CypA. CypA expression level was analyzed via immunoblotting after the establishment of adenovirus CypA/WT infectants (Ad-CypA/WT) or CypA knockdown (CypA-siRNA) in the indicated cell lines. B to D, MTT assays in HepG2 (B), HeLa (C), or HCT116 p53**+/+** and HCT116 p53**−/−** (D) cells. The established cell lines were exposed to hypoxia for 4 d and treated with 120 μmol/L cisplatin for 48 h or 400 μmol/L H2O2 for 24 h. All these experiments were repeated at least thrice. Columns, means of results from three independent experiments; bars, SE. *, P < 0.05 compared with Ad. #, P < 0.05 compared with Con-siRNA (B and C). †, P < 0.05 compared with p53**+/+**; CypA**+,** P < 0.05 compared with p53**−/−**; CypA**−/−**(D).
Cytoprotective role of CypA against cell death is not limited to a cell type. Next, to determine whether our findings are limited to human prostate DU145 cancer cells, we also tested for a suppressive role of CypA in hypoxia, cisplatin-, or \( \text{H}_2\text{O}_2 \)-induced apoptosis in HepG2 and HeLa cells. First, after establishing a CypA adenovirus or CypA knockdown in HepG2 and HeLa cells, the protein expression level of CypA was tested. In CypA infectants, exogenous CypA was expressed almost 1.5-fold more than endogenous CypA (Fig. 5A). As expected, siRNA-based CypA inhibition also suppressed CypA expression effectively both in HepG2 and HeLa cells. As shown in Fig. 4B and C, CypA infectants showed a reduced cell death compared with mock samples, whereas CypA knockdown samples gave rise to increased cell death, when subjected to hypoxia, cisplatin, or \( \text{H}_2\text{O}_2 \) treatment. These results clearly indicate that the ability of CypA to suppress apoptosis is not limited to specific cells.

We also investigated whether CypA knockdown aggravates apoptosis in p53-defective cells because p53 is one of the key regulators of apoptosis. For this study, we successfully suppressed CypA by siRNA inhibition in HCT116 p53+/+ and HCT116 p53−/− cell lines (Fig. 5A). The resistance of CypA knockdown to cellular stress was monitored in HCT116 p53+/+ and HCT116 p53−/− backgrounds. In both HCT116 p53+/+ and HCT116 p53−/− cells, CypA knockdown gave rise to increased cell death, compared with control-siRNA, when subjected to hypoxia, cisplatin, or \( \text{H}_2\text{O}_2 \) treatment. Therefore, we concluded that CypA knockdown can significantly aggravate apoptosis even without p53 (Fig. 5D).

**CypA does not affect cellular proliferation.** Recently, it has been proposed that CypA might enhance tumor growth by affecting cellular proliferation (12). To examine this hypothesis in our cell lines, we monitored the effects of CypA on cell cycle progression and on the doubling time of cells. Mock, CypA transfectant, and CypA knockdown were grown, synchronized by 2 mmol/L thymidine, and released in fresh medium for the specified periods of time and then subjected to fluorescence-activated cell sorting (FACS) analysis to determine DNA content. As presented in Fig. 6A, normal cell cycle progression was observed in each cell line. We could not see any difference in DNA content among mock, CypA transfectants, and CypA knockdown over time up to 16 h. In addition, there was no difference in the doubling time (Fig. 6B). Their doubling time was about 15 h. These data suggest that the cellular function of CypA may be related to apoptosis, rather than cellular proliferation, in our tested cell lines.

**Discussion**

To date, the role of CypA in cancer pathogenesis has not been extensively investigated, although some recent reports have shown CypA overexpression in many cancer cells (8–13). We first investigated whether CypA could be induced by HIF-1α under hypoxic conditions, a microenvironment in solid tumors. We also investigated the role of overexpressed CypA in cancer cells. In this paper, we showed that transcription of the CypA was induced by hypoxic conditions through a mechanism that involves binding to the HRE2 site located at −77 within the CypA promoter. We reproduced these results using a variety of cell lines, including DU145 and HeLa cells. Therefore, induction of CypA in response to hypoxia is not limited to a specific cell line. Interestingly, luciferase activity of pGL3-CypA-200 was lower than that of pGL3-CypA-1012 (Fig. 2). This result suggests that the promoter region from −1,012 to −201 might have other cis-acting elements required for general transcriptional induction. In fact, we found a putative GC box (−317 bp) in the region (data not shown). Because a functional GC box is a positive regulatory element in the promoter, the putative GC box is possibly responsible for the high constitutive expression of CypA gene.

Cancer cells are more resistant to stress than normal non-transformed cells. They use multiple defense systems against a variety of stresses, including hypoxia, radiation, and anticancer drugs. Under hypoxic conditions, cancer cells can survive by inducing HIF-1α expression (10, 22). Therefore, HIF-1α has been suggested as a potential regulator of hypoxia-induced apoptotic cell death (23). Induction of apoptosis by hypoxia in HIF-1α
transfected cells has been shown to be more difficult than in cells that do not express HIF-1α. This led us to speculate that the resistance of HIF-1α transfected cells to hypoxia-induced cell death may be, at least in part, due to the ability of HIF-1α to up-regulate CypA expression in cells; this was because our data showed that hypoxia induced expression of CypA through direct activation of HIF-1α, and that the induced CypA seemed to resist cellular stresses, such as hypoxia insult, cisplatin treatment, and oxidative stress. In addition, our results also showed that CypA knockdown aggravated cell death induced by hypoxia, cisplatin treatment, and oxidative stress in the tested cell lines. Because a cytoprotective role of CypA was also observed even in p53-defective cells, CypA may be a good target for new chemotherapeutic drug development to alleviate cisplatin resistance frequently observed in p53-defective cancer patients.

Interestingly, overexpression of CypA did not offer any resistance to other anticancer drugs such as paclitaxel and 5-fluorouracil (data not shown). Considering that apoptosis induced by these drugs is not directly related to the increased ROS, although ROS are generated after treatment with these drugs (28, 29), we thought that the suppression of the increased ROS generation may be involved, at least in part, in the cytoprotection of overexpressed CypA against cellular stresses, such as hypoxia and cisplatin treatment; this hypothesis turned out to be true in this case (Fig. 3C and D). Accordingly, evidence that CypA functions as an antioxidant has been recently accumulated (16, 30–32). Our previous study also showed that overexpressed CypA removed ROS generated by cyclosporin A (17). Moreover, CypA has been reported to be secreted in response to ROS in vascular smooth muscle cells (33). Therefore, it seems that the antioxidant role of CypA is at least partly critical for cytoprotection of overexpressed CypA against hypoxia and cisplatin treatment. We propose here that the antioxidant function of CypA might require PPlase activity because the CypA R55A mutation, which is defective in PPlase activity, also aggravated ROS generation and depolarization of the mitochondrial membrane (data not shown). Consistent with our proposal, cyclosporin A, a well-known inhibitor of PPlase activity in cancer, has been reported to have chemotherapeutic effects in a variety of cancer cells (34, 35). The molecular mechanism of CypA as an antioxidant should be pursued in future studies.

Recently, Howard et al. reported on the importance of CypA to tumor growth in vivo, specifically on proliferation and apoptosis (12). We also investigated the possibility that CypA may influence proliferation by monitoring cell cycle progression and doubling time of CypA transfectants and CypA knockdown samples. We found no difference in cellular proliferation among mock, CypA transfectants, and CypA knockdown samples. Our data are consistent with a recent report showing that loss of CypA in mouse embryonic stem cells had no detectable effect on cell growth or viability (36). The discrepancy between our findings and those of Howard et al. (12) concerning the involvement of CypA in proliferation is not understood at this time. Therefore, we cannot rule out the possibility that CypA might affect cellular proliferation in specific cell lines.

In conclusion, our data suggest that CypA may play a critical role in tumorigenesis by reducing apoptosis both under hypoxic conditions and with cisplatin treatment. The cytoprotective role of CypA seems to require PPlase activity. Therefore, the PPlase activity of CypA may be a good chemotherapeutic target for treatments that could facilitate to alleviate the chemoresistance in tumor cells including p53-defective tumors. We continue to study CypA to further understand its cytoprotective mechanisms, which will hopefully lead to new chemotherapeutic strategies against solid tumors.

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References

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