Mitochondrial Targeted Cyclophilin D Protects Cells from Cell Death by Peptidyl Prolyl Isomerization*

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Cyclophilin D (CyPD) is thought to sensitize opening of the mitochondrial permeability transition pore (mPTP) based on the findings that cyclosporin A (CsA), a pseudo-CyPD substrate, hyperpolarizes the mitochondrial membrane potential (ΔΨ) and inhibits apoptosis. We provide evidence that contrasts with this model. Using live cell imaging and two photon microscopy, we report that overexpression of CyPD desensitizes HEK293 and rat gliona C6 cells to apoptotic stimuli. By site-directed mutagenesis of CyPD that compromises peptidyl-prolyl cis-trans isomerase (PPIase) activity, we demonstrate that the mechanism involved in this protective effect requires PPIase activity. Furthermore, we show that, under resting conditions, ΔΨ is hyperpolarized in CyPD wild type-overexpressing mutant forms of CyPD that lack PPIase activity. Finally, in glutathione S-transferase (GST) pull-down assays, we demonstrate that CyPD binding to the adenine nucleotide translocator (ANT), which is considered to be the core component of the mPTP, is not affected by the loss of PPIase activity. Collectively, our data suggest that CyPD should be viewed as a cell survival-signaling molecule and indicate a protective role of CyPD against apoptosis that is mediated by one or more targets other than the ANT.

In multicellular organisms, programmed cell death (apoptosis) is an essential process of normal development, tissue maintenance, and aging (1, 2). Abnormal regulation of apoptosis results in multiple human diseases, including cancer, AIDS (3), and many neurological disorders (2, 4). Mitochondria provide a key regulatory role in cell death by releasing apoptogenic proteins into the cytosol that initiate the caspase cascade (5–8). Release of these factors is thought to occur via a tightly regulated increase in the permeability of the inner mitochondrial membrane, due to the opening of the mitochondrial permeability transition pore (mPTP)1 (9–12). The primary components of the mPTP include the voltage-dependent anion channel (VDAC) in the outer membrane, ANT in the inner membrane, and CyPD within the mitochondrial matrix (10, 13). Bcl-2 family members can regulate the release of apoptotic factors from mitochondria through direct interaction with VDAC and regulation of ANT activity during apoptosis (14–16).

Cyclophilins are a group of PPIases with highly conserved protein sequences (17). These proteins are thought to be important for protein folding (18). CyPD is a mitochondrial-targeted PPIase (19). Although its specific physiological function remains largely unknown, CyPD is considered critical for the opening of the mPTP (20, 21). This view is based on the observations that CsA, a potent inhibitor of mitochondrial-mediated apoptosis (9), blocks the mPTP at concentrations similar to those needed to inhibit the enzymatic activity of CyPD. This suggested that the PPIase activity was a necessary step in the opening of the mPTP (22, 23). Cyclophilins bind CsA via a conserved hydrophobic pocket, which is critical for PPIase activity (24–26). Thus, early models of CsA inhibition of the permeability transition suggested that CsA acted as a pseudo-substrate of CyPD that prevented it from interacting with the mPTP. However, the addition of CsA was shown not to disrupt the binding of CyPD and ANT (27). In addition, Scorrano et al. (28) reported that CsA inhibited mPTP opening, whereas diethylpyrocarbonate (DPC) induced mPTP opening. Because both CsA and DPC inhibit PPIase activity, the authors argued that CyPD must regulate the mPTP independently of this enzymatic activity.

In this manuscript, we examine the role of CyPD in the regulation of cell death. Overexpression of CyPD was used to ascertain its physiological function in response to oxidative stress and staurosperin-induced cell death. Two-photon imaging was employed to reduce photo-bleaching of fluorophores and photo-toxicity in live cells, which was important for the prolonged imaging periods in our experiments. The collapse of ΔΨ and the loss of membrane integrity were monitored to assess the initiation of cell death (29, 30). In contrast to the current view of CyPD acting as a key accessory protein that sensitizes the mPTP to opening, we demonstrate that overexpression of CyPD delays both the onset of ΔΨ collapse under oxidative stress and the loss of membrane integrity induced by staurosperin. The protective effect of CyPD was dependent on its PPIase activity as demonstrated by site-directed mutagenesis. Furthermore, under resting conditions, the ΔΨ was significantly higher in cells overexpressing CyPD. Taken together, these data suggest that CyPD is an important factor with functional consequences in the regulation of programmed cell death.

DPC, diethylpyrocarbonate; FCS2, Foch Chamber System 2; PI, propidium iodide; ANOVA, analysis of variance.

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1 The abbreviations used are: mPTP, mitochondrial permeability transition pore; ANT, adenine nucleotide translocator; CsA, cyclosporin A; CyFD, cyclophilin D; DIC, differential interference contrast; ETFF, enhanced yellow fluorescent protein; GST, glutathione S-transferase; PPIase, peptidyl-prolyl cis-trans isomerase; t-BuOOH, tert-butyl hydroperoxide; TMRE, tetramethyl rhodamine ethyl ester; VDAC, voltage-dependent anion channel; ΔΨ, mitochondrial membrane potential.

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MATERIALS AND METHODS

Construction of Expression Vectors—The coding fragment of rat CyPD cDNA (gift of Dr. Halestrap, University of Bristol, UK) was amplified using a forward primer, 5′-gggatccagtctagctggctggc-3′, containing a BamHI site and a reverse primer, 5′-agttcgaggtgagctgttggatc-3′, containing an XbaI site. The PCR product was subcloned into the Xenopus expression vector pGEM-HN between the BamHI and XbaI sites (31). The CyPD coding region was subsequently subcloned into pcDNA3.1/zeo+ (+) (Invitrogen, Carlsbad, CA) between the BamHI and XbaI sites. All restriction enzymes are from Life Technologies (Rockville, MD). CyPD R96G and H167Q mutants were generated by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) using plasmid pGEM-HN-CyPD as template. The forward primer for the R96G mutant was 5′-cttctcgagatgcatctggtggc-3′ and the reverse primer was 5′-gagctggtgctggatc-3′. The forward primer for the H167Q was 5′-ggctggatggcaaggagcgaccagtggcag-3′ and the reverse complement primer was 5′-cagccaaacacaagtctggagaac-3′. Both mutants were subsequently subcloned into pcDNA3.1/zeo+ between the BamHI and XbaI sites.

To generate the GST-CyPD fusion vector, the CyPD fragment was subcloned into pGEX-4T-2 (Amersham Biosciences, Piscataway, NJ) between BamHI and NotI sites from pGEM-HN-CyPD vector. The CyPD-enhanced yellow fluorescent protein (EYFP) fusion fragment was engineered by a two-stage PCR strategy. First, full-length coding regions of CyPD and EYFP were amplified in two separate reactions. The 3′ primer for CyPD had 21 bp of the 5′ of EYFP, and the 5′ primer for EYFP contained 20 bp of the 3′ of CyPD. These complementary sequences were annealed in the second PCR stage to generate the CyPD plus EYFP fusion. The CyPD primer was 5′-cttctcgagatgcatctggtggc-3′ and the reverse primer contained the forward CyPD primers. A BamHI site was attached to the forward primer of CyPD, and an XbaI site was added to the reverse primer of EYFP. The fusion PCR fragment was then subcloned into pcDNA3.1/zeo+ between BamHI and XbaI sites. The CyPD and EYFP bicistronic expression vector was generated by digesting pGEM-HN-CyPD vector with Smal and NotI sites. The CyPD fragment was subsequently subcloned into pIRE2-EYFP (CLONTECH, Palo Alto, CA) between EcoRV and NotI sites.

The rat ANT1 cDNA (gift of Dr. Y. Shinozawa, University of Tokushima, Japan) was subcloned into the pGEM-HN vector by PCR with a forward primer, 5′-agtctcgagatgcatctggtggc-3′ and a reverse primer, 5′-ggctggatggcaagggacg-3′. An Smal site was attached to the 5′-end of the forward primer and an EcoRI site was attached to the 3′-end of the reverse primer. The PCR fragment was subcloned into pGEM-HN between Smal and EcoRI sites.

All vectors were automatically sequenced by the University of Texas Health Science Center at San Antonio sequencing core facility to demonstrate correct engineering. The oligonucleotides used were purchased from Operon Technologies (Alameda, CA).

Cell Culture and the Generation of Stable Cell Lines—Cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen, Rockville, MD) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 200 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂/95% air. Cells were subcultured 1:10 by incubating at 0.05% Trypsin, 0.53 mM EDTA (Invitrogen, Rockville, MD) for 5 min when they were 70% confluent. DNA was transfected into cells by LipofectAMINE reagent (Invitrogen, Rockville, MD) according to the instructions of the manufacturer. After 72 h of transfection, HKE293 cells were trypsinized and transferred from a six-well dish to a 100-mm dish with medium containing 200 μg/ml Zeocin (Invitrogen, Carlsbad, CA). Surviving cells were grown for 2–3 weeks, and symmetrical colonies were marked under a microscope. A grease boundary was drawn around marked colonies. A solution of 5 μl of 0.05% Trypsin and 0.53 mM EDTA was briefly applied to the colony, and the cell suspension was transferred to 96-well plates. 24 colonies were picked for each construct to examine their protein expression level.

Image Acquisition and Analysis—Cells transiently expressing CyPD-EYFP were transferred to a 35-mm dish with a coverslip bottom 24 h after transfection and allowed to re-attach for another 24 h. Five minutes prior to imaging, the growth medium was replaced with recording buffer (25 mM Hepes, pH 7.4 containing 30 mM potassium acetate, and 0.5 mM KCl) and kept at 37°C for 45 min. At the end of the 45-min incubation period, 2 mM magnesium ATP was added (final concentration) to prevent PPIase activity. PPIase activity was estimated according to the method of Kofron et al. (32). Briefly, purified GST-CyPD or GST (10 μM final concentration) was pre-equilibrated with buffer (50 mM Hepes, 100 mM NaCl, pH 8.0). Immediately before assay activity, chymotrypsin (60 mg/ml) was added to the reaction mixture 5 min to be removed by centrifugation. Citrate concentration was determined by the BCA assay kit (Pierce, Rockford, IL). For SDS-PAGE, 10 μg of total protein was loaded per lane. CyPD was detected with polyclonal rabbit anti-CyPD antibody (Pocono Rabbit Farm & Laboratory, Inc., Canadensis, PA), Horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and visualized by chemiluminescence (PerkinElmer Life Sciences, Boston, MA). Goat polyclonal anti-actin antibody sc-1615 and donkey anti-goat IgG sc-2304 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used to show the equal loading of proteins.

GST Fusion Protein Purification—BL21 (DE3) bacteria transformed with pGEX-4T-2-CyPD construct were grown until an 0.6 OD at 600 nm was reached. Isopropylthio-β-D-galactoside (Invitrogen, Rockville, MD) was added to a final concentration of 1 mM to induce GST-CyPD expression for 4 h at 37°C. Bacteria were harvested and lysed by sonication in 1× phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, 100 mM EDTA. Bacterial lysate was centrifuged at 22,000 × g, and the supernatant was collected. Binding of GST or GST fusion protein to glutathione-Sepharose 4B (Amersham Biosciences) was performed according to the method of Kofron et al. (32). The GST-CyPD was eluted with 5 mM reduced glutathione (1× GST elution buffer, 0.5 mM Tris-Cl, pH 8.0, 4 mM EDTA, 0.1% 2-mercaptoethanol, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride). Elution of bound protein was performed in elution buffer with 15 mM glutathione.

In Vitro Translation of ANT1 and Binding to GST Fusion Protein—Preparation of synthetic mRNAs and in vitro translations were performed as previously described (34, 35). Briefly, mRNAs were diluted to
FIG. 1. Overexpression of CyPD prolongs the time until △Ψ collapses following oxidative stress. A, localization of transiently transfected CyPD in HEK293 cells. A fusion protein of CyPD and EYFP (green, left panel) that has also been stained with the mitochondrial marker TMRE (red, middle panel). An overlay of the two images shows that CyPD fluorescence co-localizes with mitochondria (yellow, right panel). Scale bar, 5 μm. B, Western blot of CyPD protein expression levels in stable cell lines of HEK293. The upper panel shows expression for endogenous levels (HEK), transfection with vector alone (Vector), and overexpression of CyPD (CyPD). Actin levels were used to ensure equal loading of protein (lower panel). C, overlay of two-photon images of cells during perfusion with 100 μM t-BuOOH. The DIC image is shown in gray, and △Ψ is monitored with TMRE fluorescence (red). Loss of TMRE fluorescence indicates loss of △Ψ. The time after exposure to t-BuOOH is indicated in hours (h). Vertical scale bar, 10 μm. D, spatio-temporal patterns of △Ψ during exposure to t-BuOOH (100 μM). The loss of TMRE fluorescence (△Ψ collapse) is significantly delayed in cells overexpressing CyPD (bottom stack). Each stack is composed of 200 images that were acquired at 5-min intervals. The vertical scale bar represents 10 μm. E, histogram showing the average time of △Ψ collapse in the presence of t-BuOOH for control (Vect) and CyPD overexpressing cell lines (CyPD). The asterisk denotes statistical significant difference (p < 0.0005, t test).

RESULTS

Overexpression of CyPD Protects Cells from Oxidative Stress—CyPD is thought to be an accessory protein that controls the opening of the mPTP in response to apoptosis-inducing factors (36). Because CsA is known to bind CyPD and, thereby, reduce the probability of mPTP opening, we hypothesized that overexpression of CyPD would increase sensitivity of cells to oxidative stress. Our approach to test this hypothesis was 2-fold. First, we tested whether cells overexpressing CyPD exhibited the correct targeting of this protein to the mitochondria. For this purpose, we created a fusion protein consisting of CyPD tagged with EYFP at its COOH-terminal and then transiently transfected this construct into HEK293 cells. A cell expressing CyPD-EYFP is presented in Fig. 1A. When cells expressing this fusion protein were also labeled with the mitochondrial marker TMRE (Molecular Probes, Eugene, OR), we found that CyPD-EYFP was correctly targeted to the mitochondria (Fig. 1A). The second step in our experimental design was to create stable cell lines overexpressing wild type CyPD (non-tagged). A Western blot analysis of CyPD expression in one of these cell lines is presented in Fig. 1B. The physiological effects of CyPD overexpression were tested in this stable cell line and compared with a control cell line, expressing vector alone. Oxidative stress was induced by exposure to t-BuOOH and measured by monitoring the △Ψ (37). Briefly, cells were seeded on glass coverslips and allowed to adhere for 24 h. Plated cells were initially labeled with TMRE (50 nM) in a culture incubator for 5 min. The coverslip was then sealed in a closed perfusion chamber, and the cells were imaged on a two-photon confocal microscope for 12 h. Two-photon imaging was used to simultaneously excite the membrane potential indicator TMRE and to produce a DIC image. An overlay of both the DIC image and the TMRE-labeled mitochondria is presented in Fig. 1C. Throughout the course of the experiment, a complete z section (six images in 1-μm steps) of the cells was taken every 5 min. The cells were then monitored for △Ψ collapse, which was measured by the loss of TMRE fluorescence. Simultaneous imaging with DIC optics insured that a loss of fluorescence was not due to a change in the focal plane or to the loss of the cell. Spatial-temporal stacks were created with only a single image from each z section (Fig. 1D). The chosen image from each z section was selected on the basis of brightest TMRE fluorescence, which generally coincided with the middle image. These data reveal that cells overexpressing CyPD maintain their mitochondrial membrane potentials in the presence of t-BuOOH for significantly longer periods of time compared with cells expressing vector alone (p < 0.0005, t test). A histogram of the average time of △Ψ collapse is presented in Fig. 1E. The average time until the △Ψ collapsed for CyPD-overexpressing cells was 9.9 ± 0.3 h (n = 69 cells, pooled from four separate experiments). The average time until △Ψ collapsed was 6.2 ± 0.1 h in control cells expressing vector alone (n = 85 cells, pooled from five separate experiments). We conclude from these data that overexpression of CyPD significantly prolongs the time until △Ψ collapses. Thus, rather than sensitizing cells to oxidative stress, CyPD appears to significantly protect the cells.

CsA Inhibits the Collapse of △Ψ in Cells Exposed to Oxidative Stress—CsA is a well-established inhibitor of mPTP opening (38). Because the action of CsA on mPTP is thought to be mediated by CsA binding to CyPD, we examined how CsA exposure affected the collapse of △Ψ in response to oxidative stress. HEK293 cells were plated on glass coverslips, labeled with TMRE, sealed in the perfusion chamber, and imaged as described above. In addition, cells were pre-treated with CsA (10 μM) for 30 min and then continuously perfused with Dulbecco’s modified Eagle’s medium containing CsA (10 μM),

0.1 μg/μl and translated for 45 min in a rabbit reticulocyte lysate translation system (Promega, Madison, WI) supplemented with L-[35S]methionine (PerkinElmer Life Sciences, Boston, MA). Binding of in vitro translated ANT1 to GST-CyPD fusion proteins was performed in phosphate-buffered saline, pH 7.2, at room temperature for 1 h, followed by three washes in equilibration buffer containing 0.5 M NaCl, 1% Triton X-100. Proteins bound to glutathione-Sepharose were eluted in modified Eagle’s medium (39). Briefly, cells were seeded on glass coverslips and allowed to adhere for 24 h. Plated cells were initially labeled with TMRE (50 nM) in a culture incubator for 5 min. The coverslip was then sealed in a closed perfusion chamber, and the cells were imaged on a two-photon confocal microscope for 12 h. Two-photon imaging was used to simultaneously excite the membrane potential indicator TMRE and to produce a DIC image. An overlay of both the DIC image and the TMRE-labeled mitochondria is presented in Fig. 1C. Throughout the course of the experiment, a complete z section (six images in 1-μm steps) of the cells was taken every 5 min. The cells were then monitored for △Ψ collapse, which was measured by the loss of TMRE fluorescence. Simultaneous imaging with DIC optics insured that a loss of fluorescence was not due to a change in the focal plane or to the loss of the cell. Spatial-temporal stacks were created with only a single image from each z section (Fig. 1D). The chosen image from each z section was selected on the basis of brightest TMRE fluorescence, which generally coincided with the middle image. These data reveal that cells overexpressing CyPD maintain their mitochondrial membrane potentials in the presence of t-BuOOH for significantly longer periods of time compared with cells expressing vector alone (p < 0.0005, t test). A histogram of the average time of △Ψ collapse is presented in Fig. 1E. The average time until the △Ψ collapsed for CyPD-overexpressing cells was 9.9 ± 0.3 h (n = 69 cells, pooled from four separate experiments). The average time until △Ψ collapsed was 6.2 ± 0.1 h in control cells expressing vector alone (n = 85 cells, pooled from five separate experiments). We conclude from these data that overexpression of CyPD significantly prolongs the time until △Ψ collapses. Thus, rather than sensitizing cells to oxidative stress, CyPD appears to significantly protect the cells.
TMRE (50 nM), and t-BuOOH (100 μM). Under these experimental conditions, we found that the average time for ΔΨ to collapse in CsA treated cells was 20.5 ± 0.1 h (n = 52 cells, pooled from two experiments) (Fig. 2). In comparison, the average time to ΔΨ collapse in control cells (vehicle only) exposed to t-BuOOH was 6.2 ± 0.1 (n = 54, pooled from two experiments). Because it is known that CsA also has non-mitochondrial targets that can affect cell viability (39), we also tested how the immunosuppressant FK506 affected the collapse of ΔΨ in response to oxidative stress. FK506 is an immunosuppressant that, like CsA, prevents activation of calcineurin. However, FK506 has no effect on mitochondria (40). Pre-treatment of cells with FK506 (1 μM, 30 min) had no protective effect on the collapse of ΔΨ in cells exposed to t-BuOOH (Fig. 2). The average time to ΔΨ collapse was 6.0 ± 0.1 h (n = 58 cells, pooled from two experiments). We conclude from these data that CsA inhibits the collapse of ΔΨ when cells are exposed to oxidative stress. However, the effects of CsA are unlikely to be mediated by calcineurin inhibition, because treatment of cells with FK506 did not affect the collapse of ΔΨ.

The Protective Effect of CyPD on ΔΨ is Independent of Cell-type—To determine whether the protective effect of CyPD on ΔΨ collapse was dependent on the cell-type, we re-examined the effects of CyPD overexpression in another cell line. Rat C6 glioma cells were transiently transfected with a bicistronic vector for independent expression of CyPD and EYFP (see “Materials and Methods”). Cells were allowed to express the proteins for 48 h, and positively transfected cells were identified by EYFP fluorescence (Fig. 3A, left panels). Plated cells were then labeled with TMRE, treated with t-BuOOH, and imaged as described above. Consistent with our findings in the HEK293 cells, we found that C6 glioma cells overexpressing CyPD maintained their ΔΨ for significantly longer periods than non-transfected cells when exposed to t-BuOOH (Fig. 3A, upper panels). C6 glioma cells transfected with the bicistronic vector containing EYFP alone exhibited no significant difference from control, non-transfected cells (Fig. 3A, lower panels). The average time until the ΔΨ collapsed in the presence of t-BuOOH was 2.2 ± 0.1 h in control, non-transfected cells (n = 10 experiments); 3.5 ± 0.2 h in CyPD and EYFP expressing cells (n = 5 experiments) and 2.2 ± 0.1 h for EYFP alone expressing cells (n = 5 experiments) (Fig. 3B). We conclude from these data that, under oxidative stress, overexpression of CyPD prolongs the time until ΔΨ collapses in both HEK293 and rat C6 glioma cells, suggesting that it might be a generalized mechanism operating in many cell types.
Plated cells were treated with staurosporin in the presence of PI and imaged as described above following 48 h of expression. Positively transfected cells were identified by EYFP fluorescence. A spatio-temporal stack of PI staining during the course of the experiment demonstrated that C6 glioma cells overexpressing CyPD maintained their plasma membrane integrity for a significantly longer period than non-transfected cells when exposed to staurosporin (Fig. 4B, upper panels). C6 glioma cells expressing EYFP alone were not significantly different from control, non-transfected cells (Fig. 4B, lower panels). The average time when PI-positive staining occurred in the presence of staurosporin was 7.4 ± 0.1 h in control, non-transfected cells (n = 8 experiments) and 7.6 ± 0.2 h for EYFP alone expressing cells (n = 4 experiments). In contrast, this value was increased to 10.1 ± 0.5 h (n = 4 experiments) in CyPD and EYFP expressing cells (Fig. 4C). We conclude from these data that overexpression of CyPD protects rat C6 glioma cells from staurosporin-induced cell death.

**PPIase Activity Is Required for the Protective Effect of CyPD—**PPIase enzymatic activity in cyclophilins catalyzes the rotation of prolyl peptide bonds in target proteins (42). The crystal and NMR structures of cyclophilin A in complex with CsA revealed the presence of a hydrophobic pocket that subsequently was found to play a critical role both in substrate binding and PPIase activity (24–26). A point mutation within this pocket, H126Q, completely abolished substrate binding and consequently, enzymatic activity (43). In contrast, a mutation near the mouth of the pocket, R55G, abolished PPIase activity without affecting substrate binding. We engineered the corresponding mutants in CyPD to test whether PPIase activity was required for the protective effect of CyPD on ΔΨ (Fig. 5A). CyPD wild type and mutants, R96G and H167Q, were created as GST fusion proteins, purified from bacteria, and assayed for PPIase activity (Fig. 5B). We found the mutation at both residues abolished PPIase activity from 100% in CyPD and 3.0 ± 1.9% in mutant R96G and 3.8 ± 1.5% in mutant H167Q (n = 3 experiments). Finally, GST alone did not display PPIase activity (3.4 ± 1.5%) (Fig. 5C). These experiments demonstrate that the CyPD mutants, R96G and H167Q, do not exhibit detectable PPIase activity above background levels (GST), similar to the corresponding mutants in CyPA that

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**Overexpression of CyPD Protects Cells from Staurosporin-induced Cell Death**—To determine whether CyPD protects cells against other apoptotic stimuli, the effects of CyPD overexpression were examined in staurosporin-treated cells. To induce cell death, rat C6 glioma cells were treated with 1 μM staurosporin for 6 h. Apoptosis was confirmed by staining the cells with Annexin V-FITC (Molecular Probes), which detects phosphatidylserine in the outer leaflet of the plasma membrane. Phosphatidylserine flips to the outer leaflet of the plasma membrane preceding the loss of membrane integrity and is an early indication of apoptosis (41). Co-staining with propidium iodide (PI, Molecular Probes) identified those cells with compromised plasma membrane integrity and that had progressed through apoptosis (Fig. 4A). These data demonstrate that staurosporin induces apoptosis in those cells. To test whether CyPD confers protection under these conditions, rat C6 glioma cells were transiently transfected with the bicistronic vector for independent expression of CyPD and EYFP.
abrogate PPIase activity. These data corroborate models of the structural requirements for substrate binding and PPIase enzymatic activity in the cyclophilins (24–26).

As described above, we first tested whether cells overexpressing the PPIase mutants exhibited the correct targeting to the mitochondria. Fusion proteins consisting of CyPD R96G and H167Q were tagged with EYFP at their respective COOH terminals and then transiently transfected into HEK293 cells. Cells expressing these EYFP-tagged CyPD mutants are presented in Fig. 6A. Both mutants were correctly targeted to the mitochondria (Fig. 6A). Stable cell lines expressing these EYFP-tagged CyPD mutants were then created and selected for protein expression levels that were comparable to the CyPD wild type cell line (Fig. 6C). To test whether PPIase activity was required for the action of CyPD, plated cell cultures were prepared, labeled, and then imaged while exposed to t-BuOOH (100 μM). Spatio-temporal stacks of a single field of cells are presented for each cell type for the duration of the recording (Fig. 6B). We found that, in response to oxidative stress, mutagenesis of CyPD abolished the protective effect observed with wild type CyPD as measured by the latency to ΔΨ collapse. Specifically, the average time until ΔΨ collapse was 6.3 ± 0.1 h for R96G mutant cells (n = 73 cells, from four experiments) and 6.2 ± 0.1 h for H167Q mutant cells (n = 85 cells, from five experiments) (Fig. 6D). These values were not significantly different from cells transfected with vector alone 6.2 ± 0.1 (Fig. 6D, cf. Fig. 1E). We conclude from these data that the protective effect of CyPD during oxidative stress requires PPIase activity.

Overexpression of CyPD Increases Resting ΔΨ—ΔΨ is significantly affected by the open probability of the mPTP, and, at the same time, the mPTP open probability is tightly regulated by ΔΨ (44–46). The lower the ΔΨ, the higher the probability that the mPTP opens and the more likely it becomes that this positive feedback loop collapses ΔΨ. Given the interdependence of mPTP and ΔΨ, we examined whether overexpression of CyPD wild type and its mutants affected ΔΨ under resting conditions. Plated cells were obtained from the stable cell lines expressing CyPD wild type and its mutants affected ΔΨ used above (Fig. 6C). The cells were labeled with TMRE and imaged at higher magnification (Fig. 7A). ΔΨ was estimated as the log ratio of mitochondrial (F_{mito}) to cytosolic (F_{cyt}) fluorescent intensity according to the Nernst equation: ΔΨ = \log_{10}(F_{mito}/F_{cyt}) (47). F_{mito} was estimated by selecting the highest pixel value within a 5 × 5 pixel region surrounding a single mitochondrion. In a given cell, the fluorescence intensity of the five brightest mitochondria was averaged. F_{cyt} was also averaged from five measurements for each individual cell. The single-cell log ratios were then pooled to provide the average \log_{10}(F_{mito}/F_{cyt}) for a particular cell line. We found that cells overexpressing CyPD wild type protein exhibited significantly higher log ratios of 1.96 ± 0.2 (n = 145 cells, p < 0.0005), as compared with 1.84 ± 0.03 for mutant R96G (n = 103 cells); 1.83 ± 0.02 for mutant H167Q (n = 157 cells) and 1.81 ± 0.02 for control cells transfected with vector alone (n = 166 cells) (Fig. 7B). These data demonstrate that the overexpression of CyPD increases the resting ΔΨ and provides a potential mechanism for the protective effect of CyPD.

CyPD Binding to ANT Is Not Affected by Mutations in the PPIase Binding Pocket—The binding of CyPD to ANT is considered important for mPTP regulation (48). CsA does not disrupt the binding of CyPD and ANT (27), and pharmacolog-
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Fig. 8. Cyclophilin D binding to ANT is not affected by mutations in the PPIase hydrophobic pocket. A, autoradiograph of in vitro translated ANT that binds to GST alone (G), GST-CyPD wt (CW), GST-CyPD R96G (CR), and CyPD H167Q (CH). ANT was labeled with [35S]methionine. The input amount of in vitro translated ANT is signified by I. B, histogram of densitometric analysis of ANT binding to different GST fusion proteins in A normalized to the intensity of GST-CyPD wt (CW, 100%). There is no statistical difference between CW, CR, and CH (single factor ANOVA).

There is no statistical difference between CW, CR, and CH (single factor ANOVA).

## DISCUSSION

The mPTP is generally depicted as a core protein complex composed of VDAC, ANT, and CyPD (10, 13). The importance of CyPD in the mPTP regulation was initially recognized by pharmacological studies involving the immunosuppressant CsA. CsA was shown to inhibit the mPTP and PPIase activity at similar concentrations (22, 23). Biochemical data supported a tight interaction between CyPD and ANT (27, 48). Thus, CsA was envisioned as a pseudosubstrate for the PPIase enzyme that removed CyPD binding from ANT, thereby inhibiting mPTP opening. However, Crompton and colleagues (27) recently reported that CsA does not displace CyPD from ANT, suggesting that the binding site important for PPIase activity does not directly involve in the ANT binding. Furthermore, Scorrano and co-workers showed that the PPIase inhibitor DPC induced mPTP opening, suggesting that CyPD affects the mPTP open-close transition independently of its PPIase activity (28). Lemasters and He (49) have recently presented evidence suggesting that the mPTP may be formed by aggregation of misfolded membrane proteins, which cluster to form a large conductance transmembrane pore. Moreover, CyPD was hypothesized to bind to these clusters in their model, possibly to inhibit pore formation by refolding damaged proteins to their native state. Taken together, these data indicate that the specific role of CyPD in mPTP function is different than previously assumed.

We have used overexpression studies to investigate the role of CyPD in the regulation of the mPTP. Based on previous models of CyPD interaction with the ANT, we postulated that overexpression of CyPD would sensitize mitochondria to oxidative stress. In fact, we found that CyPD overexpression results in a protective effect, increasing significantly the time it takes for the ΔΨ to collapse under oxidative stress. CyPD overexpression also protected cells against staurosporin-induced cell death. Point mutations in CyPD that eliminated PPIase activity did not have similar protective effects, clearly demonstrating that CyPD requires functional PPIase activity. Interestingly, suppression of cyclophilin A, the cytosolic isoform of the family of PPIases that bind cyclosporin A, has been reported in markedly sensitize cells to oxidative stress (50). An involvement of PPIase activity in oxygen defense has been reported for cyclophilin 18 in rabbit blastocysts (51). The thiol-specific antioxidant protein Aop1 has also been reported to bind human cyclophilin 18 (52). Taken together, these reports and our data indicate that cyclophilins are important components of our oxygen defense system.

The traditional view of the mPTP regulation is that CyPD binds to the ANT sensitizing the pore to open by stimuli such as Ca$^{2+}$, pH, and reactive oxygen species (45, 53, 54). Our overexpression studies, on the other hand, indicate that CyPD can desensitize and close the mPTP. This view of mPTP function is consistent with recent work reported by Bauer and colleagues (55). These authors showed that transient overexpression of ANT1 induced apoptosis in HEK293 cells and that this effect could be inhibited by co-expression of CyPD. The authors suggested that CyPD might mask an interaction domain of ANT1 with an unknown repressor. This view is inconsistent with our data, because overexpression of CyPD, in the absence of ANT1 overexpression, would be predicted to increase mPTP opening by blocking repressor binding to the ANT. The alternative explanation proposed by the authors was that overexpression of ANT1 may titrate out endogenous inhibitors of mPTP, thereby inducing apoptosis. In this case, Bauer and colleagues suggested that CyPD would act as the endogenous inhibitor of the mPTP. However, Bauer and colleagues did not investigate the protective effects of CyPD expression on apoptosis, because ANT1 expression itself was used as their apoptotic stimulus. Our data are consistent with the second conceptual alternative (i.e. that CyPD acts as an inhibitor of the mPTP) and further demonstrate that CyPD protects cells against apoptotic stimuli.

Several possible mechanisms could account for the protective effect of CyPD against cell death. CyPD has been shown to directly bind ANT, a primary component of the mPTP, which could inhibit mPTP opening. This mechanism appears unlikely, because mutation of the PPIase binding pocket of CyPD removes the protective effect but does not affect ANT binding. Published reports are also consistent with our finding that ANT binding is not through the PPIase binding pocket of CyPD (27). Although the protective effect of CyPD appears unlikely to be mediated solely through ANT binding, it is possible that one or more other binding targets of CyPD are recruited to the ANT, which in turn directly inhibits mPTP opening. In fact, He and Lemasters (49) have recently proposed that CyPD and an unidentified chaperone-like protein bind to clusters of damaged proteins to inhibit mPTP opening. Our data are consistent with this new paradigm of mPTP pore structure and regulation. The protective effect of CyPD could also be mediated by an unknown target, which indirectly inhibits mPTP opening. For
example, any protein that can functionally hyperpolarize ΔΨ could clearly account for the protective effect of CyPD against apoptotic stimuli given the ΔΨ dependence of mPTP opening. In support of this mechanism of action, mitochondrial respiration in yeast has been reported to decrease when the CyPD homolog CPR3 is mutated (56). Furthermore, our data demonstrate that overexpression of CyPD hyperpolarizes ΔΨ, whereas overexpression of the two PPIase mutants that do not provide oxidant protection do not increase ΔΨ. Irrespective of the precise underlying mechanism of action, our data underscore the importance of CyPD in the regulation of mPTP and programmed cell death and suggest CyPD can be viewed as a cell survival molecule.

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