Suppression of Apoptosis by Cyclophilin D via Stabilization of Hexokinase II Mitochondrial Binding in Cancer Cells

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The permeability transition pore is involved in the mitochondrial pathway of apoptosis. Cyclophilin D, a pore component, has catalytic activity as a peptidyl prolyl cis, trans-isomerase (PPIase), which is essential to the pore opening. It has been reported that cyclophilin D overexpression suppresses apoptosis in cancer cells. To clarify the mechanism of this effect, we generated glioma cells overexpressing wild-type or a PPIase-deficient mutant of cyclophilin D. Interestingly, we found that the PPIase-dependent apoptosis suppression by cyclophilin D correlated with the amounts of mitochondrial-bound hexokinase II, which has anti-apoptotic activity. Inactivation of endogenous cyclophilin D by small interference RNA or a cyclophilin inhibitor was found to release hexokinase II from mitochondria and to enhance Bax-mediated apoptosis. The anti-apoptotic effects of cyclophilin D were canceled out by the detachment of hexokinase II from mitochondria, demonstrating that mitochondrial binding of hexokinase II is essential to the apoptosis suppression by cyclophilin D. Furthermore, cyclophilin D dysfunction appears to abrogate hexokinase II-mediated apoptosis suppression, indicating that cyclophilin D is required for the anti-apoptotic activity of hexokinase II. Based on the above, we propose here that cyclophilin D suppresses apoptotic cell death via a mitochondrial hexokinase II-dependent mechanism in cancer cells.

During apoptosis, the permeability of the mitochondrial outer membrane is up-regulated, inducing a release of pro-apoptotic factors such as cytochrome c from the intermembrane space into the cytosol. Once cytochrome c is released from mitochondria, it binds to Apaf-1 and promotes the assembly of apoptosis multiprotein complex, which induces activation of the caspase-9 cell death protease (1). It has been believed that a mitochondrial protein complex called the permeability transition (PT) pore, which is composed of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), cyclophilin D, and hexokinase, mediates the permeabilization of the mitochondrial membrane and cytochrome c release (2).

Cyclophilin D, which is a peptidyl prolyl-cis, trans-isomerase (PPIase), has been considered to be a promoter of pore opening. It has been reported that cyclophilin inhibitors such as cyclosporin A (3) and sanguilerin A (4) block the opening of the PT pore. Moreover, recent reports clearly show that mitochondria isolated from cyclophilin D null mice are resistant to the permeability transition (5–8). Thus, cyclophilin D is now regarded as a key factor in the regulation of pore function.

When the PT pore opens in vitro, mitochondria swell, the outer membrane is disrupted, and cytochrome c is released (9, 10). However, during apoptosis cytochrome c release is observed without the permeability transition (11, 12). Indeed, consistent with these observations, cyclophilin D null fibroblasts are not protected from Bax-dependent apoptosis (5, 6). Therefore, the opening of the PT pore appears not to be essential to Bax-induced apoptosis. However, although the opening of the PT pore is not a trigger of apoptosis, components of the pore are involved in apoptosis as targets or suppressors against pro-apoptotic factors. For instance, VDAC, which is a primary component of the pore, is recognized as a target of pro-apoptotic protein Bax (13). VDAC may play a role in regulating cytochrome c release by forming, together with Bax, a highly conductive channel (14). It has also been reported that Bax promotes cytochrome c release and apoptosis through interactions with the ANT (15). Hexokinase II, which catalyzes glucose phosphorylation, is overexpressed in cancer cells and plays a pivotal role in cellular proliferation and survival (16). Hexokinase II appears to antagonize the mitochondrial translocation of Bax by inhibiting the molecular interaction between Bax and VDAC (17). Therefore, the mitochondrial binding of hexokinase II seems to play an essential role in the anti-apoptotic mechanism of cancer cells.

It has been reported that cyclophilin D suppresses apoptosis when it is overexpressed (18–20). However, whether its PPIase activity is essential remains unknown, as is the mechanism of the anti-apoptotic effect of cyclophilin D.

In the present study, we investigated the influences of cyclophilin D overexpression or its dysfunction on constituents of the PT pore complex. We found that cyclophilin D stabilizes hexokinase II mitochondrial binding by its PPIase activity. Our findings demonstrate that hexokinase II mitochondrial binding is essential to apoptosis suppression by cyclophilin D.

MATERIALS AND METHODS

Reagents—Cyclosporin A (CsA) and FK506 were purchased from Calbiochem. Adriamycin, A23187, DEVD-AMC, clotrimazole (CTZ), a protease inhibitor mixture (P8340), and digitonin were from Sigma. Bax-inhibiting peptide V5 and negative control peptide were from Calbiochem. Succinyl-Ala-Ala-Pro-Phe-4-nitroanilide was from Peptide Inc. (Osaka, Japan).

Cell Cultures and the Generation of Stable Cell Lines—HeLa and C6 cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum, 200 μg/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 5%
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CO₂, C6 cell lines expressing HA-tagged fusion cyclophilin D proteins were obtained by plasmid transfection using Effectene reagents (Qiagen, Hilden, Germany). After 48 h of transfection, the cells were transferred to medium containing 800 μg/ml of G418 (Invitrogen). Surviving cells were grown for 2–3 weeks, and colonies were picked. The protein expression of HA-cyclophilin D was analyzed by Western blotting using anti-HA antibody.

**Bax-induced Apoptosis**—HeLa cells (6 × 10⁴ cells/well of a 12-well plate) were preincubated for 24 h, and then the cells were transfected with pCI-Bax or empty vector using transfection reagent Trans-It-LT1 (Mirus Bio Corp., Madison, WI). At 18 h post-transfection, the cells were treated with or without reagents (1 μM CsA, 10 μM CTZ, and 1 μM FK506) for 6 h. Cells were then collected, and caspase-3 activity was measured. Reagents were dissolved in Me₂SO, and the concentration of Me₂SO in culture medium was 0.1% (v/v).

**Measurement of Caspase-3 Activity**—Cells were solubilized in buffer (20 mM Pipes, pH 7.2, 100 mM NaCl, 1 mM EDTA, 10% sucrose, 0.1% CHAPS) at 4 °C for 10 min. The caspase-3-like protease activity of each sample was measured as described (21). Substrate (DEVD-aminomethylcoumarin) cleavage to release free AMC (excitation 355 nm, emission 460 nm) was monitored with a spectrophotometer Wallac 1420 ARVO SX (GE Healthcare Life Science). Data are represented as the means ± S.D. of separate experiments. Statistical significance was determined utilizing a one-way analysis of variance followed by the Student-Newman-Keuls test. A value of p < 0.01 was considered statistically significant.

**Cytochrome c Release and Bax Mitochondrial Translocation**—Cytochrome c release and Bax translocation to mitochondria during apoptosis were analyzed as described (22). Briefly, cells were suspended in the buffer (70 mM Tris-HCl, pH 7.0, 250 mM sucrose, 0.05 mg/ml of digitonin) and incubated for 10 min. The cells were centrifuged at 12,000 × g for 10 min. Precipitation (mitochondria-rich fraction) and cytosolic supernatant were analyzed by Western blotting using anti-cytochrome c antibody or anti-Bax antibodies.

**RNA Interference**—A 21-nucleotide RNA duplex with 3’-deoxythymidine overhang corresponding to human cyclophilin D (NM_000189) was purchased from Ambion, Inc. (Austin, TX). The RNA sequences (siRNA 1) were as follows: sense 5’-GGCAGAAGUGCUUCAGAAAT-3’, antisense 5’-CUUUGGACGACUUCGCT-3’. For specific down-regulation of endogenous cyclophilin D, we used another RNA duplex that binds the 3’-untranslated region of cyclophilin D mRNA. The RNA sequences (siRNA 2) were as follows: sense 5’-GGACUCUAUAACCUCGUGUAA-3’, antisense 5’-UAAACAGGAUAUUGAGGGCC-Ct-3’. Endogenous Bax was down-regulated by Stealth siRNA duplex oligoribonucleotides from Invitrogen. The RNA sequences were as follows: sense 5’-UUGA-GCACGGAGUUUUGCGGCAAAGU-3’, antisense 5’-ACUUGGCAGC-GCAAUCUGGUGUGC-3’.

A luciferase GL2 siRNA duplex (Dharmacon Research, Inc., Lafayette, CO) was used as a control siRNA. Each siRNA (50 nM) was introduced into HeLa cells with Oligofectamine reagent (Invitrogen). Each siRNA (50 nM) was introduced into HeLa cells with Oligofectamine reagent (Invitrogen). The RNA sequences were as follows: sense 5’-GGACUCUAUAACCUCGUGUAA-3’, antisense 5’-UAAACAGGAUAUUGAGGGCC-Ct-3’. The luciferase activity was measured with a luciferase reporter assay system (Promega, Madison, WI). After 48 h transfection, the cells were treated with or without reagents (1 μM CsA, 10 μM CTZ, and 1 μM FK506) for 6 h. Cells were then collected, and caspase-3 activity was measured. Reagents were dissolved in Me₂SO, and the concentration of Me₂SO in culture medium was 0.1% (v/v).

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**Subcellular Fractionation**—Mitochondrial and cytosolic fractions were isolated from C6 or HeLa cells as described (17). Briefly, cells were lysed in buffer (70 mM Tris-HCl, pH 7.0, 250 mM sucrose, 0.05 mg/ml of digitonin) and incubated for 10 min. The supernatant was further fractionated into mitochondrial and cytosolic fractions using a sucrose gradient (GE Healthcare Life Science). Mitochondrial and cytosolic fractions were analyzed by Western blotting using antibodies against cytochrome c, cyclophilin D, and hexokinase II, respectively.

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**RESULTS**

**Establishing a Stable Cell Line with Overexpressed Wild-type or PPlase-deficient Cyclophilin D**—We generated PPlase-deficient mutants (R97A and H168Q) of cyclophilin D to address whether the PPlase activity of cyclophilin D is essential. In accordance with previous reports (18), both R97A and H168Q mutants completely lost their PPlase activity (Fig. 1A). It has been reported that cyclophilin D...
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FIGURE 1. Overexpression of wild-type (WT) or PPIase-deficient cyclophilin D in glioma cells. A, PPIase activities of recombinant cyclophilin D proteins were measured by the chymotrypsin-coupled assay as described under “Materials and Methods.” B, the ANT binding activities of glutathione S-transferase-cyclophilin D fusion proteins were analyzed by Western blotting using anti-ANT antibodies. C and D, C6 glioma cells stably expressing HA-tagged fusion cyclophilin D (wild-type, WT; PPIase-deficient mutants, R97A and H168Q) and control cells transfected with empty vector (Vec) were generated. Mitochondrial and cytosolic fractions were obtained from cells expressing WT (clone 1), R97A (clone 6), and H168Q (clone 7), and mitochondrial localization of HA-cyclophilin D proteins was analyzed by immunofluorescent staining for HA tag and HSP60 (D). Bax binds to the ANT in vitro (25, 26), indicating that cyclophilin D may associate with the ANT and thus regulate the opening of the PT pore. As shown in Fig. 1B, binding of cyclophilin D to the ANT was independent of its PPIase activity, which is consistent with previous observations (18). Therefore, we expected that the cyclophilin D mutants (R97A and H168Q) could inhibit endogenous cyclophilin D when they were overexpressed in cancer cells. The C6 rat glioma cell line had smaller amounts of endogenous cyclophilin D protein than other tumor cell lines (data not shown). We therefore generated the C6 glioma cell line stably overexpressing wild-type or mutant cyclophilin D in their mitochondria. We confirmed that the introduced cyclophilin D proteins were successfully transferred to mitochondria (Fig. 1, C and D). It was observed that total protein levels of PPIase-deficient mutants were less than that of wild-type cyclophilin D (Fig. 1C and supplemental Fig. S1). These observations indicate that PPIase-deficient cyclophilin D proteins are unstable or slightly toxic to tumor cells.

Cyclophilin D Suppressed Apoptosis, whereas PPIase-deficient Cyclophilin D Sensitized Cells against Apoptosis—We investigated the effects of cyclophilin D overexpression on apoptosis induction by an anti-tumor reagent, adriamycin. This anti-cancer reagent has been reported to induce Bax-dependent cytochrome c release and apoptosis (27). As shown in Fig. 2A, overexpression of wild-type cyclophilin D protected the cells from adriamycin-induced caspase-3 activation. Expectedly, overexpression of the PPIase-deficient mutants enhanced the caspase-3 activation (Fig. 2B), cytochrome c release, and the Bax mitochondrial translocation induced by adriamycin (Fig. 2C). We confirmed that other stable clones with PPIase-deficient cyclophilin D were sensitive to adriamycin (see supplemental Fig. S1). These results demonstrate that the anti-apoptotic activity of cyclophilin D is dependent on its PPIase activity.

To clarify the role of Bax in apoptotic cell death, we investigated the effects of Bax-inhibiting peptide, which is derived from the Bax-binding domain of Ku70 (28), on the caspase-3 activation by adriamycin. We pre-treated the cells expressing PPIase-deficient cyclophilin D (R97A and H168Q) with Bax-inhibiting peptide (BIP) or its negative control peptide (NC) on apoptosis induced by adriamycin. Cells with PPIase-deficient cyclophilin D (R97A and H168Q) were pretreated with 200 μM BIP or control peptide for 60 min and then treated with (filled column) or without (empty column) 1 μM adriamycin for 16 h. Caspase-3 activation was measured as described under “Materials and Methods.” The data are the means ± S.D. of three independent experiments. *, p < 0.05; NS, nonsignificant (p > 0.05) in comparison with Me2SO (DMSO) treatment in adriamycin-treated cells.

Cyclophilin D Stabilized Mitochondrial Binding of Hexokinase II—Because cyclophilin D is a component of the PT pore, we next analyzed the pore constitution among the cyclophilin D-overexpressed cells. Fig. 3A shows that overexpression of wild-type cyclophilin D increases the levels of mitochondrial-bound hexokinase II, whereas that of mutant cyclophilin D reduces these levels. Other pore components (VDAC and ANT) and a mitochondrial chaperon, HSP60, are not affected by overexpression of cyclophilin D. These observations suggest that cyclophilin D maintains hexokinase II mitochondrial binding by its PPIase activity. As shown in Fig. 3B, CTZ, which has been reported to inhibit mitochondrial binding of hexokinase II (17), dissociated hexokinase II from mitochondria. When the cells were treated with CTZ, the anti-apoptotic
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FIGURE 3. Overexpression of cyclophilin D affected the mitochondrial-bound hexokinase II. A, mitochondrial fractions were isolated from C6 cells expressing HA-cyclophilin D proteins (WT, R97A, and H168Q) and control cells (Vec). Amounts of hexokinase II, α-tubulin, and HSP60 were analyzed by Western blotting. B, HeLa cells were transfected with pCI-Bax (filled column) or empty vector (empty column) (0.4 μg each). At 18 h post-transfection, the cells were treated with or without adriamycin (1 μM adriamycin) for 4 h. C, amounts of hexokinase II, α-tubulin, and HSP60 were analyzed by Western blotting. D, mitochondria and cytosolic fractions were isolated from HeLa cells cultured in the presence or absence of 10 μM clotrimazole (CTZ) for 6 h, and the cells were then collected. Mitochondrial and cytosolic fractions were obtained, and amounts of hexokinase II, α-tubulin, and HSP60 in each sample were analyzed by Western blotting. C, cells were treated with (filled column) or without (empty column) 1 μM adriamycin in the presence or absence of 10 μM CTZ for 20 h. The cells were then collected, and caspase-3 activity was measured. The data are the means ± S.D. of three independent experiments. *, p < 0.01; NS, nonsignificant (p > 0.05) versus control cells (Vec) treated with adriamycin.

FIGURE 4. Cyclophilin inhibitor released hexokinase II from mitochondria. A, mitochondrial and cytosolic fractions were isolated from HeLa cells cultured in the presence or absence of 1 μM cyclosporin A (CSA), 10 μM clotrimazole (CTZ), and 1 μM FK506 for 4 h. Amounts of hexokinase II, α-tubulin, and HSP60 were analyzed by Western blotting. B, HeLa cells were transfected with pCI-Bax (filled column) or empty vector (empty column) (0.4 μg each). At 18 h post-transfection, the cells were treated with or without reagents (1 μM CSA, 10 μM CTZ, and 1 μM FK506) for 6 h. The cells were then collected, and the caspase-3 activity of each sample was measured as described under "Materials and Methods." The data are the means ± S.D. of three independent experiments. *, p < 0.01; NS, nonsignificant (p > 0.05) in comparison with Me2SO control in Bax-expressing cells.

activity of cyclophilin D was diminished (Fig. 3C). These results indicate that hexokinase II mitochondrial binding is important to the anti-apoptotic activity of cyclophilin D.

Cyclosporin A Released Hexokinase II from Mitochondria—We investigated the effects of CSA, a specific inhibitor of cyclophilin D, on hexokinase II mitochondrial binding. Expectedly, CSA released hexokinase II from mitochondria as CTZ did, but FK506 did not (Fig. 4A). CSA also inhibits calcineurin, a serine/threonine protein phosphatase. Because FK506, an inhibitor of calcineurin, did not release hexokinase II from mitochondria, the effects of CSA on calcineurin can be excluded from the hexokinase II release induction. We next investigated the effects of CsA on Bax-induced apoptosis. It has previously been reported that CTZ enhances Bax-induced apoptosis by detaching hexokinase II from mitochondria (17). Fig. 4B shows that CsA, but not FK506, enhanced the caspase-3 activity induced by transient expression of Bax. Moreover, CsA enhanced the Bax mitochondrial translocation (Fig. 4C). These results indicate that CsA enhances Bax-induced apoptosis through inhibition of hexokinase II mitochondrial binding.

Down-regulation of Endogenous Cyclophilin D Released Hexokinase II from Mitochondria and Sensitized Cells to Apoptosis—To determine whether endogenous cyclophilin D is essential to hexokinase II mitochondrial binding, we investigated the effects of RNA interference-mediated gene silencing on cyclophilin D. We treated cells with a small interfering (si) RNA designed to target human cyclophilin D (siRNA 1). As shown in Fig. 5A, cyclophilin D siRNA reduced the levels of endogenous cyclophilin D protein without any effects on cyclophilin A or HSP 60. As shown in Fig. 5B, at 26 h after transient transfection with siRNA, levels of endogenous cyclophilin D were reduced and hexokinase II was released from mitochondria into the cytosol. Other components of the PT pore such as VDAC and ANT were unaffected. At 36 h, the amounts of cytosolic hexokinase II protein were decreased. This observation may reflect the predicted protein degradation of hexokinase released from mitochondria (29). The down-regulation of endogenous cyclophilin D enhanced the adriamycin-induced caspase-3 activation (Fig. 5C). Fig. 5D shows that inactivation of endogenous Bax by siRNA suppressed caspase-3 activation in cyclophilin D down-regulated cells. This result suggests that Bax is
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FIGURE 6. Endogenous cyclophilin D maintained mitochondrial binding of hexokinase II by its PPlase activity. A, designs of siRNAs for the specific down-regulation of endogenous cyclophilin D. siRNA 1 was targeted on the open reading frame of cyclophilin D, whereas siRNA 2 was targeted on the 3' untranslated region of cyclophilin D mRNA. siRNA 2 could specifically silence the endogenous cyclophilin D. B, siRNAs were introduced into HeLa cells (3 × 10⁵ cells) as described under "Materials and Methods." At 24 h post-transfection, the cyclophilin D expression vectors or empty vector (0.6 μg) were introduced into the cells. At 24 h, mitochondrial and cytosolic fractions were isolated from the cells. Hexokinase II mitochondrial binding was analyzed by Western blotting.

FIGURE 7. Cyclophilin D function was required for the anti-apoptotic effect of hexokinase II. A, human hexokinase II with Myc tag at the C-terminal was overexpressed in HeLa cells. At 2 h post-transfection, cells were collected and mitochondrial expression of Myc-tagged fusion hexokinase II was analyzed by Western blotting. B, the cells overexpressing hexokinase II were transfected with the Bax expression vector (filled column) or empty vector (empty column). At 18 h post-transfection, caspase-3 activity of the cells was measured as described under "Materials and Methods." The data are the means ± S.D. of three independent experiments. *, p < 0.01 versus Bax expression in control vector transfected cells. C, the cells overexpressing hexokinase II were treated with or without reagents (10 μM CTZ, 1 μM CsA, and 1 μM FK506) for 6 h. The cells were then collected, and hexokinase II mitochondrial binding was analyzed by Western blotting. D, the cells overexpressing hexokinase II were transfected with the Bax expression vector (filled column) or empty vector (empty column). At 18 h post-transfection, the cells were treated with or without reagents (10 μM CTZ, 1 μM CsA, and 1 μM FK506) for 6 h. The cells were then collected, and caspase-3 activity was measured as described under "Materials and Methods." The data are the means ± S.D. of three independent experiments. *, p < 0.01; NS, nonsignificant (p > 0.05) in comparison with M₆SO treatment in hexokinase II-Myc-expressing cells. E, the hexokinase II expression vector (0.5 μg) was introduced into HeLa cells (5 × 10⁵ cells). At 24 h post-transfection, siRNAs were introduced into the cells. At 24 h, mitochondrial and cytosolic fractions were isolated from the cells. Hexokinase II mitochondrial binding was analyzed by Western blotting. F, the cells (8 × 10⁴ cells) were transfected with hexokinase II expression vector or empty vector (0.2 μg each). At 24 h post-transfection, the cells were further treated with siRNAs for 24 h. The cells were then transfected with the Bax expression vector (filled column) or empty vector (empty column) (0.5 μg each). At 18 h post-transfection, the cells were collected and caspase-3 activity was measured. The data are the means ± S.D. of three independent experiments. *, p < 0.01 versus Bax expression in control vector and control siRNA-introduced cells; NS, nonsignificant (p > 0.05) versus Bax expression in control vector and control siRNA-1-introduced cells.

DISCUSSION

Cyclophilin D has been considered to be an essential factor of the PT pore opening (5–8). Once the PT pore opens in vitro, mitochondrial swelling, outer membrane disruption, and cytochrome c release are induced. Based on these in vitro observations, the opening of the PT pore has been regarded as a trigger of apoptosis. However, recent studies have shown that cyclophilin D and the opening of the PT pore are required to mediate calcium- and oxidative stress-induced necrotic cell death but are not essential to Bax-induced apoptotic cell death (5, 6). These results...
clearly demonstrate that cyclophilin D is not a trigger of Bax-induced apoptosis. Accordingly, it has been suspected that cyclophilin D is a suppressor, but not a promoter, of apoptosis (18–20). Though cyclophilin D has PPIase activity, the role of enzymatic activity in the anti-apoptotic effect remains unknown. One investigation (18) has shown that cyclophilin D suppresses apoptotic cell death by its enzymatic activity, whereas another indicates the presence of a PPIase-independent anti-apoptotic mechanism against ANT1-mediated apoptosis (19). Thus, the role of PPIase activity in the cyclophilin D-mediated anti-apoptotic effect remains controversial.

To clarify the role of cyclophilin D in apoptosis, we generated a glioma cell line overexpressing wild-type or a PPIase-deficient mutant of cyclophilin D (Fig. 1). Overexpression of cyclophilin D protected the cells from Bax-mediated apoptosis, whereas that of mutant cyclophilin D enhanced Bax mitochondria translocation, cytochrome c release, and apoptotic cell death (Fig. 2). Because Bax-inhibiting peptide or Bax-specific siRNA attenuated apoptosis, Bax seems to play a central role in mediating apoptosis in tumor cells with defective cyclophilin D (Figs. 2 and 5). Moreover, inhibition of cyclophilin D function by cyclosporin A or siRNA also sensitized the cells against apoptosis (Figs. 4 and 5). These findings clearly demonstrate that cyclophilin D is a suppressor of apoptosis and that its PPIase activity is required for this anti-apoptotic effect.

We also found that overexpression of cyclophilin D increases the levels of mitochondrial-bound hexokinase II, whereas overexpression of cyclophilin D mutant and dysfunction of endogenous cyclophilin D significantly reduced these levels (Figs. 3–5). The cyclophilin D-mediated stabilization of hexokinase II mitochondrial binding is dependent on its PPIase activity (Fig. 6). Because detachment of hexokinase II from mitochondria by clotrimazole abrogates the anti-apoptotic effect of cyclophilin D (Fig. 3), it seems that mitochondrially bound hexokinase II plays an essential role in the cyclophilin D-mediated anti-apoptotic effect.

Hexokinase II binds to the transmembrane channel formed by a protein called VDAC. VDAC is a primary component of the PT pore complex, and it also is a binding partner of a pro-apoptotic protein, Bax. Hexokinase II appears to antagonize the mitochondrial translocation of Bax by inhibiting the interaction between Bax and VDAC (17). It has recently been reported that activation of the Akt/protein kinase B survival signal pathway increases the mitochondrial binding of hexokinase II and prevents apoptotic cell death (30, 31). Therefore, the mitochondrial binding of hexokinase II appears to be essential to the anti-apoptotic mechanism of cancer cells. We also observed that overexpressed hexokinase II protects cells from Bax-mediated apoptotic cell death (Fig. 7). When endogenous cyclophilin D was inactivated, the anti-apoptotic activity of hexokinase II was reduced (Fig. 7). These observations suggest that cyclophilin D is required for the hexokinase II-mediated anti-apoptotic effect. We therefore propose that cyclophilin D inhibits Bax-induced apoptotic cell death through stabilization of the mitochondrial binding of hexokinase II by its PPIase enzymatic activity.

Hexokinase II is thought to interact with VDAC in the outer membrane, whereas cyclophilin D is localized in matrix. Thus, it appears that cyclophilin D cannot directly interact with hexokinase II. Cyclophilin D possibly stabilizes the hexokinase II mitochondrial binding via interactions with another component of the PT pore such as the ANT. It has been believed that cyclophilin D regulates pore opening by interacting with the ANT (32). However, a recent study (33) questions this hypothesis; it reports that mitochondrial swelling is still observed in hepatocyte mitochondria from (ANT1/ANT2) knock-out mice. The authors also show that normal mitochondria are sensitive to the ANT ligand-induced swelling whereas the ANT null mitochondria are insensitive. These observations suggest that the ANT possibly regulates the pore function, at least under "normal" conditions, although it is not essential to the opening of the PT pore. In accordance with this consideration, it was reported that ANT-1 overexpression-mediated apoptosis is suppressed by co-expression of cyclophilin D (19, 34). Furthermore, the ANT ligands affect both the conformation of mitochondria-bound type I hexokinase (35) and the binding of hexokinase to the VDAC/ANT complexes in vitro (36). Taken together, the molecular interaction between cyclophilin D and the ANT can still be considered an important factor in pore function. Cyclophilin D seems to regulate the PT pore structure and to maintain hexokinase II mitochondrial binding by its PPIase enzymatic activity, even though its binding partner remains unclear (it may be the ANT).

In several cancer cells, up-regulation of cyclophilin D expression has been observed (19, 37). These observations suggest that cyclophilin D plays an important role in cancer cells. Unexpectedly, recent knock-out studies of cyclophilin D have demonstrated that cyclophilin D null mice grow normally and show no apparent anomalies (5–8). Moreover, cyclophilin D knock out did not sensitize primary hepatocytes and fibroblasts to pro-apoptotic stimuli (5, 6). These conflicting observations may be explained by differences in the amounts of hexokinase II between primary cultured cells and tumor cells. In supporting this hypothesis, it has been reported that hexokinase II is highly expressed in tumor cells whereas it is found normally in muscle and adipose tissue in low amounts (16). It seems that cyclophilin D is not essential to normal cell growth and differentiation but plays a pivotal role in the survival of cancer cells. Therefore, it is implied that specific inhibitor of cyclophilin D might be not toxic for human cells and cyclophilin D may be able to successfully act as target molecules in anti-cancer chemotherapy. However, known cyclophilin D inhibitors such as cyclosporin A and sanglifehrin A likely inhibit all cellular cyclophilin family proteins, which maintain various cellular physiological functions. If specific inhibitors of cyclophilin D are developed, they will become powerful drugs functioning as anti-cancer reagents.

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