Biochemical and Biophysical Research Communications 426 (2012) 590–595

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# PKCδ promotes etoposide-induced cell death by phosphorylating Hsp27 in HeLa cells

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ARTICLE INFO

*Article history:*
Received 24 August 2012
Available online 4 September 2012

*Keywords:*
PKCδ
Hsp27
Phosphorylation
Cytochrome c
Caspase activation
Apoptosis

ABSTRACT

We investigated the regulation of Hsp27 phosphorylation by protein kinase C δ (PKCδ) during etoposide-induced apoptosis. The phosphorylation of Hsp27 at Ser78 was temporally correlated with the proteolytic activation of PKCδ during apoptosis. Hsp27 phosphorylation was dependent on the activity of PKCδ since treatment with rottlerin, a chemical inhibitor of PKCδ, or overexpression of a PKCδ dominant negative mutant abolished the phosphorylation. In addition, recombinant PKCδ phosphorylated Hsp27 at Ser78 *in vitro*. Moreover, caspase-3 was specifically activated following Hsp27 phosphorylation at Ser78. Pull-down assays using a phosphomimetic Hsp27 mutant revealed that binding between Hsp27 and cytochrome c was abolished by the phosphorylation. These results suggest that Hsp27 dissociates from cytochrome c following PKCδ-mediated phosphorylation at Ser78, which allows formation of the apoptosome and stimulates apoptotic progression.



## 1. Introduction

Apoptosis, or programmed cell death, is involved in various physiological processes, and the failure to regulate apoptosis is implicated in several diseases including cancers, immune diseases, and neurodegenerative disorders [1]. During DNA damage-induced apoptosis induced by a topoisomerase II inhibitor, etoposide, cytochrome c is released from mitochondria into the cytosol. This release triggers a caspase cascade [2,3], and cytosolic cytochrome c binds to apoptotic protease-activating factor-1 and procaspase-9 to form the apoptosome complex [4,5]. Regulatory mechanisms involving bcl-2 family proteins control the release of cytochrome c from mitochondria [6]. In addition, the formation of the apoptosome is also regulated after cytochrome c release [4,7]. Hsp27 functions as a chaperone in normal physiological conditions [8]. However, during apoptosis Hsp27 interacts with cytosolic cytochrome c to prevent the formation of the apoptosome [9,10], and binds procaspase-3 to prevent the activation of procaspase-3 by caspase-9 [11]. Hsp27 has three known phosphorylation sites at serine residues, Ser15, Ser78, and Ser82, which show different levels of phosphorylation in different conditions [12–15]. Protein kinase C δ (PKCδ) is one of a number of protein kinases reported to phosphorylate Hsp27 [16]. PKCδ is activated by various apoptotic stimuli including DNA damage induced by etoposide [17]. Following apoptotic stimulation, caspase 3 cleaves PKCδ to generate a catalytically active fragment [18]. If this activation of PKCδ is blocked, apoptosis cannot proceed [19,20]. While it is known that PKCδ activation is important for the progression of apoptosis [17,21], few targets of PKCδ have been reported.

In this study, we examined PKCδ-mediated phosphorylation of Hsp27 during etoposide-induced apoptosis in HeLa cells. The phosphorylation of Hsp27 correlated with the activation of PKCδ; the activation of PKCδ resulted in Hsp27 phosphorylation, and the phosphorylation of Hsp27 at Ser78 was blocked by inhibition of PKCδ. Moreover, we attempted to elucidate whether phosphorylation of Hsp27 by PKCδ regulates the progression of apoptosis.

## 2. Materials and methods

### 2.1. Materials

Rottlerin, His-Bind® resin, and recombinant PKCδ were from Merck Millipore (Germany), and SB203580 was from Cell Signaling Technology (Danvers, MA). Etoposide, cytochrome c from horse heart, anti-Flag antibody, and other chemical reagents were from Sigma–Aldrich (St. Louis, MO), unless stated otherwise. Chemicals

*Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethyleneglycoltetraacetic acid; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; PKCδ, protein kinase C δ; PMSF, phenylmethylsulfonyl fluoride.

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http://dx.doi.org/10.1016/j.bbrc.2012.08.132

used to treat cells were dissolved in dimethyl sulfoxide (DMSO), and the amount of DMSO added to cell medium was kept below 0.1% in all experiments. Anti-phosphoserine-Hsp27 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Hsp27 antibody was from Stressgen (Victoria, BC, Canada). Other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Micro BCA™ protein assay reagent was from Thermo Fisher Scientific (Rockford, IL). ECL reagent was from GE healthcare (Piscataway, NJ). [$\gamma$-$^{32}$P] ATP was from Perkin Elmer (Boston, MA). Polyfect® was from QIAGEN (Valencia, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics–antimycotics solution were from Invitrogen (Grand Island, NY). Plastic wares were from Nalge Nunc International (Rochester, NY). Complete™ Protease inhibitor cocktail was from Roche (Mannheim, Germany). Ac-DEVD-AMC was from Peptron (Daejeon, Korea).

### 2.2. Cell culture and sample preparation

HeLa cells were cultured in DMEM containing 10% FBS and 1% antibiotics–antimycotics at 37 °C, and 5% $CO_2$ as a monolayer culture. $1 \times 10^6$ cells were seeded on 100 mm dishes and incubated for 24 h before etoposide treatment (85 μM).

### 2.3. Plasmids

cDNAs for PKCδ and Hsp27 were kind gifts from Dr. Yuspa (National Cancer Institute, Bethesda, MD) [22], and Dr. Kim (Seoul National University, Republic of Korea) [23], respectively. These cDNAs were used as PCR templates for the preparation of the plasmids used in the study. For the expression of PKCδ, pCS2 + PKCδ (wild-type, full length) and pCS2 + PKCδ-CF (catalytic fragment of PKCδ) were prepared by inserting PCR products into the pCS2 + myc vector (XhoI/XbaI). A dominant negative mutant of PKCδ (PKCδ-DN) was generated by site-directed mutagenesis of pCS2 + PKCδ. For the expression of Hsp27, pCS2 + Hsp27 was generated by inserting a PCR product into the pCS2 + Flag vector (BamHI/EcoRV). Site-directed mutagenesis of pCS2 + Hsp27 was performed to generate pCS2 + Hsp27-DDD, a mutant of Hsp27 in which residues Ser15, Ser78, and Ser82 were replaced with aspartate residues. For the bacterial expression of Hsp27, pET-Hsp27 was generated by inserting a PCR product into the pET-28a(+) vector (BamHI/HindIII). pET-Hsp27-DDD was generated using the pCS2 + Hsp27-DDD vector as a PCR template and inserting the product into the pET-28a(+) vector. All DNA constructs were confirmed by DNA sequencing.

### 2.4. Transient transfection and immunoblot analysis

HeLa cells were seeded on 35 mm culture dishes ($5 \times 10^4$ cells/mL) and grown for 24 h before transfection with 3 μg of the appropriate plasmid DNA using Polyfect® in accordance with manufacturer's instructions. After the indicated times, cells were harvested and lysed in lysis buffer (10 mM HEPES (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 25 μg/mL leupeptin, 5 μg/mL pepstatin A, 40 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cell extracts were resolved by SDS–PAGE and immunoblotted.

### 2.5. PKCδ kinase reaction

The enzymatic activity of PKCδ was analyzed by an immunoprecipitation kinase assay as follows: 200 μg of protein prepared in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, and Complete™ Protease inhibitor cocktail was immunoprecipitated with 1 μg of anti-PKCδ antibody at 4 °C for 2 h. The antigen–antibody complexes were collected by incubation with protein A agarose beads, and the beads were washed three times with lysis buffer and then twice with kinase assay buffer (50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 5 mM NaF, 20 μM $Na_3VO_4$, 1 mM DTT, 0.1 μg/mL antipain, 0.1 μg/mL leupeptin, 0.1 μg/mL pepstatin and 0.1 mM PMSF). After washing, the beads were resuspended in 25 μL of kinase assay buffer, after which 25 μL of reaction buffer containing 1 μg of histone H1, 2.5 μL of lipid activators (0.5 mg/mL phosphatidylserine, 0.05 mg/mL diacylglycerol, 1 mM $Na_3VO_4$, 1 mM DTT and 1 mM $CaCl_2$) and 50 μM of ATP was added and samples were incubated for 20 min at 30 °C. Reactions were terminated by adding SDS sample loading buffer, and the reaction products were resolved by SDS–PAGE and analyzed by autoradiography. For the *in vitro* phosphorylation of Hsp27, 5 μg of Hsp27 (1 mg/mL) was treated with PKCδ in a reaction buffer containing lipid activators and 100 μM ATP, in accordance with manufacturer's instructions.

### 2.6. Pull-down assay of His-tagged proteins

*Escherichia coli* BL21(DE3) cells containing a bacterial expression vector (pET-28a(+), pET-Hsp27 or pET-Hsp27-DDD) were grown in 1 L of LB at 37 °C until $A_{600} \sim 0.6$, and were induced with 0.1 mM IPTG. After induction, cells were harvested and resuspended in 20 mL of lysis buffer (20 mM HEPES (pH 7.9), 500 mM KCl and 10 mM imidazole). The bacterial lysate was prepared by lysing cells with sonication on ice, followed by centrifugation at 4 °C, 10,000×*g* for 10 min. Recombinant N-terminal His-tagged wild-type Hsp27 and Hsp27-DDD were purified using His-Bind® resin. For the pull-down assay, 100 μL of 50% His-Bind® resin was incubated with 400 μL of the bacterial lysate with gentle shaking at 4 °C for 1 h. The beads were washed twice with 800 μL of wash buffer I (20 mM HEPES (pH 7.9), 500 mM KCl and 20 mM imidazole), and twice with 800 μL of wash buffer II (20 mM HEPES (pH 7.9), 100 mM KCl and 20 mM imidazole). The washed beads were incubated with 1 μg of recombinant cytochrome c in 200 μL of wash buffer II at 4 °C for 2 h. After washing twice with 800 μL of wash buffer II and twice with 800 μL of wash buffer III (20 mM HEPES (pH 7.9), 100 mM KCl, 40 mM imidazole and 0.1% Tween-20), the beads were boiled in SDS–PAGE sample buffer and samples were resolved by SDS–PAGE and immunoblotted.

### 2.7. Preparation of S-100 fractions and in vitro caspase-3 activation assay

S-100 fraction immunodepleted of cytochrome c was prepared from HeLa cells as described [24]. In the *in vitro* caspase activation assay the following component were sequentially combined: ice-cold buffer A (20 mM HEPES-NaOH (pH 7.4), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 μg/mL aprotinin, and 1 mM PMSF); 0.1 mM dATP, 1 μM cytochrome c, protease inhibitors prepared in buffer A; freshly thawed S-100 fraction. The mixture was incubated at 30 °C for 30 min, before caspase-3 activity was assessed using Ac-DEVD-AMC, a fluorogenic caspase-3 substrate.

## 3. Results

### 3.1. Proteolytic activation of PKCδ is temporally correlated with the activation of caspase-3 and phosphorylation of Hsp27

To study the relationship between PKCδ activation and the phosphorylation of Hsp27, we used etoposide-induced apoptosis in HeLa cells as a model system. In HeLa cells, treatment with 85 μM etoposide triggered the activation of caspase-3 and cleavage of poly(ADP-

ribose) polymerase (PARP) indicating that apoptosis had been induced. Activation of caspase-3 was detected 6 h after treatment, and the activity increased markedly 18–24 h after treatment (Fig. 1A, upper panel). In addition, PARP cleavage, an indication of caspase-3 activation, was first observed 12 h after treatment (Fig. 1A, lower panel). Proteolytic activation of PKCδ was also observed. The catalytic fragment of PKCδ was detected by immunoblotting following etoposide treatment, and the kinase activity of PKCδ increased over the time as determined by an increase in histone H1 phosphorylation (Fig. 1B). These data imply that the activation of PKCδ by proteolytic cleavage and the activation of caspase-3 are correlated during etoposide-induced apoptosis in HeLa cells.

To determine whether there was a positive feedback in which caspase-3 activity was regulated by PKCδ activity during etoposide-induced apoptosis, we measured caspase-3 activity in HeLa cells transfected with vector control, pCS2 + PKCδ-CF, or pCS2 + PKCδ-DN. Cells were harvested 24 h after etoposide treatment and caspase-3 activity was measured. The overexpression of PKCδ-CF enhanced caspase-3 activity by 38%, whereas the overexpression of PKCδ-DN suppressed the activity by 31% (Fig. 1C).

To examine whether the phosphorylation of Hsp27 changes during etoposide-induced apoptosis in HeLa cells, we monitored the phosphorylation levels of Hsp27 by immunoblotting. The levels of phosphorylated Hsp27 increased following etoposide treatment, while the total level of Hsp27 remained constant. Importantly, phospho-Ser78 was not detected before treatment but appeared during the progression of apoptosis. In contrast, phospho-Ser15 and phospho-Ser82 were detected prior to treatment and their levels only increased slightly following etoposide treatment (Fig. 1D).

### 3.2. The phosphorylation of Hsp27 at Ser78 is dependent on the activity of PKCδ

To investigate if the phosphorylation of Hsp27 is dependent on PKCδ, we studied the effects of treatment with chemical inhibitors on the levels of the various phosphorylated forms of Hsp27, as well as on total Hsp27 levels, during etoposide-induced apoptosis. The PKCδ inhibitor rottlerin reduced the phosphorylation of Hsp27 at Ser78 and Ser82, whereas SB203580, an inhibitor of p38 MAP ki-

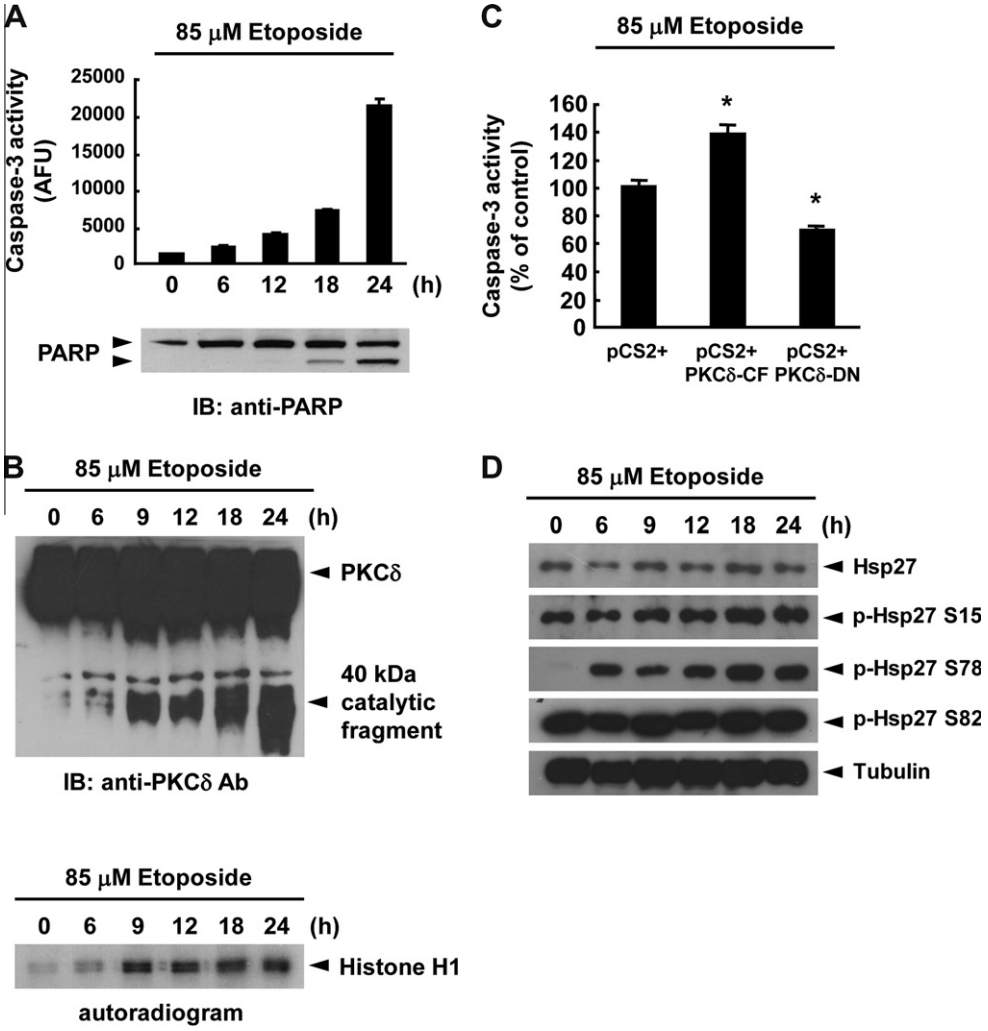


**Fig. 1.** Proteolytic activation of PKCδ is temporally correlated with caspase-3 activation and phosphorylation of Hsp27. (A) Effect of etoposide treatment on caspase-3 activity in HeLa cells. Caspase-3 activity was measured with Ac-DEVD-AMC, and the mean arbitrary fluorescence units (AFU) ± SD of three wells from one representative experiment is shown (upper panel). PARP cleavage was detected by immunoblot analysis (lower panel). (B) Proteolytic activation of PKCδ following etoposide treatment. PKCδ and the catalytic fragment were detected by immunoblot analysis (upper panel). The *in vitro* kinase activity of PKCδ was analyzed by monitoring histone H1 phosphorylation after immunoprecipitation of PKCδ (lower panel). (C) Overexpression of PKCδ-DN (pCS2 + PKCδ-DN) reduced caspase-3 activity, indicating that apoptosis was inhibited. In contrast, the overexpression of PKCδ-CF (pCS2 + PKCδ-CF) increased caspase-3 activity and enhanced apoptotic progression. The relative percentage activity of caspase-3 in comparison to the vector control (pCS2+) 9 h after etoposide treatment is shown. *Significant difference from vector control, $P < 0.05$, determined using a Student's *t*-test. (D) Effect of etoposide treatment on Hsp27 phosphorylation in HeLa cells. The level of Hsp27 phosphorylation at Ser15, Ser78 and Ser82 residues at various time-points following etoposide treatment was analyzed by immunoblotting with anti-phosphoserine-Hsp27 antibodies. The levels of total Hsp27 and tubulin are also shown.

nase, did not. However, the level of Ser15 phosphorylation remained similar with all treatments (Fig. 2A).

Similarly, the overexpression of PKCδ-DN affected the phosphorylation of Hsp27 during etoposide-induced apoptosis. Phosphorylation of Ser78 and Ser82, but not Ser15, was abolished when PKCδ-DN was overexpressed (Fig. 2B).

Since the phosphorylation of Hsp27 at Ser78 and Ser82 was dependent on the kinase activity of PKCδ during apoptosis, we wanted to determine if active PKCδ alone can phosphorylate Hsp27. The overexpression of PKCδ-DN or PKCδ-CF in HeLa cells was detected by immunoblotting (Fig. 2C, upper panel). Overexpression of PKCδ-CF increased PKCδ kinase activity in untreated cells, whereas overexpression of PKCδ-DN did not (Fig. 2C, lower panel). The levels of Hsp27 phosphorylation at Ser78 and Ser82 increased concomitantly with the increase in PKCδ kinase activity (Fig. 2D). These data suggest that the phosphorylation of Hsp27 at Ser78 and Ser82 is dependent on the kinase activity of PKCδ.

### 3.3. PKCδ phosphorylates recombinant human Hsp27 in vitro

To confirm whether PKCδ directly phosphorylates Hsp27, an *in vitro* kinase assay was performed using purified recombinant

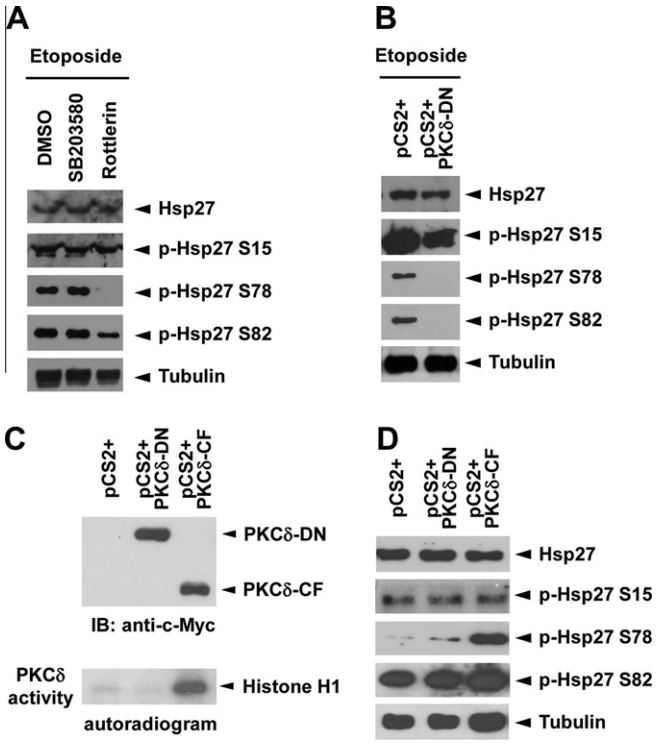


**Fig. 2.** The phosphorylation of Hsp27 at Ser78 is dependent on the activity of PKCδ. (A) Effects of SB203580 and rottlerin on the phosphorylation of Hsp27. After pretreatment with SB203580 (20 μM) or rottlerin (10 μM) for 30 min, HeLa cells were incubated with etoposide for 9 h before lysis. Immunoblotting with anti-phosphoserine-Hsp27 antibodies was performed. The levels of total Hsp27 and tubulin are also shown. (B) Effect of PKCδ-DN (pCS2 + PKCδ-DN) expression on the phosphorylation of Hsp27 in etoposide-treated HeLa cells. HeLa cells were transfected with pCS2 + or pCS2 + PKCδ-DN and incubated with etoposide for 9 h before lysis. Immunoblotting with anti-phosphoserine-Hsp27 antibodies was performed. The levels of total Hsp27 and tubulin are also shown. (C) Overexpression of PKCδ in HeLa cells. HeLa cells were transfected with pCS2+, pCS2 + PKCδ-DN, or pCS2 + PKCδ-CF. The overexpression of PKCδ-DN or PKCδ-CF was confirmed by immunoblotting (upper panel). The *in vitro* kinase activity of PKCδ was analyzed by monitoring the phosphorylation of histone H1 (lower panel). (D) Effect of PKCδ overexpression on the phosphorylation of Hsp27. HeLa cells transfected with pCS2+, pCS2 + PKCδ-DN, or pCS2 + PKCδ-CF were lysed and Hsp27 phosphorylation was analyzed by immunoblotting with anti-phosphoserine-Hsp27 antibodies. The levels of total Hsp27 and tubulin are also shown.

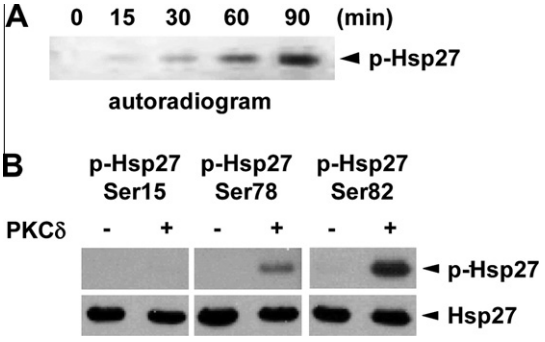


**Fig. 3.** PKCδ phosphorylates recombinant human Hsp27 *in vitro*. (A) *In vitro* phosphorylation of recombinant Hsp27 by PKCδ. (B) Immunoblotting of phosphorylated Hsp27. Protein samples from the *in vitro* kinase assay were analyzed by immunoblotting with anti-phosphoserine-Hsp27 antibodies. Each membrane was stripped and reprobed with an anti-Hsp27 antibody to determine the level of total Hsp27.

Hsp27 and PKCδ. PKCδ-mediated phosphorylation of recombinant Hsp27 was observed in an autoradiogram, and the level of phosphorylation increased in a time-dependent manner (Fig. 3A). Phosphorylation of Hsp27 at Ser78 and Ser82, but not at Ser15, was detected (Fig. 3B).

### 3.4. The anti-apoptotic binding of Hsp27 to cytochrome c is regulated by PKCδ-mediated phosphorylation

To investigate the role of phosphorylated Hsp27 in activating the caspase cascade, the phosphomimetic Hsp27 mutant, Hsp27-DDD [25], was overexpressed in HeLa cells. While the overexpression of wild-type Hsp27 inhibited the activation of caspase-3 by 38%, the overexpression of Hsp27-DDD did not alter caspase-3 activity indicating that the progression of apoptosis was unaffected (Fig. 4A). Similarly, recombinant Hsp27-DDD did not affect caspase-3 activity in the cell-free *in vitro* system, whereas recombinant wild-type Hsp27 inhibited the activation of caspase-3 (Fig. 4B). These results indicate that caspase-3 is specifically activated following phosphorylation of Hsp27.

To investigate if the binding of Hsp27 to cytochrome c is regulated by phosphorylation, His-tagged wild-type Hsp27 or Hsp27-DDD was mixed with cytochrome c *in vitro*. After incubation for 2 h, the mixture was pulled down with protein A agarose beads linked to an anti-cytochrome c antibody, and samples were analyzed by immunoblotting. Wild-type Hsp27 bound to cytochrome c, whereas Hsp27-DDD did not. This indicates that phosphorylated Hsp27 does not bind cytochrome c (Fig. 4C). To determine whether binding of Hsp27 to cytochrome c is involved in the activation of caspase-3, we studied the activation of caspase-3 in the presence of recombinant Hsp27 *in vitro*. Wild-type Hsp27 inhibited the activation of caspase-3, while phosphorylated Hsp27 prepared by incubation with PKCδ, did not (Fig. 4D). These data indicate that the PKCδ-mediated phosphorylation of Hsp27 abolishes its binding to cytochrome c.

## 4. Discussion

In this study, we established a relationship between PKCδ activity and the association of Hsp27 with cytochrome c during etoposide-induced apoptosis in HeLa cells. Since Hsp27 is reported to be a negative regulator of cytochrome c-dependent activation of procaspase-3 [9,10] and is a substrate of PKCδ [16], we hypothesized that PKCδ regulates the progression of apoptosis by phosphorylating Hsp27.

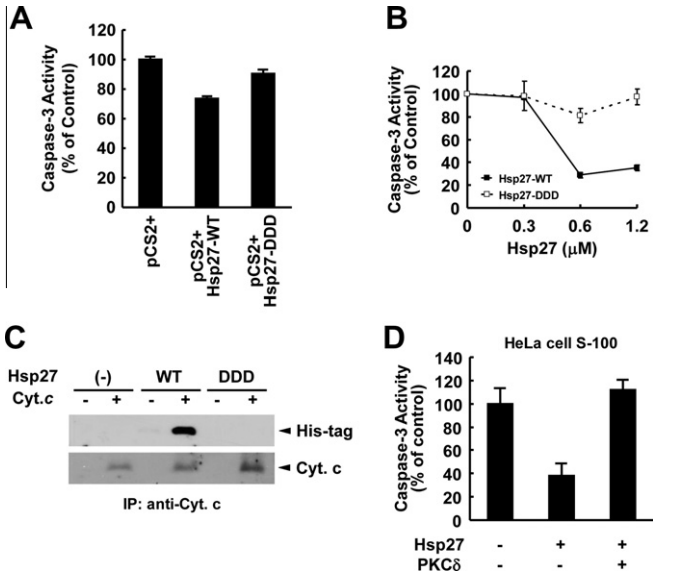


**Fig. 4.** The anti-apoptotic binding of Hsp27 to cytochrome c is regulated by PKCδ-mediated phosphorylation. (A) The activation of caspase-3 is regulated by phosphorylation of Hsp27. The activity of caspase-3 in etoposide-treated HeLa cells overexpressing wild-type Hsp27 or Hsp27-DDD is shown. HeLa cells transfected with pCS2+, pCS2 + Hsp27, or pCS2 + Hsp27-DDD were treated with etoposide for 24 h, lysed, and caspase-3 activity was measured with Ac-DEVD-AMC. The relative percentage activity of caspase-3 in comparison to the vector control ± SD is shown. (B) Caspase-3 activation in the presence of recombinant Hsp27 proteins in a cell-free reaction. S-100 fractions prepared from HeLa cells were sequentially mixed with recombinant wild-type Hsp27 or Hsp27-DDD, and then with additives containing dATP and cytochrome c. Samples were incubated for 30 min at 30 °C. Mean ± SD is shown. (C) *In vitro* pull-down assay. Recombinant wild-type Hsp27 or Hsp27-DDD protein was incubated in the presence or absence of cytochrome c, and immunoprecipitation with an anti-cytochrome c antibody was performed. (D) Caspase activation in the presence of phosphorylated Hsp27 in a cell-free reaction. S-100 fractions prepared from HeLa cells were sequentially mixed with recombinant Hsp27 (0.5 μM), and then with additives containing dATP and cytochrome c. Samples were incubated for 30 min at 30 °C. Mean ± SD is shown.

We observed proteolytic activation of PKCδ during etoposide-induced apoptosis in HeLa cells (Fig. 1B), and a positive feedback (Fig. 1C), as previously reported [17]. As phosphorylation regulates the activity of Hsp27 [26], we analyzed the phosphorylation states of three serine residues, Ser15, Ser78, and Ser82. Ser15 and Ser82 had basal levels of phosphorylation, while Ser78 phosphorylation was not detected in untreated HeLa cells. The level of phosphorylation at all three residues was higher following etoposide treatment (Fig. 1D). We suspect that the basal phosphorylation of Ser15 and Ser82 in untreated cells is due to the activities of kinases, such as MAPKAP kinases-2 and -3 [25].

The increased level of Hsp27 phosphorylation following etoposide treatment, especially at Ser78, may be related to the activity of PKCδ. The phosphorylation of Hsp27 at Ser78 and Ser82 was reduced in the presence of a PKCδ inhibitor (Fig. 2A), whereas overexpression of PKCδ-CF increased the phosphorylation of these residues, even without apoptotic stimulation (Fig. 2D). In contrast, the phosphorylation of Ser15 did not appear to be affected by PKCδ, since there was little change in the phosphorylation of this residue in the presence of PKCδ-CF or PKCδ-DN (Fig. 2D).

Since the phosphorylation of Hsp27 was dependent on PKCδ in HeLa cells, we also determined whether PKCδ phosphorylates Hsp27 *in vitro*. Radioactive kinase assays confirmed that PKCδ phosphorylated Hsp27 at Ser78 and Ser82 (Fig. 3B). We next addressed the role of Hsp27 phosphorylation in the progression of apoptosis. Overexpression of Hsp27-DDD did not alter the activation of caspase-3, whereas overexpression of wild-type Hsp27 reduced caspase-3 activity and thereby inhibited apoptotic progression (Fig. 4A). It is likely that the overexpressed wild-type Hsp27 protein was not efficiently phosphorylated by endogenous PKCδ, and consequently apoptosis was inhibited by the high level of unphosphorylated Hsp27. The negative regulation of apoptosis by Hsp27 was also observed by monitoring the *in vitro* activation of caspase-3. The activation of caspase-3 was inhibited by wild-type Hsp27, but not by Hsp27-DDD (Fig. 4B).

It has been reported that Hsp27 negatively regulates cell death by binding to cytochrome c [9,10], and we hypothesized that this binding is regulated by phosphorylation. Whereas recombinant wild-type Hsp27 bound to cytochrome c in an *in vitro* pull-down assay, Hsp27-DDD did not (Fig. 4C). Furthermore, unphosphorylated Hsp27 inhibited the activation of caspase-3 in a cell-free reaction, while caspase-3 activity was unaffected by phosphorylated Hsp27 (Fig. 4D).

The data indicate that the negative regulation of cytochrome c by Hsp27 is switched off by PKCδ-mediated phosphorylation of Hsp27 mainly at Ser78. We suspect that Hsp27 is phosphorylated at Ser15 and Ser82 in untreated cells, and that the phosphorylation at Ser78 plays a key role in preventing Hsp27 binding to cytochrome c. In summary, the phosphorylation of Hsp27 at Ser78 is mediated by PKCδ during etoposide-induced apoptosis. Following phosphorylation at Ser78, Hsp27 releases cytochrome c, thereby triggering formation of the apoptosome and the progression of apoptosis.

## Acknowledgment

This research was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) to S.H. Joo (2010-0003008).

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