HSP25 Inhibits Protein Kinase Cδ-mediated Cell Death through Direct Interaction*

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Yoon-Jin Lee‡§, Dae-Hoon Lee‡§§, Chul-Koo Cho, Sangwoo Bae‡, Gil-Ja Jhon†, Su-Jae Lee, Jae-Won Soh**, and Yun-Sil Lee‡‡‡

From the ‡Laboratory of Radiation Effect and §Laboratory of Experimental Radiation Therapeutics, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, §Division of Molecular Life Science, Ewha Woman’s University, Seoul 120-750, and **Laboratory of Signal Transduction, Department of Chemistry, Inha University, Incheon, 402-751 Seoul, Korea

Heat shock protein 25 (HSP25) interferes negatively with apoptosis through several pathways that involve its direct interaction with cytochrome c or Akt. Here we show that HSP25 inhibits protein kinase C (PKC) δ-mediated cell death through direct interaction. HSP25 binds to kinase-active PKCδ to inhibit its kinase activity and translocation to the membrane, which results in reduced cell death. Deletion constructs of HSP25 and PKCδ identified amino acids 90–103 of HSP25 as the C-terminal V5 region of PKCδ as binding sites. In addition, the interaction between HSP25 and PKCδ induced HSP25 phosphorylation at Ser-15 and Ser-86, and these phosphorylations permitted HSP25 release from PKCδ. Based on these observations, we propose that after PKCδ activation, HSP25 binds to the exposed V5 region of PKCδ. This novel function of HSP25 accounts for its cytoprotective properties via the inhibition of PKCδ and the enhancement of HSP25 phosphorylation.

Small heat shock protein (HSP)1 has been suggested to protect cells against apoptotic cell death triggered by hyperthermia, ionizing radiation, oxidative stress, Fas ligand, and cytotoxic drugs (1–5). Several mechanisms have been proposed to account for HSP27-mediated apoptotic protection, for example, via its specific interaction with cytochrome c released from mitochondria into the cytosol, which prevents apoptosis formation (6, 7). The elimination of unfolded protein via the extralysosomal, energy-dependent, ubiquitin-proteasome degradation pathway is another mechanism that contributes to cell protection from stressful stimuli (8). HSP27 binds to polyubiquitin chains as well as 26 S proteasomes, and the ubiquitin-proteasome pathway is involved in the activation of transcription factor NF-κB by degrading its main inhibitor I-κBα (8).

Moreover, phosphorylated HSP27 has been shown to bind an adaptor protein Daxx and then to inhibit Fas-mediated apoptosis (9). Interaction between HSP27 and Akt is necessary for Akt activation, and this is followed by dissociation of phosphorylated HSP27 from Akt (10).

We reported recently that the radioprotective effect of HSP25 involves delayed cell growth (11, 12) and HSP25-mediated Mn-SOD gene expression (13, 14). HSP25 overexpression down-regulates ERK1/2 expression, and HSP25-mediated ERK2 suppression is involved in HSP25-induced radioresistance and cell cycle delay (15). In addition, attenuated oxidative stress-induced apoptosis by HSP25 overexpression was found to be due to the inhibition of the PKCδ-mediated ERK1/2 pathway and to the induction of the Mn-SOD gene (14), and HSP25 also inhibits radiation-induced PKCδ-mediated reactive oxygen species production (16).

Radiation and many anticancer drugs are known to kill tumor cells by inducing apoptosis. However, a defect in the apoptotic process can lead to resistance to anticancer drugs or radiotherapy. Several members of the protein kinase C (PKC) family serve as substrates for caspases, and the PKCδ isozone has been intimately associated with apoptosis. The activation of PKCδ was found to be associated with cell cycle progression inhibition (17), and PKCδ down-regulation was found to be associated with tumor promotion (18), suggesting that PKCδ may have a negative effect on cell survival. In addition, the proteolytic activation of PKCδ has since been associated with the apoptosis induced by DNA damage, including that caused by UV light, ionizing radiation, cisplatin, etoposide, and doxorubicin (19–24).

Moreover, several investigators have confirmed that the ectopic expression of the PKCδ catalytic fragment results in cell death (25–28). Mizuno et al. (26) showed that the kinase activity of a PKCδ catalytic fragment during apoptosis may be a key participant in the late stages of apoptosis.

In the present study, we observed for the first time that HSP25 binds directly to the V5 region of kinase-active PKCδ. This results in a dual form of HSP25-mediated cytoprotection, namely through the inhibition of PKCδ activity, thereby blocking apoptosis, and via the phosphorylation of HSP25, which increases its cytoprotective effect. In addition, these two types of HSP25-mediated apopotic effect were found to be triggered by radiation or oxidative stress.

MATERIALS AND METHODS

Reagents—Rottlerin (PKCδ inhibitor), phorbol 12-myristate 13-acetate, and H2O2 were purchased from Calbiochem. Anti-HSP27, anti-phospho-HSP27 (Ser-15), anti-phospho-HSP27 (Ser-82), anti-HA, anti-PKCδ, anti-
lamin B, anti-β-actin, and anti-GFP antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-PKCα (Ser-634) and phospho-PKCθ (pan) were from Cell Signaling Technology (Beverly, MA). Anti-phosphoryostryl antibody, PKCδ protein (recombinant protein expressed in E. coli), and PKCδ polyclonal antibody was from BD Biosciences.

**Plasmids**—Wild type mouse HSP25 (GenBank™ accession number XM124655) was cloned into pcDNA4HisMaxC (Invitrogen), which contains a N-terminal His tag. The phosphorylation mutant constructs HSP25-S15A, HSP25-S15D, HSP25-S643A, HSP25-S643D, HSP25-S662A, and HSP25-S662D were constructed by PCR (29) by using the overlap extension primers. Serine was replaced by alanine or aspartate to generate the phosphorylation-deficient or phosphorylation-mimicking mutants of HSP25, respectively. The dimerization mutant HSP25-C141A was constructed using the same method. PCR products were digested with EcoRI and cloned into the EcoRI site of pcDNA4HisMaxC to construct expression vectors of the His-tagged mutant HSP25 protein. PCR products were also cloned into pEGFP-N1 vector (Clontech) to express GFP-tagged mutant HSP25 proteins. The internal deletion mutants were constructed in HSP25-containing pcDNA4HisMaxC vector by introducing two Sall sites in the HSP25-coding sequence using deletion primers. This was followed by restriction digestion. For the HSP25 construct, HSP25-(91–187) was amplified by PCR, and the PCR products obtained were digested and cloned into pEGFP-N1 vector (Clontech).

Wild type PKCθ (GenBank™ accession number AY545076), the regulatory domain (amino acids 2–333, REG), the catalytic domain (amino acids 334–674, CAT), and the dominant-negative catalytic domain (amino acids 334–674, CAT-KR) were cloned into pHACE that contains a N-terminal FLAG tag. To construct the pHACE-goal (1:100) against anti-HA antibody and 30 μl of aprotinin (Roche Diagnostics), 1.25 mg/ml lysozyme, and 11.2 μg/ml pepstatin A, 10 μl (1/100) of cell extracts were immunoprecipitated with 3 μg of anti-HA antibody and 30 μl of protein G-Sepharose for 3 h at 4 °C. The immunoprecipitates were washed twice with PCR extraction buffer and then incubated with the PCR buffer (50 mM HEPES (pH 7.5) containing 0.5 mM dithiothreitol, 2.5 mM EGTA, and 10% glycerol) containing protease inhibitors (10 μg/ml each of aprotinin and leupeptin and 0.1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM NaF, 0.1 mM Na3VO4, and 10 mM β-glycerophosphate). HA-tagged PKCθ proteins from 300 μg of cell extracts were immunoprecipitated with 3 μg of an anti-HA antibody and 30 μl of protein G-Sepharose for 3 h at 4 °C. The immunoprecipitates were washed twice with PCR extraction buffer and then incubated with the PCR buffer (50 mM HEPES (pH 7.5) containing 0.5 mM dithiothreitol, 2.5 mM EGTA, 1 mM NaF, 0.1 mM Na3VO4, and 10 mM β-glycerophosphate) and then resuspended in 20 μl of PCR reaction buffer. The kinase assay was initiated by adding 40 μl of PCR reaction buffer containing 5 μg of dephosphorylated MBP protein (Upstate Biotechnology, Inc.) or His-HSP25 protein and 5 μl of [γ- 32P]ATP. Reactions were carried out for 30 min at 30 °C and then added to SDS-PAGE buffer. Western blots were then boiled for 5 min before the reaction products were analyzed by SDS-PAGE and autoradiography.

**Preparation of Recombinant HSP25 Proteins**—Vectors for the expression of recombinant HSP25 were prepared by inserting the HSP25 wild type into the EcoRI site of respective expression vectors pProExHTa vector (Invitrogen). The resulting plasmid was transformed into Escherichia coli host strain (strain H9252) containing pProExHTa vectors (His-HSP25) after treating with 0.15 M isopropyl-1-thio-D-galactopyranoside for 4 h at 30 °C. Bacteria were lysed in a buffer containing 50 mM HEPES (pH 7.5), 0.45 mM NaCl, 5 mM dithiothreitol, Complete protease inhibitor (Roche Diagnostics), 1.25 mg/ml lysozyme, and 11.2 μg/ml DNase I (Sigma). His-HSP25 proteins were extracted from inclusion bodies by incubating for 1 h followed by vigorous shaking at 37 °C in 25 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 2% (v/v) Triton X-100, 5 mM dithiothreitol, and Complete protease inhibitor. The solubilized His-HSP27 proteins were affinity-purified using nickel-nitrioltriacetic acid-agarose beads (Qiagen).

**Immunoprecipitation**—Cells (1 × 10^7) were lysed in immunoprecipitation buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40). After centrifuging (10 min at 15,000 × g) to remove particulate material, the supernatant was incubated with antibodies (1:100) against anti-HS27 or PKCθ with constant agitation at 4 °C. Immune complexes were precipitated with protein A-Sepharose (Sigma) and analyzed by SDS-PAGE by enhanced chemiluminescence (Amer sham Biosciences).

**Cell Transfection**—Pre-designed siRNA for human PKCθ (HSP25-C141A) was purchased from Ambion, Inc. (Austin, TX). Cells were transfected with the siRNAs for 48 h using Lipofectamine TM 2000 (Invitrogen). A mixture of 250 μl of Opti-MEM medium (Invitrogen) and 20 μl of Lipofectamine™ 2000 was incubated for 5 min at room temperature and then combined with 100 μl of siRNA diluted with 250 μl of Opti-MEM. The resulting mixture (500 μl) was incubated for 20 min at room temperature to allow complex formation and then overlaid on each well containing the cells to a final concentration and then overlaid on each well containing the cells to a final concentration.
volume of 2.5 ml/60-mm dish. The transient transfection of all cell types was carried out using Plus Lipofectamine™ reagent (Invitrogen) and Lipofectamine™ reagent.

Histidine Pull-down Assay in Vitro—The *in vitro* transcription and translation of PKCδ was performed using a TnT T7 Quick Master Mix kit (Promega, Madison, WI) in the presence of [35S]methionine, according to the manufacturer’s protocol. An aliquot of His-tagged HSP25 protein, bound to nickel-nitriilotriacetic acid-agarose beads, was incubated with 5 μl of *in vitro* translated PKCδ in 500 μl of NETN buffer (100 mM NaCl, 1 mM EDTA (pH 8.0), 20 mM Tris-HCl (pH 8.0), 0.2% Nonidet P-40, 10 mM imidazole) for 2 h. Beads were washed five times with 1 ml of NETN buffer, resuspended in Laemmli sample buffer, and subjected to SDS-PAGE before autoradiography.

Histidine Pull-down Assay—Histidine-tagged DNA constructs were transfected into the cells by using Lipofectamine Plus reagent. After incubation for 48 h, cells were harvested, and whole cell extracts were prepared as described previously. Cell extracts were mixed and incubated with nickel-nitriilotriacetic acid-agarose beads for 30 min at 4 °C in the presence of 10 mM imidazole. After washing the resin with buffer containing 10 mM imidazole, proteins were recovered by suspension in Laemmli sample buffer and then subjected to SDS-PAGE and Western blot analysis using anti-PKCδ or anti-HA antibodies.

**RESULTS**

Interaction between HSP25 and the Catalytic Domain of PKCδ and PKCδ Kinase Activity Is Necessary for Its Interaction with HSP25—As HSP25 overexpression was found to inhibit PKCδ activity in our previous study (14), and another HSP, HSP70, was reported to bind PKCδ (32), we examined possible binding between HSP25 and PKCδ. HSP25 was found to coimmunoprecipitate with PKCδ in whole cell lysates of L929 and Jurkat T cells overexpressing HSP25 (Fig. 1A). To confirm binding between PKCδ and HSP25, several deletion mutants of PKCδ were made (Fig. 1B). Immunoprecipitation revealed that the catalytic domain of PKCδ (CAT), but not the regulatory domain of PKCδ (REG), is an HSP25 binding target. When rotterlin, a specific PKCδ inhibitor, was pretreated, HSP25 binding disappeared, suggesting that the kinase activity of PKCδ is important for the interaction between HSP25 and PKCδ (Fig. 1C). A loss of binding activity after HSP25 antisense transfection confirmed the specific interaction between HSP25 and PKCδ (Fig. 1C), which was further confirmed by probing histidine pull downs of HSP25 using several domain mutants of PKCδ fused with a HA tag. Consistently, wild type (WT) PKCδ interacted with HSP25, and PKCδ-CAT mediated this binding (Fig. 1D). TR cells, which were derived as a thermoresistant clone of RIF and which show high expression levels of HSP25, were used to confirm the binding potential of endogenous HSP25 with PKCδ. Moreover, transfection of PKCδ-CAT into TR cells was found to increase the interaction between HSP25 and PKCδ-CAT (Fig. 1E). However, we did not detect any interaction between HSP70 and PKCδ (data not shown). Because rotterlin pretreatment completely inhibited the interaction between HSP25 and PKCδ, we hypothesized that kinase-active PKCδ is required for this interaction. Thus we transfected PKCδ-CAT-K376R (CAT-KR), which lacks PKCδ kinase activity, and performed immunoprecipitation experiments. Neither PKCδ-CAT-KR (Fig. 1F, 5th lane) nor CAT-KR interacted with HSP25 (Fig. 1F, 4th lane). In contrast, PKCδ-CAT bound to HSP25 (Fig. 1F, 3rd lane). Moreover, pretreatment with PKCδ activators such as PMA or H2O2 increased the interaction between HSP25 and PKCδ, and this was accompanied by an increase in the MBP kinase activity of PKCδ; however, rotterlin pretreatment reduced HSP25-PKCδ binding, even though MBP phosphorylation by PKCδ was not completely inhibited (Fig. 1G) (H2O2 treatment data not shown). Because PKCδ-CAT-KR did not interact with HSP25, we used ATP and ATPβS to confirm the importance of the kinase activity of PKCδ. An interaction was observed between HSP25 and PKCδ after treating with H2O2, and this binding was dramatically increased by cotreating H2O2 with ATP. However, co-treatment with ATPβS (often regarded as a nonhydrolyzable ATP analogue and is used as an inhibitor of phosphatases and ATPases (33)) blocked this interaction (Fig. 1H), suggesting that PKCδ kinase activity (in terms of the binding and hydrolysis of ATP) is important for the interaction between HSP25 and PKCδ.

Interaction between PKCδ-CAT and HSP25 Inhibits PKCδ-mediated Cell Death—Because PKCδ has been reported to induce apoptosis (34), we examined if the interaction between HSP25 and PKCδ affects PKCδ-mediated apoptosis. In L929 cells cotransfected with PKCδ and HSP25, PKCδ activation by H2O2 was lower than in cells transfected with only PKCδ (Fig. 2A). In addition, the tyrosine phosphorylation of PKCδ, which correlates with its kinase activity (35) and which induces many PKCδ-mediated effects like the induction of apoptosis (36), was also inhibited by HSP25 overexpression (Fig. 2B). In addition, we observed that H2O2 treatment increased PKCδ kinase activity and that the addition of HSP25 protein to the cell lysates reduced PKCδ kinase activity (Fig. 2C), which shows the inhibition of PKCδ kinase activity by HSP25. PKCδ-WT increased cell death due to H2O2 treatment, and PKCδ-CAT appeared to mediate this effect, although PKCδ-REG and PKCδ-CAT-KR, which do not have PKCδ kinase activity, reduced H2O2-mediated cell death (Fig. 2D). Moreover, radiation-induced DNA fragmentation (Fig. 2E) and cytochrome c release to the cytosol were inhibited when cells were pretreated with rotterlin. The effect of HSP25 overexpression was similar to that of rotterlin in terms of cytochrome c release (Fig. 2F).

Amino Acids 90–103 of HSP25 and V5 Region Located in the Catalytic Domain of PKCδ Are the Binding Sites of Each Molecule—To provide definitive evidence that HSP25 prevents cell death by binding to PKCδ, several mutants of HSP25 were prepared. L929 cells were transiently transfected with an empty plasmid (control) or a plasmid containing wild type or a mutated HSP25 cDNA (refer to Fig. 3A for details of the mutants used). Immunoprecipitation studies demonstrated that deletion mutants HSP25-(Δ90–103) and HSP25-(Δ32–49) did not bind to PKCδ-CAT, whereas HSP25 mutants Δ53–91, Δ106–132, and Δ133–142 were able to bind to PKCδ-CAT as efficiently as wild type HSP25 (Fig. 3B). His pull-down analysis also confirmed that amino acids 90–103 of HSP25 are necessary for PKCδ-CAT binding (data not shown). When H2O2-induced cell death was examined, a protective effect was observed after transfecting HSP25 Δ53–91, Δ106–132, or Δ133–142 mutants, which interacted with PKCδ-CAT like the wild type (Fig. 3C). However, cell death remained at the control level after transfection with HSP25-(Δ90–103) or HSP25-(Δ32–49) mutant. To identify the PKCδ binding locus, deletion constructs of PKCδ-CAT were made (refer to Fig. 3D) and transfected into HSP25-overexpressing L929 cells. Immunoprecipitation analysis revealed that the deletion mutants Δ343–629, Δ344–500, and Δ403–629 did not bind to HSP25, whereas mutant Δ373–647 bound to HSP25 as efficiently as PKCδ-CAT (Fig. 3E), suggesting that the amino acid sequence 630–674 (the V5 region) of PKCδ is a binding site. To determine whether the V5 region of PKCδ binds directly to the amino acid sequence 90–103 of HSP25, immunoprecipitation was performed using a GFP-tagged 90–103-amino acid sequence of HSP25 or an RFP-fused PKCδ V5 region. HSP25 was found to bind to PKCδ-CAT and to the V5 region, and the amino acid 90–103 sequence of HSP25 also directly bound to the PKCδ-V5 region (Fig. 3F). Confocal analysis also revealed the colocalization of the V5 region of PKCδ and of the amino acid 90–103 sequence of HSP25 in cytosol (data not shown). These findings demonstrate that...
FIG. 1. Interaction between HSP25 and kinase-active PKCδ catalytic domain. A, immunodetection of HSP25 or PKCδ in control and in HSP25-overexpressing L929 cells or Jurkat T cells, with previous immunoprecipitation (IP) of PKCδ. B, schematic drawing of PKCδ point or deletion mutants. Wild type PKCδ (PKCδ-WT), Lys-Arg point mutant of wild type PKCδ (PKCδ-WT-KR), regulatory domain deleted mutant of PKCδ (PKCδ-CAT), catalytic domain deleted mutant (PKCδ-REG). C, after immunoprecipitation of PKCδ in lysates from control or HSP25-overexpressed L929 cells transfected with the indicated HA-tagged PKCδ point or deletion mutants or the HSP25 antisense construct (HSP25AS), or pretreated with rottlerin (5 μM), the immunodetection of HSP25 and PKCδ was performed. D, the indicated HA-tagged PKCδ deletion mutants were transfected to L929 cells, and cell lysates were incubated with immobilized His-HSP25 or His vector. Retained HA proteins were detected by Western blotting using an anti-HA antibody. His fusion proteins are also shown. The transfection efficiencies of HA-tagged or HSP25 vectors were confirmed by Western blotting using anti-HA or anti-HSP25 antibody. E, PKCδ was immunoprecipitated in lysates from control or HSP25-overexpressing L929 cells, RIF, or TR (thermosensitive clone of RIF) cells with or without PKCδ-CAT transfection, and HSP25 was immunodetected. F, immunoprecipitation of HA in lysates from control or HSP25-overexpressing L929 cells after transfecting HA-tagged PKCδ-CAT, PKCδ-CAT-KR, or PKCδ-CAT-REG vectors. HSP25 protein was immunodetected using anti-HSP25 antibody. Cellular proteins were extracted after lysis with PKC extraction buffer. HA-tagged PKC proteins from 300 μg of cell extracts were immunoprecipitated using an anti-HA antibody and protein G-Sepharose. Immune complex kinase reactions were performed in the presence of GST-MBP substrate and [γ-32P]ATP. The transfection efficiencies of HA-tagged or HSP25 vectors were confirmed by Western blotting using anti-HA or anti-HSP25 antibody. G, immunoprecipitation of PKCδ in lysates from control or HSP25-overexpressing L929 cells 30 min after adding PMA (100 nM) with or without pretreatment with rottlerin (5 μM) for 30 min, followed by HSP25 or PKCδ immunodetection using anti-HSP25 or anti-PKCδ antibodies. Cellular proteins were extracted after lysis with PKC extraction buffer. HA-tagged PKC proteins from 300 μg of cell extracts were immunoprecipitated using an anti-HA antibody and protein G-Sepharose. Immune complex kinase reactions were performed in the presence of GST-MBP substrate and [γ-32P]ATP. H, immunoprecipitation of PKCδ in lysates of control or HSP25-overexpressing L929 cells pretreated with H2O2 (2 mM) with or without ATP or ATPγS (100 μM) pretreatment. Protein extracts (500 μg) were immunoprecipitated with anti-PKCδ antibody, and immunodetection was performed using antiphosphotyrosine (p-Tyr) antibody. IB, immunoblot.
amino acids 90–103 of HSP25 are a binding site for the V5 region of PKC8.

HSP25 Binds to the Unphosphorylated PKC8-V5 Region and Inhibits PKC8 Membrane Translocation—Binding between HSP70 and PKC8 II is dependent on the phosphorylation status of PKC8 II (32), and several autophosphorylation sites have been identified in the V5 region of PKC8 (37–40). Moreover, PKC activation has been reported to regulate the autophosphorylation of these sites (37, 38). To elucidate whether autophosphorylation status affects the interaction between HSP25 and PKC8, we generated Ser-643 and Ser-662 phosphorylation-deficient and phosphorylation-mimicking mutants of PKC8-CAT. Coimmunoprecipitation revealed that the phosphorylation-deficient mutants (S643A, S662A, and S643A/S662A) bound to HSP25, whereas the phosphorylation-mimicking mutants (S643D, S662D, and S643D/S662D) did not, suggesting that the nonphosphorylated form of PKC8 V5 is important for HSP25 binding (Fig. 4A).

In the case of PKC8-CAT-KR, which does not have kinase activity, all mutants failed to bind HSP25 (Fig. 4A). His pull-down analysis was used to confirm the above results. Because it has been reported that the autophosphorylation of the V5 region affects the membrane translocation of PKC8 (41), we examined PKC8 phosphorylation status at Ser-643 and Ser-662. After treating the cells with H2O2 for 30 min, phosphorylated PKC8 at Ser-643 and Ser-662 was translocated to the particulate fraction, and this was sustained 60 min later. However, in the case of HSP25-overexpressing cells, no phosphorylated PKC8 was translocated to the particulate fraction in accompany with the reduced total amount of phosphorylated PKC8 (Fig. 4B), which suggests that the interaction...
between the V5 region of PKCδ and HSP25, which blocks the phosphorylation of PKCδ at Ser-643 and Ser-662, inhibits PKCδ translocation to the particulate fraction.

Binding of Kinase-active PKCδ with HSP25 Induces HSP25 Phosphorylation—PKCδ is a family of the phospholipid-dependent serine/threonine protein kinases, members of which activate various proteins by phosphorylation. Moreover, HSP25 is known to be phosphorylated at Ser-15 and Ser-662 by PKCδ. To elucidate whether PKCδ affects HSP25 phosphorylation status after interaction, we examined the kinetics of binding between PKCδ and HSP25, PKCδ activity, and HSP25 phosphorylation. After adding H₂O₂ to HSP25-overexpressing cells, binding of HSP25 and PKCδ was observed within 10 min and peaked at 30 min, which was similar to the PKCδ kinase activity profile (using MBP as a substrate). To determine whether PKCδ can affect HSP25 phosphorylation, PKCδ kinase activity was examined using HSP25 as a substrate in the absence or presence of [γ-32P]ATP. 32P incorporation was found to increase in a time-dependent manner with maximal kinase activation 90 min after adding H₂O₂, suggesting that PKCδ acts as a specific kinase for HSP25 and that it activates HSP25 after interacting with HSP25. Western blot studies on the phosphorylation status of Ser-663 and Ser-662 of HSP25, using specific antibodies, showed that HSP25 phosphorylation peaked 90 min after adding H₂O₂ (Fig. 5A). When PKCδ and HSP25 proteins were directly mixed in the presence of [γ-32P]ATP, increased HSP25 (25 kDa) phosphorylation was accompanied by an increase in 32P-labeled PKCδ (78 kDa) protein, as shown Fig. 5B. Increased phosphorylation of Ser-663 and Ser-662 by PKCδ treatment was also inhibited by rottlerin pretreatment (Fig. 5C). Cotransfection of HSP25 with PKCδ-CAT-KR also reduced phosphorylation at these two sites, whereas PKCδ-CAT-CAT-KR increased the phosphorylation of HSP25 (Fig. 5D), which indicates that the interaction between HSP25 and PKCδ induces HSP25 phosphorylation at Ser-15 and Ser-662. Because the degree of inhibition by CAT-KR was higher at the Ser-662 site, we hypothesized that the phosphorylation of HSP25 at Ser-662 is affected more by the binding of HSP25 and PKCδ. To elucidate if the direct interaction between PKCδ and HSP25 affects HSP25 phosphorylation, S643A/S662A (serine phosphorylation sites replaced with Ala; HSP25 binding capaci-
HSP25 Binds to PKC\(\delta\)

**A.** Dephosphorylation-dependent binding of the PKC\(\delta\) by HSP25. A, immunodetection of HSP25 (in lysates after PKC\(\delta\) immunoprecipitation) of transfected L929 cells of HA-fused PKC\(\delta\)-CAT or PKC\(\delta\)-CAT-KR vectors containing the wild type and the indicated Ser-643 and Ser-662 point mutants. The transfection efficiencies of HA-tagged vectors were confirmed by Western blotting using anti-HA antibody (upper panel). Transfected L929 cells were incubated with immobilized His-HSP25 vectors. Retained HA protein was detected by Western blotting using an anti-HA antibody. His fusion proteins are also shown in the same gel. The transfection efficiencies of HA-tagged vectors were confirmed by Western blotting using anti-HA antibody. B, soluble and particulate fractions were isolated from control and HSP25-overexpressing L929 cells, and Western blotting was performed for PKC\(\delta\) phosphorylation at Ser-643 or Ser-662 using specific antibodies. IP, immunoprecipitation; IB, immunoblot.

**B.** HSP25 binds to PKC\(\delta\)-CAT has a cytoprotective effect. HSP25 (S15D/S86D) also exhibited a cytoprotective effect even though it did not bind PKC\(\delta\). Moreover, the binding of the phosphorylation-deficient mutant S15A/S86A to PKC\(\delta\) inhibited H\(_2\)O\(_2\)-induced PKC\(\delta\) activation, whereas the phosphorylation-mimicking mutant (S15D/S86D), which did not bind PKC\(\delta\), did not affect H\(_2\)O\(_2\)-induced PKC\(\delta\) activation (Fig. 6E), suggesting that unphosphorylated HSP25 binds kinase-active PKC\(\delta\) and that this interaction inhibits PKC\(\delta\) activity. Moreover, the interaction between unphosphorylated HSP25 and PKC\(\delta\)-CAT inhibited the cell death triggered by H\(_2\)O\(_2\) (Fig. 6F), suggesting that unphosphorylated HSP25 binds kinase-active PKC\(\delta\) and that this interaction inhibits PKC\(\delta\) activity. Moreover, the interaction between unphosphorylated HSP25 (S15A/S86A) and PKC\(\delta\)-CAT has a cytoprotective effect. HSP25 (S15D/S86D) also exhibited a cytoprotective effect even though it did not bind to PKC\(\delta\)-CAT, suggesting the importance of the PKC\(\delta\)-independent protective activity of HSP25, which has been reported previously (44).

Interaction between HSP25 and PKC\(\delta\) Correlates with Radiosensitivity in Lung Carcinoma Cell Lines—To determine the physiological relevance of the HSP25 and PKC\(\delta\) interaction, three types of lung cancer cells with different HSP27 expression and radiosensitivities were examined; NCI-H1299 showed highest HSP27 expression, and NCI-H460 least. HSP27 expression levels were found to correlate with radiation survival (Fig. 7A). When the interaction between HSP27 and PKC\(\delta\) was checked by coimmunoprecipitating PKC\(\delta\) and HSP27, a greater interaction between PKC\(\delta\) and HSP27 and lower PKC\(\delta\) kinase activity were found for NCI-H1299 than for NCI-H460 (Fig. 7B). When NCI-H1299 cells were treated with siRNA to HSP27, the HSP27-PKC\(\delta\) interaction disappeared, and PKC\(\delta\) kinase activity was restored (Fig. 7C). Cell death also increased after treating NCI-H1299 with HSP27 siRNA (Fig. 7D), sug-
FIG. 5. Interaction of PKCδ with HSP25 induces HSP25 phosphorylation. A, immunoprecipitation (IP) of PKCδ in lysates from control or HSP25-overexpressing L929 cells after H2O2 (2 mM) pretreatment at the indicated time points. HSP25 or PKCδ protein was detected using anti-HSP25 or anti-PKCδ antibodies. Cellular proteins were extracted after lysis with PKC extraction buffer. PKCδ proteins from 300 μg of cell extracts were immunoprecipitated using anti-PKCδ antibody and protein G-Sepharose. Immune complex kinase reactions were performed in the presence of GST-MBP or His-HSP25 substrate and [γ-32P]ATP. Western blotting was performed using anti-HSP25, anti-phospho-HSP25 (Ser-15), and anti-phospho-HSP25 (Ser-86) antibodies. B, the indicated amounts of recombinant PKCδ protein were immunoprecipitated using an anti-PKCδ antibody and protein G-Sepharose. Immune complex kinase reactions were performed in the presence of His-HSP25 substrate and [γ-32P]ATP with or without PKC lipid activator. C, immunoprecipitation of PKCδ in lysates from control or HSP25-overexpressing L929 cells treated with PMA (100 nM) and with or without rottlerin (5 μM). HSP25 or PKCδ proteins were detected using anti-HSP25 or PKCδ antibody. Cellular proteins were extracted after lysis using PKC extraction buffer. PKCδ proteins from 300 μg of cell extracts were immunoprecipitated using an anti-PKCδ antibody and protein G-Sepharose. Immune complex kinase reactions were performed in the presence of His-HSP25 substrate and [γ-32P]ATP. Western blotting was performed using anti-HSP25, anti-phospho-HSP25 (Ser-15), and anti-phospho-HSP25 (Ser-86) antibodies after immunoprecipitating HSP25 with anti-HSP25 antibody. The light chain band of immunoglobulin G overlapped the phospho-HSP25 band. Cellular proteins were lysed and extracted using PKC extraction buffer. PKCδ proteins from 300 μg of cell extracts were immunoprecipitated using an anti-PKCδ antibody and protein G-Sepharose. Immune complex kinase reactions were performed in the presence of His-HSP25 substrate and [γ-32P]ATP. Western blotting was performed using anti-HSP25, anti-phospho-HSP25 (Ser-15), and anti-phospho-HSP25 (Ser-86) antibodies after immunoprecipitating HSP25 with anti-HSP25 antibody. The light chain band of immunoglobulin G overlapped with the phospho-HSP25 band.

**DISCUSSION**

The coordinated interactions of kinases, phosphatases, and other regulatory molecules with scaffolding proteins is emerging as a major theme in intracellular signaling networks (45–47). Increasing numbers of types of PKC-binding proteins are now believed to play a role in directing the location and function of individual PKC isoforms to particular subcellular locations. In this study, we identified HSP25 as such a PKCδ-binding protein.

Small heat shock proteins are pleiotropic inhibitors of cell death whose physiological protective effects are observed mainly in stressed cells (11, 48–50). Several mechanisms have been proposed to account for this anti-apoptotic activity. HSP25 could raise defenses against oxidative stress by increasing glutathione content (51) or Mn-SOD enzyme activity (13). HSP27 also binds to activated Akt, a protein that generates a survival signal in response to growth factor stimulation (10, 52). Akt also inhibits cell death by phosphorylating and inactivating procaspase-9 (48) or by preventing the release of cytochrome c from mitochondria (6). HSP25 was also found to inhibit cell growth via the inhibition of PKCδ-mediated ERK1/2 activation (12, 14). Moreover, the present study identifies other mechanisms by which HSP25 interferes with cell death pathways. This cytoprotective activity is potentiated in the following two ways: by interaction between HSP25 and the V5 region of the catalytic domain of PKCδ, thereby preventing PKCδ-mediated cell death; and by interaction between kinase-active PKCδ and HSP25, which induces HSP25 phosphorylation at Ser-15 and Ser-86 and potentiates HSP25 cytoprotection.

In a previous study, we found that HSP25 inhibits PKCδ translocation to the membrane, the kinase activity, and tyrosine phosphorylation of PKCδ (14). In the present study, immunoprecipitation experiments were performed using HSP25-overexpressing L929 and Jurkat T cells, which do not express endogenous HSP25 or HSP27 protein. Most interestingly, HSP25 directly bound PKCδ in HSP25-overexpressing cells. HSP25/27 shares several properties with HSP70, another inducible HSP. When overexpressed, both stress proteins inhibit apoptosis in vitro and in vivo, induce resistance to most chemotherapeutic agents, and enhance tumorigenesis in rodents.
HSP25 Binds to PKCα

FIG. 6. Unphosphorylated HSP25 preferentially binds to PKCα and phosphorylated HSP25 produced by the HSP25-PKCα interaction is translocated to the nucleus. A, cytosolic (c) and nuclear (n) fractions of control and HSP25-overexpressing L929 cells were isolated; PKCα was immunoprecipitated (IP) in lysates, and HSP25 protein was immunodetected using anti-HSP25 antibody. Nucleus specific protein lamin B was detected by Western blotting. B, cytosolic and nuclear fractions of HSP25-overexpressing L929 cells were isolated; PKCα was immunoprecipitated from lysates, and HSP25 protein was immunodetected using anti-HSP25 antibody. Nucleus specific protein Ref-1, and cytosol-specific proteins IκB, and PKCα were detected by Western blotting. C, localization changes of phospho-HSP25 (Ser-15), and phospho-HSP25 (Ser-86) were detected by Western blotting. D, localization changes of phospho-HSP25 (Ser-15) and phospho-HSP25 (Ser-86) in L929 cells which stably overexpress HSP25 after treatment of H2O2 (2 mM). Cells were fixed with formaldehyde and immunostained with either anti-phospho-HSP25 (Ser-15) or anti-phospho-HSP25 (Ser-86) antibodies. The results shown are representative of two independent experiments. E, PKCα was immunoprecipitated from the lysates of transfected L929 cells of control or vector containing either wild type HSP25 cDNA or the indicated point mutants and PKCα-crystalline domain of HSP25/27 (indispensable for the interaction between HSP25 and PKCα). Amino acids 92–145 of HSP25 are overlapping with the cytochrome c-binding site (7) and with the 26 S proteasomal PA700-binding site (8), which suggests that the β-sheets of the α-crystalline domain of HSP25/27 (indispensable for the HSP25/27 chaperone function in vitro (55)) might also be important for the interaction between HSP25 and PKCα. Moreover, the deletion of amino acids 90–103 in the HSP25 sequence inhibited HSP25-mediated cytoprotection, suggesting that binding between HSP25 and PKCα is required for the HSP25-mediated cytoprotection (Fig. 3). Because the 90–103-amino acid region of HSP25 overlaps the cytochrome c-binding...

(49) However, several differences between these two chaperones have been identified. The first concerns ATP hydrolysis dependence. The second is that HSP70 as an early response gene and HSP25/27 is a late response gene. The third is that different molecular mechanisms are required for their anti-apoptotic effects (48). The present study also suggests that they differ in terms of their interaction with the PKCα protein, although it should be noted that HSP70 did not bind PKCα (data not shown). The PKCα-binding site for HSP25 was PKCα-CAT, and PKCα kinase activity was important for the PKCα-HSP25 interaction, because treatment with the PKCα kinase inhibitor rottlerin, PKCα-CAT-KR, or ATPγS inhibited the interaction between PKCα and HSP25. An in vitro translation assay also confirmed the interaction between PKCα-CAT and HSP25.

Because PKCα has been reported to induce cell death with the concomitant activation of PKCα or PKCα tyrosine phosphorylation (36, 53, 54) and HSP25 was found to inhibit cell death (Fig. 2), we concluded that the interaction between kinase-active PKCα-CAT and HSP25 inhibits PKCα activity and PKCα-mediated cell death. When the binding sites of HSP25 and PKCα-CAT were investigated using deletion mutants of PKCα or HSP25, we found that amino acids 90–103 of HSP25 and the V5 region (amino acids 630–674 of PKCα) of PKCα (Figs. 4 and 5) are essential for the interaction between HSP25 and PKCα. Amino acids 92–145 of HSP25 are overlapping with the cytochrome c-binding site (7) and with the 26 S proteasomal PA700-binding site (8), which suggests that the β-sheets of the α-crystalline domain of HSP25/27 (indispensable for the HSP25/27 chaperone function in vitro (55)) might also be important for the interaction between HSP25 and PKCα. Moreover, the deletion of amino acids 90–103 in the HSP25 sequence inhibited HSP25-mediated cytoprotection, suggesting that binding between HSP25 and PKCα is required for the HSP25-mediated cytoprotection (Fig. 3). Because the 90–103-amino acid region of HSP25 overlaps the cytochrome c-binding...
lysates of NCI-H1299 after transfection with control siRNA or HSP27 siRNA; PKC
immunoprecipitated (IP) in lysates from NCI-H460, and -H1299 with anti-PKC
Moreover, it appears reasonable to conclude that the inhibition
Immune complex kinase reactions were performed in the presence of GST-MBP substrate and 
Phosphorylation-deficient mutants but not phosphorylated, and fully activated. As soon as the V5 region is
is involved in the inhibition of cell death by HSP25.
Oxidative stress or ionizing radiation permits PKC to translocate to the membrane in an open conformation, allowing its
site (amino acids 51–141 of HSP27) and the PA700-binding site (amino acids 88–141 of HSP27), this cytoprotective effect represents more than an inhibition of PKCδ-mediated activity. Moreover, it appears reasonable to conclude that the inhibition of PKCδ-mediated cell death by HSP25 interaction with PKCδ is involved in the inhibition of cell death by HSP25.
PKCδ is involved in the inhibition of cell death by HSP25.
PKCδ is involved in the inhibition of cell death by HSP25.
PKCδ is involved in the inhibition of cell death by HSP25.
PKCδ is involved in the inhibition of cell death by HSP25.
...
in cytosol (Fig. 6), and phosphorylated HSP25 was translocated to the nucleus. The results of our kinetic experiments involving H$_2$O$_2$ additions and phosphorylation-deficient mutants of HSP25 at Ser-15 and Ser-86 suggest that exposed V5 regions of PKC8 after activation interact with unphosphorylated HSP25 in the cytosol and that after phosphorylation by PKC8, phosphorylated HSP25 translocates to the nucleus.

The physiological importance of the correlation between PKC8-HSP25 binding and radioresistance in lung carcinoma cell lines implies that HSP27 overexpression, which is related to radioresistance (Fig. 7), is in part determined by the HSP25/PKC8 interaction. Because HSP27 expression in lung carcinoma cells is well correlated with radioresistance, the V5 region of PKC8 might be therapeutically useful for inhibiting radioresistance by HSP27.

PKC8 activity plays an essential role in the apoptosis of cells, and small HSP is constitutively expressed in many cancer cells to negatively regulate apoptotic induction. The small HSP-PKC8 interaction could thus indicate the physiologic importance of this small HSP (Fig. 8). Moreover, this property might account for the observed protective effect of this protein when induced in response to radiation or oxidative stress.

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HSP25 Inhibits Protein Kinase Cδ-mediated Cell Death through Direct Interaction
Yoon-Jin Lee, Dae-Hoon Lee, Chul-Koo Cho, Sangwoo Bae, Gil-Ja Jhon, Su-Jae Lee,
Jae-Won Soh and Yun-Sil Lee

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