Serine/threonine kinase Akt is thought to mediate many biological actions toward anti-apoptotic responses. Screening of drugs that could interfere with the Akt signaling pathway revealed that Hsp90 inhibitors (e.g. geldanamycin, radicicol, and its analogues) induced Akt dephosphorylation, which resulted in Akt inactivation and apoptosis of the cells. Hsp90 inhibitors did not directly affect Akt kinase activity \textit{in vitro}. Thus, we examined the effects of Hsp90 inhibitors on upstream Akt kinases, phosphatidylinositol-3-kinase (PI3K) and 3-phosphoinositol-dependent protein kinase-1 (PDK1). Hsp90 inhibitors had no effect on PI3K protein expression. In contrast, treatment of the cells with Hsp90 inhibitors decreased the amount of PDK1 without directly inhibiting PDK1 kinase activity. We found that the kinase domain of PDK1 was essential for complex formation with Hsp90 and that Hsp90 inhibitors suppressed PDK1 binding to Hsp90. PDK1 degradation mechanisms revealed that inhibition of PDK1 binding to Hsp90 caused proteasome-dependent degradation of PDK1. Treatment of proteasome inhibitors increased the amount of detergent-insoluble PDK1 in Hsp90 inhibitor-treated cells. Therefore, the association of PDK1 with Hsp90 regulates its stability, solubility, and signaling. Because Akt binding to Hsp90 is also involved in the maintenance of Akt kinase activity, Hsp90 plays an important role in PDK1-Akt survival signaling pathway.

The characterization of the survival signal transduction pathways stimulated by growth factors and cytokines has revealed that phosphatidylinositol-3-kinase (PI3K) is involved in the pathway (1–3). PI3K is a heterodimeric lipid kinase, which consists of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. After stimulation with growth factors and cytokines, PI3K is activated by the interaction of the 85-kDa subunit with phosphorytrosines of activated intracellular domain of growth factor receptors or with the receptor-associated adapter proteins. Subsequently, PI3K associates with the plasma membrane where the 110-kDa catalytic subunit phosphorylates phosphatidylinositols. The generated phospholipid second messenger molecule, phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3), raises a diverse set of cellular responses (4–6). The major targets of PtdIns(3,4,5)P_3 are pleckstrin homology (PH) domain-containing proteins, including serine/threonine kinase Akt (also known as protein kinase B (PKB) or RAC-PK) (7, 8).

The interaction of PtdIns(3,4,5)P_3 with the PH domain of Akt recruits Akt to the plasma membrane, where it is phosphorylated at two key regulatory residue sites, Thr^{308} and Ser^{473}. Phosphorylation at both residues is necessary for full activation of Akt and the subsequent regulation of many PI3K-regulated biological responses, including glucose uptake, protein synthesis, and apoptosis inhibition (7, 8). Akt phosphorylation at Thr^{308} is catalyzed by the ubiquitously expressed 3-phosphoinositide-dependent protein kinase-1 (PDK1) (9–11). The kinase responsible for phosphorylation of Akt at Ser^{473} residue is called PDK2. Recently, Balendran et al. (12) reported that interaction of the fragment of protein kinase C-related kinase-2 with PDK1 converted PDK1 from a kinase that could phosphorylate only the Thr^{308} residue of Akt to one that could phosphorylate both Thr^{308} and Ser^{473} residues. Akt itself was reported to be associated with the Akt phosphorylation at the Ser^{473} residue (13).

The 90-kDa heat-shock protein (Hsp90) was highly conserved and played an important role in refolding certain denatured proteins under stress conditions. Unlike the more general Hsp70 and Hsp60 chaperones, Hsp90 appeared to have substrate-specific folding activity. Hsp90 has an additional role in the conformational regulation of certain signal transduction molecules, such as steroid hormone receptors and some kinases. Recent reports, including ours, indicated that Hsp90 is involved in the inhibition of apoptosis by suppressing cytochrome c-mediated oligomerization of Apaf-1 (14) or by stabilizing Akt kinase activity (15). These data suggest that Hsp90 plays an important role in cell survival. This notion was supported by the facts that Hsp90 inhibitors could induce apoptosis in various types of cell lines (reviewed in Refs. 16 and 17).
We thus investigated whether Hsp90 inhibitors (i.e. geldanamycin, radicicol, and oxime derivatives of radicicol) possess the ability to modulate the Akt signaling pathway. We found that Hsp90 inhibitors induced Akt dephosphorylation and inactivation in vivo, although they had no direct effects on Akt kinase activity in vitro. Recent reports also documented the down-regulation of phospho-Akt level and Akt signaling by Hsp90 inhibitors (18, 19). We then examined the effects of Hsp90 inhibitors on the upstream Akt kinases, PDK1 and PI3K, and found that they down-regulated the expression level of PDK1 but did not affect the amount of PI3K. Hsp90 inhibitors had no direct effects on PDK1 kinase activity in vitro. Because binding to Hsp90 has been shown to prevent some signaling proteins from self-association or becoming insoluble (16, 17), we investigated the binding capability of PDK1 to Hsp90. We found that PDK1 bound to Hsp90 via its kinase domain and that Hsp90 inhibitors suppressed the binding. Inhibition of PDK1 binding to Hsp90 led to the proteasome-dependent degradation of PDK1. Therefore, we concluded that Hsp90 is involved in the signaling and stability of PDK1.

EXPERIMENTAL PROCEDURES

Reagents—Radicicol, oxime derivatives of radicicol (KF25706 and KF58333), and UCN-01 were kindly provided by Kyowa Hakko Kogyo (Tokyo, Japan). Geldanamycin and LY294002 were purchased from Sigma Chemical Co. (St. Louis, MO). Caspase inhibitors benzoyloxycarbonyl-Asp-CH₂OOC-2,6-dichlorobenzene (Z-Asp) and benzoyloxycarbonyl-Val-Ala-Asp-CH₂OOC-2,6-dichlorobenzene (Z-YAD) were purchased from Funakoshi (Tokyo, Japan). Proteasome inhibitors benzoxycarbonyl-Leu-Leu-Leu-aldehyde (MG132), benzoyloxycarbonyl-Ille-Glu(γ-Glu)-Ala-Val-Leu-aldehyde (PSI), and lactacystin were obtained from the Peptide Institute (Osaka, Japan) or Biomol (Plymouth Meeting, PA). Serine and cysteine protease inhibitor acetyl-Leu-Leu-Leu-Val-Tamoxifen was purchased from the Peptide Institute. Chymotrypsin-like serine protease inhibitor N-acetyl-L-phenylalanine chloromethyl ketone (TPCK) and trypsin-like serine protease inhibitor acetyl-Leu-Leu-aldehyde (PSI), and lactacystin were kindly provided by Dr. M. Thelen (Istituto di Ricerca in Biomedicina, Switzerland) (23). The V5- and (His)6-epitope-tagged human wild-type hsp90β cDNA containing the membrane-targeting CAAX motif (active-hsp90β) and its inactive mutant cDNA (KD-P3K) in pcDNA3 vectors were kindly provided by Dr. M. Thelen (Istituto di Ricerca in Biomedicina, Switzerland) (23). The V5- and (His)6-epitope-tagged human wild-type hsp90β cDNA (WT-hsp90β) in a pCDNA3.1/HisG vector was purchased from Invitrogen (San Diego, CA) (15). All the plasmid DNAs for transfection were purified using a Qiagen plasmid Maxi kit, according to the manufacturer’s protocol (Qiagen, Chatsworth, CA).

Transient Transfection, Immunoprecipitation, and Western Blot Analysis—Cells were transfected with appropriate plasmids using SuperFect transfection reagent (Qiagen) according to the manufacturers’ instructions.

For immunoprecipitation, cells were harvested and solubilized in lysis buffer for immunoprecipitation (20 mM Tris-HCl, pH 7.5, 0.2% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1.5 mM magnesium chloride, 137 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium vanadate, 12 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM aprotinin). The cell lysates were centrifuged at 17,000 × g for 10 min. The supernatants were incubated with agarose conjugated with an anti-FLAG M2 monoclonal antibody (mAb) (anti-FLAG M2-agarose, Sigma), agaroase conjugated with an anti-Myc mAb (clone 9E10) (anti-Myc agarose, Santa Cruz Biotechnology, Santa Cruz, CA), agaroase conjugated with an anti-HA mAb (clone F-7) (anti-HA-agarose, Santa Cruz Biotechnology), or protein G-agarose that had been conjugated with control sheep IgG (Upstate Biotechnology) or sheep anti-PDK1 antibody (Upstate Biotechnology). Immunoprecipitated proteins were subjected to kinase assays or electrophoresed in a 4–20% gradient polyacrylamide gel. The electrophoresed proteins were transblotted onto a nitrocellulose membrane. After blocking, the membrane was subjected to Western blot analysis, as described below.

Western blot analysis was performed, as described previously with a slight modification (15, 21). Cells were solubilized in lysis buffer for Western blot (50 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 3 mM EGTA, 12 mM β-glycerophosphate, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium vanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, and 0.1% 2-mercaptoethanol). The cell lysates were centrifuged at 17,000 × g for 30 min. The supernatants were electrophoresed and transblotted onto a nitrocellulose membrane. In some experiments, the supernatants were recovered as soluble fractions, and the precipitates were solubilized by directly adding the diluted SDS sample buffer to obtain insoluble fractions (24). After blocking, the membranes were incubated with one of the following: antibodies to phospho-Akt (Thr^{320}, PISK p85 subunit, or GSK3 (Upstate Biotechnology); antibodies to Akt, phospho-Akt (Ser^{473}), 89-kDa cleaved fragment of PARP, or phospho-GSK3β (Ser^{9}) (Cell Signaling Technology, Beverly, MA); an antibody to phospho-MAPK (Protegna, Madison, WI); an antibody to PARP (PharMingen, San Diego, CA); antibodies to PDK1 or c-Raf1 (Transduction Laboratories, Lexington, KY); antibodies to MAPK, Myc tag, or HA tag (Santa Cruz Biotechnology); an antibody to Hsp90 (Stressgen, Victoria, Canada); an antibody to V5 tag (Invitrogen); or an antibody to FLAG tag (Sigma). Blots were scanned with a Phosphoimager or a Chemiluminescence scanner supported by Adobe Photoshop 5.5 and quantified with IMAGE 1.62 software (National Institutes of Health).

Kinase Assay—HT1080 cells were transfected with a pFLAG-CMV-2 vector containing WT-akt cDNA and were cultured for 24 h in medium containing inhibitors. Akt kinase activity of the immunoprecipitated FLAG-tagged Akt was estimated using GSK3 peptide (RPRATTF) as a substrate by immunoprecipitating an Akt kinase assay kit according to the manufacturer’s instructions (Upstate Biotechnology). In some experiments, recombinant human active PDK1 (Upstate Biotechnology) was incubated with inhibitors for 30 min at 30°C and was then subjected to Akt kinase assay.

We measured PDK1 kinase activity using an assay kit according to the manufacturer’s instructions (Upstate Biotechnology). Briefly, recombinant human active PDK1 (Upstate Biotechnology) protein was first incubated with inhibitors for 30 min at 30°C, followed incubation with inactive SGK for 30 min at 30°C. Then the PDK1-dependent SGK kinase activity was estimated by incubating the reaction with GSK3 kinase peptide as a substrate for 10 min at 30°C in the presence of γ-[32P]ATP.

To examine the direct effects of Hsp90 inhibitors on PI3K activity, we transfected pcDNA3-p110α-CAXX and pcDNA3-p110α-CAXX-KD into 293T cells. Then Hα-tagged p110α-CAXX or p110α-CAXX-KD protein was immunoprecipitated with agarose conjugated with anti-HA-agarose following incubation with Hsp90 inhibitors or LY294002 for 30 min at 30°C. Next, the immunoprecipitated p110α was subjected to Western blot analysis in the presence of these drugs, as described previously (23).
cells were further incubated in nonradioactive, complete medium for various times indicated before lysis. Exposure to KF58333 was maintained in the treated culture. The amount of radiolabeled PDK1 was determined by immunoprecipitation with anti-Myc agarose and SDS-PAGE. The relative amounts of incorporated radioactivity were visualized and quantified with a BAS1500 Bio-Imaging analyzer (Fuji Film, Tokyo, Japan).

**Estimation of Apoptosis**—The caspase activity in the cell lysates was measured, as described previously (15). In brief, cells were harvested and lysed with lysis buffer (10 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% CHAPS, and 5 mM dithiothreitol). The cell lysate (5 μg) was then incubated with 20 μM acetyl-L-aspartyl-glu-taryl-L-valyl-alanyl-L-aspartic acid–amino-4-methylcoumarin (DEVD-AMC) (Peptide Institute) in ICE buffer (20 mM HEPES (pH 7.4), 10% glycerol, and 2 mM dithiothreitol) for 1 h at 37°C. The aminomethylcoumarin released from the fluorogenic substrate was excited at 380 nm, and the emission was measured at 460 nm using an Hitachi fluorescence spectrophotometer, model F-2000 (Hitachi, Tokyo, Japan).

**RESULTS**

**Hsp90 Inhibitors Induced Apoptosis with Caspase Activation**—We have recently identified that Akt is associated with Hsp90 (15). To clarify the role of Hsp90 on apoptosis and Akt survival signaling pathway, we examined the effects of several inhibitors of Hsp90. We used benzoquinone ansamycin geldanamycin (16, 17), antifungal antibiotic radicicol (25), and analogues of radicicol, KF25706 and KF58333 (26, 27), as Hsp90 inhibitors. When 293T cells were treated with Hsp90 inhibitors, 293T cells underwent apoptosis with nuclear fragmentation (data not shown). The caspase family of aspartate-specific cysteine proteases has been demonstrated to be critical mediators in the cell death pathway (28). Among the members of the caspase family, caspase-3/CPP32/Yama/Apopain is a common and important effector of the apoptotic process. To clarify the activation of caspase-3 in vivo, we performed Western blot analysis using antibodies specifically recognizing the p85 cleaved fragment of poly(ADP-ribose) polymerase (PARP).

PARP was one of the substrates of caspase-3 and produced the 85-kDa fragment after caspase-3-mediated cleavage (29, 30). Treatment of 293T cells with 10 μM geldanamycin or 1 μM radicicol, KF25706, or KF58333 for 24 h increased the amount of cleaved fragment of PARP (Fig. 1A), indicating that caspase-3 is activated in vivo in 293T cells after inhibition of Hsp90 function. We further examined the activity of caspase-3 (like) proteases in the KF58333-treated human HT1080 cells using fluorogenic-labeled tetrapeptide DEVD-AMC. KF58333 promoted the activation of caspase-3 (like) proteases in HT1080 cells in a dose-dependent manner (Fig. 1B). The PARP cleavage in HT1080 cells was also induced by KF58333 in a dose-dependent manner (Fig. 1B). Activation was suppressed by adding caspase inhibitors Z-Asp and Z-VAD (data not shown). These results indicate that inhibition of Hsp90 can initiate the cells to undergo apoptosis.

**Inhibition of Akt Kinase Activity in Vivo but Not in Vitro by Hsp90 Inhibitors**—Hsp90 inhibitors were known to suppress MAPK signaling pathway by down-regulation of Raf-1 protein expression (31). However, transfection of active MEK1 could not diminish the Hsp90 inhibitor-induced apoptosis (data not shown). Therefore, inhibition of one or more other signaling pathways might be critical for the induction of apoptosis by Hsp90 inhibitors. Akt is recently reported to suppress apoptosis by phosphorylating some apoptosis-relating proteins (i.e., pro-apoptotic Bcl-2 family member Bad at Ser136, caspase family member caspase-9 at Ser206, the Forkhead transcription factor protein family, and IκB kinase) (7, 8). We thus examined the Akt kinase activity after treatment with Hsp90 inhibitors.

Akt was continuously phosphorylated at Ser473 and Thr308 residues when 293T cells were cultured in serum-containing medium. Incubation of 293T cells with radicicol, KF25706, or KF58333 induced the dephosphorylation of endogenous Akt at Ser473 in a dose-dependent manner (Fig. 2A). The decrease in endogenous phospho-Akt (at Ser473) level was also found in human fibrosarcoma HT1080 and mouse colon adenocarcinoma NL-17 cells treated with KF58333 (data not shown), suggesting that Akt dephosphorylation induced by Hsp90 inhibitors is not restricted to one particular cell line. We also examined the change of phospho-Akt (Thr308) level in 293T cells. Because the anti-phospho-Akt (Thr308) antibody is not sensitive enough to detect the phosphorylated form of endogenous Akt protein in 293T cells, we transfected the FLAG-tagged akt cDNA into 293T cells and then examined the effects of KF58333 on phospho-Akt (Thr308) levels. KF58333 treatment promoted the dephosphorylation of Akt at Thr308 residue in a dose-dependent manner (Fig. 2B). These results indicate that Hsp90 inhibitors suppress the phosphorylation at both Thr308 and Ser473 residues, although they also slightly down-regulate the expression of Akt protein itself (Fig. 2, A and B).

To confirm that the dephosphorylation of Akt was associated with the decrease in Akt-mediated signaling, we investigated the change of phosphorylated form of GSK3. Akt is known to phosphorylate GSK3α at the Ser21 residue and GSK3β at the Ser9 residue (7, 8). Western blot analysis using an anti-phospho-GSK3α/β (Ser21) antibody revealed that treatment of 293T cells with Hsp90 inhibitors decreased the endogenous phospho-GSK3β level (Fig. 2C), consistent with the decrease in phospho-Akt level (Fig. 2, A and B). We also confirmed the decrease in Akt kinase activity by measuring the activity by immunoprecipitating FLAG-tagged Akt from 293T cells that had been transfected with the FLAG-tagged akt cDNA. The transfected 293T cells were treated with Hsp90 inhibitors for 24 h and then harvested. As shown in Fig. 2D, treatment of Hsp90 inhibitors suppressed the Akt kinase activity. Examination of the change...
substrates. The cell lysates were electrophoresed and immunoblotted with an anti-phospho-Akt (Ser473) antibody, an anti-Akt antibody, an anti-phospho-MAPK antibody, or an anti-MAPK antibody. B, 293T cells were transfected with pFLAG-CMV-2 vector encoding WT-Akt (pFLAG-Akt). After transfection for 24 h, cells were treated with the indicated concentrations of KF58333. After incubation for 24 h, cells were harvested and lyzed in lysis buffer for Western blot analysis. The cell lysates were electrophoresed and immunoblotted with an anti-FLAG antibody, or an anti-MAPK antibody. C, 293T cells were treated as described in Fig. 1A. The cell lysates were electrophoresed and immunoblotted with an anti-phospho-GSK3β (Ser9) antibody, an anti-GSK3 antibody, or an anti-Akt antibody. D, 293T cells were transfected with pFLAG-CMV-2 vector encoding WT-Akt (pFLAG-Akt) or an anti-Akt antibody. E, recombinant active Akt protein (500 ng) was preincubated with the indicated concentrations of KF58333 for 30 min at 30°C in the presence of [γ-32P]ATP, as described under “Experimental Procedures.” Each point represents a mean ± S.D. of triplicate determinations. The immunoprecipitated proteins were also subjected to immunoblot analysis with an anti-FLAG antibody (7). F, recombinant active Akt protein (500 ng) was preincubated with the indicated concentrations of KF58333 for 30 min at 30°C in vitro. Then Akt kinase activity was evaluated by incubating with GSK3 peptide (RPRAATF) as a substrate for 10 min at 30°C in the presence of [γ-32P]ATP, as described under “Experimental Procedures.” In the control experiment, the reaction was performed without Akt protein (solid column). Each point represents a mean ± S.D. of triplicate determinations. cmn ± S.D. of the control Akt activity (open column) was 210,789 ± 10,717, and this value was taken as 100%.

Because treatment of Hsp90 inhibitors down-regulate the phospho-Akt level, Hsp90 inhibitors might interfere with the kinase activity of the upstream Akt kinases P13K and PDK1. We first examined the change of endogenous P13K and PDK1 protein expression after drug treatment. As shown in Fig. 3A, the treatment of 293T cells with KF58333 decreased the amount of endogenous PDK1 but not p85 subunit of P13K protein. Down-regulation of PDK1 protein expression was found when 293T cells were treated with KF58333 from the concentration of 30 nM. Raf-1 is well known to interact with Hsp90, and the inhibition of Hsp90-Raf-1 binding results in the decrease in Raf-1 protein expression (31, 32). Consistent with the previous report (27), KF58333 suppressed the endogenous Raf-1 protein expression in 293T cells (Fig. 3A). The minimum concentration of KF58333 that could promote Raf-1 destabilization was also 30 nM. Because the level of p85 subunit of P13K was not affected by KF58333 treatment, the depletion of PDK1 and Raf-1 proteins was not the result of nonspecific protein degradation. Destabilization of endogenous PDK1 and Raf-1 proteins was also observed in human fibrosarcoma HT1080, human prostate PC-3, and mouse colon adenocarcinoma NL-17 cells (Fig. 3B), suggesting that these effects of Hsp90 inhibitors are not restricted to one particular cell line. Moreover, destabilization of PDK1 was also observed in the transfected Myc-tagged PDK1 protein in vivo (data not shown).

To check directly if the half-life of PDK1 in cells is shortened by treatment with KF58333, we performed pulse-chase experiments. 293T cells that had been transfected with pCMV-PDK1 were pulse-labeled with [35S]methionine/[35S]cysteine for 2 h and then chased in a nonradioactive, complete medium. KF58333 (1 μM) had been added to cells before the start of pulse labeling and included during both pulse-label and chase periods. As shown in Fig. 3C, PDK1 seemed to be relatively stable in control (treated with vehicle Me6SO alone) cells. In contrast,
Fig. 4. Effect of Hsp90 inhibitors on PDK1 and PI3K activity. A, recombinant active PDK1 (10 ng) protein was preincubated with vehicle Me2SO (Control), 10 μM geldanamycin, 1 μM KF58333, or 1 μM UCN-01 for 30 min at 30 °C in vitro. Then PDK1 kinase activity was measured, as described under “Experimental Procedures.” Each point represents a mean ± S.D. of triplicate determinations. B, 293T cells were transfected with pCMV3 vector containing HA-tagged PI3K α subunit cDNA with membrane-targeting CAAX motif (Active) and its kinase-dead mutant cDNA (KD). After incubation for 24 h, cells were harvested and lysed in lysis buffer for immunoprecipitation. The immunoprecipitated HA-tagged active- and KD-PI3K was incubated with pFLAG-CMV-2 vector containing WT- or E40K-akt cDNA. After incubation for 24 h, the transfected cells were treated with medium containing vehicle Me2SO (Control), 1 μM KF58333, or 10 μM geldanamycin. After incubation for an additional 24 h, cells were harvested and lysed in lysis buffer for Western blot analysis. The cell lysates were electrophoresed and immunoblotted with an anti- phospho-Akt (Thr308) antibody or an anti-Akt antibody.

PI3K was much more unstable in the KF58333-treated cells. Measurement of radioactivity with a BAS1500 Bio-Imaging analyzer demonstrated that the half-life of PI3K was shortened down to under 2 h (Fig. 3D). These results indicate that PDK1 requires the molecular chaperone function of Hsp90 for its intracellular stability.

Hsp90 Inhibitors Had No Direct Effects on PDK1 and PI3K Kinase Activity—We then examined the direct effects of Hsp90 inhibitors on PDK1 kinase activity. The recombinant active PDK1 protein was incubated with control (vehicle Me2SO alone), 10 μM geldanamycin, or 1 μM KF58333 for 30 min at 30 °C in vitro before estimating its kinase activity. Hsp90 inhibitors exhibited no inhibitory effects on PDK1 kinase activity (Fig. 4A). In contrast, specific PDK1 inhibitor UCN-01 almost completely inhibited the PDK1 kinase activity, as we have recently found. PDK1 phosphorylates itself at the activation loop of PDK1 (Ser441), resulting in its own activation (33). Thus, KF58333 did not affect PDK1 autoactivation. We also examined the direct effects of Hsp90 inhibitors on PI3K kinase activity. Constitutive active PI3K and kinase-dead PI3K were immunoprecipitated from 293T cells that had been transfected with pcDNA3-p110αCAAX and pcDNA3-p110αCAAX-KD plasmids. The immunoprecipitated PI3K proteins were preincubated with control (vehicle Me2SO alone), geldanamycin (10 μM), radicicol (1 μM), KF25706 (1 μM), KF58333 (1 μM), or LY294002 (50 μM) for 30 min at 30 °C in vitro before estimating its kinase activity. Although PI3K inhibitor LY294002 almost completely suppressed the PI3K activity to the level that seen in KD-PI3K control, all the Hsp90 inhibitors exhibited no inhibitory effects on PI3K (Fig. 4B). These results indicate that Hsp90 inhibitors had no direct effects on both upstream Akt kinases.

We then examined the role of PDK1 down-regulation in Akt phosphorylation in vivo. Mutation of Akt protein at Glu40 with Lys (E40K) was reported to be activated in a PI3K-independent manner due to its increase in the affinity of the PH domain for phospholipids (34). We estimated the effects of Hsp90 inhibitors on 293T cells that had been transfected with E40K-akt cDNA. As shown in Fig. 4C, E40K-Akt was phosphorylated at the Thr308 residue even in the presence of PI3K inhibitor LY294002 whereas LY294002 treatment down-regulated the phospho-Akt (Thr308) level of WT-Akt. KF58333 decreased the phospho-Akt (Thr308) level in both WT-Akt and E40K-Akt, indicating that destabilization of PDK1 plays an important role in KF58333-mediated Akt dephosphorylation. These results indicate that PDK1 destabilization is the main reason for Akt dephosphorylation and inactivation induced by Hsp90 inhibitors.

Association of PDK1 with Hsp90 in Vivo—Because most Hsp90 client proteins were destabilized after inhibiting the binding to Hsp90 (16, 17), we hypothesized that PDK1 might be one of the Hsp90 client proteins. We checked the in vitro PKD1 binding to Hsp90 by immunoprecipitating endogenous PDK1 protein following Western blot analysis with an anti-Hsp90 antibody. As shown in Fig. 5A, endogenous Hsp90 protein was co-immunoprecipitated by an anti-PDK1 antibody but not by control sheep antibody. By transfection of WT-PDK1 cDNA in a pFLAG-CMV-2 vector and WT-hsp90 cDNA in a pcDNA3.1/GS vector into 293T cells, we confirmed the binding of flag-tagged PDK1 to V5-tagged Hsp90 in cells (Fig. 5C). To identify the binding site in PDK1, we prepared NH2-terminal and COOH-terminal deletion mutants, as indicated in Fig. 5B. The V5-tagged Hsp90 and WT-PDK1 or deleted PDK1 were expressed in 293T cells. The V5-tagged Hsp90 could interact with WT-, ΔN51-, ΔPH-, ΔC342-, ΔC273-, ΔC242-, and ΔN155-PDK1 (Fig. 5C). Hsp90 could hardly interact with ΔC196-PDK1 and could not bind to ΔN223-PDK1. The relationship between the Hsp90-binding ability and the structure of PDK1 deletion mutants is summarized in Fig. 5B. These results suggest that amino acid residues around 156–223 of PDK1 (active site of PDK1, dotted area in Fig. 5B) is involved in its binding to Hsp90.

Then we investigated the effects of Hsp90 inhibitors on PDK1 binding to Hsp90. We transfected WT-PDK1 cDNA in a pFLAG-CMV-2 vector, WT-hsp90 cDNA in a pcDNA3.1/GS vector, or both into 293T cells. After transfection for 24 h, cells were incubated with 1 μM KF58333 for 2 h following immunoprecipitation with an anti-Myc (PDK1) antibody. As shown in Fig. 5D, PDK1 binding to Hsp90 was inhibited by treatment with KF58333. This result indicates that Hsp90 inhibitors can suppress PDK1 binding to Hsp90. Interestingly, ΔC196-PDK1 and ΔN223-PDK1 were hardly expressed in 293T cells (Fig. 5C). We speculate that these mutants were unstable and degraded because of their inability to bind to Hsp90, as in the case of WT-PDK1 in Hsp90 inhibitor-treated cells (Fig. 3). These results indicate that PDK1 requires Hsp90 association for its stability.

Role of Proteosome-dependent Pathway in Hsp90 Inhibitor-mediated PDK1 Degradation—Several protease systems are involved in the protein degradation. To examine what kinds of proteases were involved in PDK1 destabilization after inhibiting PDK1-Hsp90 binding, 293T cells were cultured in medium containing KF58333 with caspase inhibitors (Z-Asp or Z-VAD), proteasome inhibitors (MG132, PSI, or lactacystin), serine and

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cysteine protease inhibitor (leupeptin), chymotrypsin-like serine protease inhibitor (TPCK), or trypsin-like serine protease inhibitor (leupeptin), chymotrypsin-like serine protease inhibitor (leupeptin), or trypsin-like serine protease inhibitor (TPCK), or trypsin-like serine protease inhibitor (TPCK). The soluble PDK1 amount was quantified with IMAGE 1.62 software (National Institutes of Health), as described under "Experimental Procedures." Each point represents a mean ± S.D. of three independent experiments. PDK1 level of KF58333-treated cells was taken as 100%. C. COS-7 cells were transfected with empty pUSEamp vector, pFLAG-CMV-2 vector containing ΔN51-PDK1, or pUSEamp vector containing myr-Akt cDNA. After transfection for 24 h, cells were treated with 1 μM MG132. After incubation for an additional 18 h, cells were harvested and lysed with lysis buffer for Western blot analysis. The cell lysates were electrophoresed at 17,000 g for 30 min. The supernatants were recovered as soluble fractions (Soluble), and the precipitates were solubilized by directly adding the diluted SDS-sample buffer to obtain insoluble fractions (Insoluble). Each fraction was electrophoresed and immunoblotted with an anti-PDK1 antibody, an anti-phospho-Akt (Ser473) antibody, an anti-Akt antibody, or an anti-cleaved PARP fragment antibody. B. soluble PDK1 amount was quantified with IMAGE 1.62 software (National Institutes of Health), as described under "Experimental Procedures." Each point represents a mean ± S.D. of three independent experiments. PDK1 level of KF58333-treated cells was taken as 100%. C. COS-7 cells were transfected with empty pUSEamp vector, pFLAG-CMV-2 vector containing ΔN51-PDK1, or pUSEamp vector containing myr-Akt cDNA. After transfection for 24 h, cells were treated with 1 μM MG132. After incubation for an additional 18 h, cells were harvested and lysed with lysis buffer for Western blot analysis. The cell lysates were electrophoresed and immunoblotted with an anti-cleaved PARP fragment antibody, an anti-FLAG antibody, an anti-phospho-Akt (Ser473) antibody, or an anti-Akt antibody.

was carried out by the proteasome system.

Proteasome inhibitors could not completely restore the phospho-Akt level and could not fully suppressed KF58333-induced apoptosis (Fig. 6A), whereas proteasome inhibitors drastically increased the insoluble PDK1 amount. Moreover, we found that insoluble PDK1 was dephosphorylated at the Ser473 residue (data not shown), which is essential for PDK1 activation (33). These results suggest that insoluble PDK1 is inactive. Therefore, Hsp90 is not just a shield against degradation but is a chaperone to keep PDK1 in a soluble and in an active conformational state prior to phosphorylating its downstream substrates.

We examined the effects of PDK1 overexpression on KF58333-induced apoptosis. Unfortunately, overexpression of ΔN51-PDK1 alone could not inhibit KF58333-induced apoptosis (Fig. 6C). When examining the phospho-Akt level in PDK1-

Fig. 5. Formation of PDK1-Hsp90 complex in vivo. A. 293T cells were harvested and lysed in lysis buffer for immunoprecipitation. The cell lysates were incubated with protein G-agarose that had been conjugated with control sheep IgG or sheep anti-PDK1 antibody for 2 h at 4°C. The immunoprecipitated proteins were electrophoresed and immunoblotted with an anti-Hsp90 antibody or an anti-PDK1 antibody. B, structural domains of PDK1 deletion mutants used in these experiments are represented as black bars. The predicted Hsp90 binding site in PDK1 is shown schematically (dotted column). C. 293T cells were transfected with pcDNA3.1/His vector encoding WT-Hsp90 together with empty pFLAG-CMV-2 vector (Mock) or pFLAG-CMV-2 vector containing WT-PDK1 (WT), ΔN51-PDK1 (ΔN51), ΔPH-PDK1 (ΔPH), ΔC342-PDK1 (ΔC342), ΔC273-PDK1 (ΔC273), ΔC242-PDK1 (ΔC242), ΔC196-PDK1 (ΔC196), ΔN155-PDK1 (ΔN155), or ΔN223-PDK1 (ΔN223) cDNA. After transfection for 24 h, cells were harvested and lysed with lysis buffer for immunoprecipitation. The FLAG-tagged PDK1 mutant proteins were immunoprecipitated with anti-FLAG agarose. The immunoprecipitated proteins were electrophoresed and immunoblotted with an anti-V5 antibody or an anti-Myc antibody. D. 293T cells were transfected with pcDNA3.1/His vector containing WT-PDK1 cDNA together with empty pcDNA3.1/His vector (−) or pcDNA3.1/His vector encoding WT-Hsp90 (+). After transfection for 24 h, cells were treated with or without 1 μM KF58333. After incubation for an additional 2 h, cells were harvested and lysed with lysis buffer for immunoprecipitation. The Myc-tagged PDK1 proteins were immunoprecipitated with an anti-Myc agarose. The immunoprecipitated proteins were electrophoresed and immunoblotted with an anti-V5 antibody and an anti-Myc antibody. Expression of V5-tagged Hsp90 and Myc-tagged PDK1 proteins was confirmed by immunoblot analysis with an anti-V5 antibody or an anti-Myc antibody.
transfected cells, we found that overexpression of PDK1 alone restored the phosphorylation of the Thr\textsuperscript{308} residue (data not shown) but not of Ser\textsuperscript{473} (Fig. 6C). Because phosphorylation at both Thr\textsuperscript{308} and Ser\textsuperscript{473} residues is necessary for full activation of Akt, PDK1 transfection could not inhibit KF58333-induced apoptosis. To examine the role of the PDK1-Akt signaling pathway during KF58333-induced apoptosis, we transfected the constitutive active form of akt cDNA (myr-Akt) and found that overexpression of active Akt suppressed KF58333-induced apoptosis (Fig. 6C). The result suggests that inhibition of the PDK1-Akt survival signaling pathway is involved in the KF58333-induced apoptosis.

**DISCUSSION**

The sensitivity of cells to apoptosis-inducing stimuli appears to be dependent on the balance between apoptosis-inducing signals and survival signals. Akt is well known to transmit the survival signals by phosphorylating its downstream substrates (7, 8). The akt gene was originally identified as the cellular counterpart of the retroviral oncogene v-akt, which was present in AKT8, a retrovirus that caused T-cell lymphoma in mice. The catalytically inactive Akt exists in the cytosol. By activation of PI3K, PtdIns(3,4,5)\textsubscript{P}\textsubscript{3} and PtdIns(3,4)\textsubscript{P}\textsubscript{2} are synthesized at the plasma membrane, and Akt is recruited to the plasma membrane. Interaction of Akt to the lipids induces a conformational change of Akt. Then Akt is phosphorylated at two key regulatory sites, Thr\textsuperscript{308} in the activation loop of the catalytic domain and Ser\textsuperscript{473} in the COOH-terminal domain (7, 8). Dual phosphorylation at both residues is necessary for full activation of Akt. Bad is one of the substrates of Akt and is phosphorylated at the Ser\textsuperscript{136} residue. Phosphorylation of BAD prevented apoptosis by inducing the association with 14-3-3 protein isoforms. The increase in phospho-Bad/14-3-3 interaction sequesters Bad from anti-apoptotic Bcl-2 or Bcl-xL, which leads to apoptosis by inducing the association with 14-3-3 protein isoforms. The increase in phospho-Bad/14-3-3 interaction sequesters Bad from anti-apoptotic Bcl-2 or Bcl-xL, which leads to apoptosis by inducing the association with 14-3-3 protein isoforms. Therefore, Hsp90 also plays an important role in the maintenance of protein quality by regulating the balance of ubiquitination and proteasomal degradation of the misfolded substrates (35). Akt also phosphorylates caspase family member caspase-9 at the Ser\textsuperscript{196} residue and prevents its proteolytic activity. The anti-apoptotic function of Akt was also mediated by phosphorylating and modulating the function of Forkhead transcription factors and IκB kinase.

Hsp90 is an abundant and highly conserved protein involved in a diverse array of cellular processes. It is present in all species studied, from *Escherichia coli* to humans, with 40% amino acid identity. In contrast to other heat-shock proteins, Hsp90 is not required for maturation or maintenance of most proteins in vivo. By interacting with a wide variety of signaling proteins, Hsp90 prevents these Hsp90 client proteins from degradation, self-association, becoming active at an inappropriate time, or incorrect localization (16, 17). Once Hsp90 substrates fail to be correctly folded, Hsp90 promotes the degradation of the misfolded substrates (35). Hsp90 might promote the degradation of the misfolded substrates by association with an ubiquitin ligase carboxy terminus of Hsc70-interacting protein (CHIP) (36). Therefore, Hsp90 also plays an important role in the maintenance of protein quality by regulating the balance between protein folding and degradation. Because Hsp90 is constitutively overexpressed at 2- to 10-fold higher levels in tumor cells (37), Hsp90 is thought to be a critical regulator in tumor growth and survival. Our previous work (15) demonstrated that Akt kinase activity is also regulated by binding to Hsp90. Because the binding to Hsp90 protects Akt from PP2A-mediated dephosphorylation and increases Akt-mediated survival signals, overexpression of Hsp90 might protect tumor cells from undergoing apoptosis by increasing survival signals. Hsp90 inhibitors (geldanamycin, radicicol, and their derivatives) have been reported to bind to Hsp90 and to affect Hsp90 function (16, 17, 25–27). Moreover, Hsp90 inhibitors have been shown to possess anti-tumor activity in preclinical models. Hsp90 inhibitors might exhibit their anti-tumor activity by destabilizing the Hsp90 client proteins important in cancer cell proliferation (i.e. steroid receptors, Raf-1, Src family kinases, p53 mutant, cyclin-dependent kinases, and so on). As we have shown in Fig. 1, Hsp90 inhibitor had the ability to induce apoptosis with caspase activation. Therefore, it is possible that Hsp90 protects cells from undergoing apoptosis by promoting stabilization of unidentified Hsp90 client proteins that are critical for transmitting survival signals. Because Hsp90 forms a complex with Akt, we hypothesized that Hsp90 inhibitors would interfere with the Akt signaling pathway. As we have previously reported (15), exposure of the cells to geldanamycin did not inhibit the Hsp90-Akt binding. However, long-term exposure of geldanamycin suppressed the Hsp90-Akt complex formation and decreased slightly the amount of Akt (data not shown, Fig. 2). During the experiments, we found that Hsp90 inhibitors strongly promoted dephosphorylation and inactivation of Akt (Fig. 2). Recently, similar results were also reported that Hsp90 inhibitors could down-regulate phospho-Akt level and Akt signaling (18, 19). Because Hsp90 inhibitors suppressed the phosphorylation of Akt, we investigated the amount of upstream Akt kinases, PI3K and PDK1. Many Hsp90 client proteins are degraded after treatment with Hsp90 inhibitors (16, 17). Thus, we examined the expression level of PI3K and PDK1 after Hsp90 inhibitor treatment. Screening of the Hsp90 client proteins in the Akt signaling pathway revealed that PDK1 might be the critical, missing Hsp90 client protein for transmitting survival signals, because treatment of the cells with Hsp90 inhibitors decreased PDK1 stability (Fig. 3) and overexpression of the active form of Akt suppressed Hsp90 inhibitor-induced apoptosis (Fig. 6C). We also found that a proteasome-dependent pathway was involved in the Hsp90 inhibitor-induced PDK1 degradation and that Hsp90 functioned as a chaperone to keep PDK1 in a soluble and an active conformational state (Fig. 6). In contrast, Hsp90 inhibitors did not affect PI3K protein expression (Fig. 3A). Moreover, Hsp90 inhibitors showed no direct effects on PI3K activity in vitro (Fig. 4B). Therefore, PDK1 destabilization might be the main reason for the decrease in Akt-mediated survival signaling pathway. This notion was supported by the fact that KF58333 possessed the ability to decrease the amount of phospho-E40K-Akt that preferentially localized in plasma membrane in a PI3K-independent mechanism (Fig. 4C).

It is now clear that PDK1 plays a central role in the activation of the AGC subfamily of protein kinases. PDK1 phosphorylates all members of the AGC kinases at residues equivalent to Thr\textsuperscript{308} of Akt (known as activation loop or T-loop) (8). PDK1 has been shown to be able to phosphorylate p70 S6K, PKC isoforms, SGK isoforms, and protein kinase A. PDK1 itself is also a member of the AGC subfamily of protein kinases. Like other members of the AGC kinases, PDK1 phosphorylates itself at the activation loop of PDK1 (Ser\textsuperscript{241}), which results in its own activation (33). Because Hsp90 inhibitors had no inhibitory effects on PDK1 kinase activity (Fig. 4A), Hsp90 inhibitor-induced Akt inactivation was not the cause of direct PDK1 inhibition. These results imply, again, that PDK1 degradation is the main reason for the Hsp90 inhibitor-induced Akt dephosphorylation.

Our experiments with PDK1 deletion mutants indicate that the catalytic domain of PDK1 (amino acid residues around 156–223) is responsible for binding to Hsp90 (Fig. 5). Akt is also bound to Hsp90 via its catalytic domain (amino acid residues 229–309) (15). Moreover, recent reports have also suggested that Hsp90 binds to the catalytic domain of Raf, pp60\textsuperscript{v-src}, male germ cell-associated kinase (MAK), MAK-re-
lated kinase (MRK), and MAPK/MAK/MRK overlapping kinase (MOK) (24). However, MAPK, p38, and JNK/SAPK could not bind to Hsp90 despite their high homologies to Hsp90-interacting kinases (24). Consistent with this finding, Western blot analysis of the MAPK protein revealed that treatment of Hsp90 inhibitors did not affect the expression level of MAPK while the treatment decreased the phospho-MAPK amount (Fig. 2A). Therefore, not all protein kinases could bind to Hsp90.

Four lines of evidences suggest that the Akt-mediated survival pathway is an attractive target for cancer chemotherapy (38, 39). First, the Akt pathway is relatively inactive in resting cells; second, amplification of the akt gene occurs in some tumors; third, increased Akt kinase activity contributes to tumor progression in prostate cancer; fourth, loss of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is common in tumors, and its loss constitutively activates Akt. Some recent reports, including ours, also demonstrate that several anti-tumor agents exhibit their cytotoxicity by directly or indirectly modulating Akt signaling pathway (21, 40), suggesting that the PDK1-Akt survival signaling pathway is very important for tumor growth control and providing a rationale for developing new Hsp90 inhibitors that could specifically interfere with PDK1-Hsp90 complex formation, thus causing tumor cells to undergo apoptosis.

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