Akt is stimulated by several growth factors and has a major anti-apoptotic role in the cell. Therefore, we hypothesized that a pathway leading to the inhibition of Akt might be utilized in the process of apoptosis. Accordingly, we used a yeast two-hybrid screening assay to identify the proteins that interact with and possibly inhibit Akt. We found that the C-terminal region of protein kinase C-related kinase 2 (PRK2), containing amino acids 862 to 908, specifically binds to Akt in yeast and mammalian cells. During early stages of apoptosis, the C-terminal region of PRK2 is cleaved from the inhibitory N-terminal region and can bind Akt. The protein-protein interaction between Akt and the PRK2 C-terminal region specifically down-modulates the protein kinase activities of Akt by inhibiting phosphorylation at threonine 308 and serine 473 of Akt. This inhibition of Akt leads to the inhibition of the downstream signaling of Akt in vivo. The PRK2 C-terminal fragment strongly inhibits the Akt-mediated phosphorylation of BAD, a pro-apoptotic Bcl-2 family protein, and blocks the anti-apoptotic activities of Akt in vivo. These results provide direct evidence that the products of protein cleavage during apoptosis inhibit pro-survival signaling, leading to the amplification of pro-apoptotic signaling in the cell.

Akt, also termed RAC kinase (1) or PKB (2) was originally identified as the transforming oncogene in a retrovirus from a spontaneous thymoma in an AKR mouse (3). Later, it was found that Akt is phosphorylated and activated in response to mitogens and survival factors in a phosphatidylinositol 3-kinase-dependent manner (4–12). Phosphorylation of Akt occurs on two residues; threonine 308, in the activation loop, and serine 473, within the C-terminal activation domain. Whereas the upstream kinase phosphorylating serine 473 is still unknown, phosphorylation of the activation loop site by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is dependent on the products of phosphatidylinositol 3-kinase, either phosphatidylinositol 3,4-bisphosphate or 3,4,5-trisphosphate (13–15). Additionally, phosphatidylinositol 3,4-bisphosphate and 3,4,5-trisphosphate are thought to provide docking sites for Akt and PDK1, both of which become targeted to the plasma membrane via their pleckstrin homology domains (10, 11, 16, 17).

Many results support that Akt regulates various cellular metabolic pathways. It regulates glycogen synthesis by inhibiting glycogen synthase kinase-3 (6, 18) and glucose uptake and metabolism by promoting translocation of the glucose transporter GLUT4 to the plasma membrane (19–21). In addition, Akt has been implicated in the regulation of protein synthesis through indirect activation of the p70 ribosomal S6 kinase (p70S6K) (5, 7) and inactivation of eIF4E-binding protein (4E-BP) (22). Furthermore, Akt is necessary for cell survival and the prevention of apoptosis (23–27). Akt exerts its anti-apoptotic effects in a variety of ways (28). It phosphorylates and inactivates pro-apoptotic proteins, such as BAD (29, 30) and caspase-9 (31). In addition, two reports demonstrated that Akt can influence the production of nitric oxide, which is linked to the pathways that regulate apoptosis (32, 33). Most recently, Akt has been found to yield anti-apoptotic signals via the nuclear factor-kB (NF-kB) and forkhead transcription factor pathways (34–38).

However, apoptosis often occurs despite the presence of cytokines and growth factors that stimulate the anti-apoptotic pathway modulated by phosphatidylinositol 3-kinase (39). Moreover, the death signal molecule tumor necrosis factor (TNF) induces apoptosis despite transiently activating Akt (37). Based on these results, we hypothesized that cells undergoing apoptosis should possess a mechanism that inhibits Akt to secure cell death processes.

In the present study, we show that Akt binds to the C-terminal fragments of PRK2 that is generated during the early stages of apoptosis. This protein-protein interaction specifically down-modulates the protein kinase activities of Akt by inhibiting phosphorylation at its threonine 308 and serine 473 residues and strongly inhibits the anti-apoptotic function of Akt. These results support a novel mechanism where PRK2 is involved in the regulation of Akt and its downstream targets in the cell.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum, cell culture media, and LipojectAMINE were obtained from Life Technologies, Inc. DNA restriction

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and modifying enzymes were purchased from New England Biolabs and Roche Molecular Biochemicals. [α-32P]dATP and γ-[32P]P-ATP were purchased from PerkinElmer Life Sciences. Protein G-Sepharose, glutathione-Sepharose, and DEAE-dextran were purchased from Amersham Pharmacia Biotech. TNF and other chemicals were obtained from Sigma.

Yeast Two-hybrid Assays—A full-length cDNA fragment encoding mouse Akt was inserted into the EcoRI/SoI sites in the pEG202 vector. The sequence of the bait construct was verified by DNA sequencing, and the construct was introduced into EGY048 (PS0P-LacZ) yeast cells using a lithium acetate transformation protocol (40). The transformants were streaked to screen a HeLa cDNA library (a gift from Dr. P. J. Coffer, University of Cambridge) in EGY048 (PS0P-LacZ; pEG202-Akt) cells were transformed with the library plasmids and selected by plating on an SD medium lacking histidine, uracil, and tryptophan (SD/-His/-Ura/-Trp). Expression of proteins encoded by pJG4–5 library vectors was induced by growing the yeast in the presence of galactose (SD/-His/-Ura/-Trp). Positive yeast colonies, as indicated by activation of both reporter genes (LEU2 and loc2), were independently identified and isolated. We transformed Escherichia coli KC8 cells with the rescued plasmids and selected bacteria containing the pJG4–5 vectors by growing them in a medium lacking tryptophan. The pJG4–5 plasmids were isolated from E. coli KC8 and restriction (EcoRI/XhoI)-mapped. Identities of the plasmid-encoding genes were verified by DNA sequencing.

TdT-mediated dUTP Nick End-labeling (TUNEL) Assay—Cells were transfected with pEG202 and pJG4–5 expression vectors encoding various deletion mutants of Akt and PRK2 as described in figure legends.

Plasmids—cDNAs for wild type PRK2 and its deletion mutants were obtained by high fidelity polymerase chain reaction and cloned into yeast expression vector pEBG. PRK2 tagged at its C terminus with AU1 epitope was also obtained by polymerase chain reaction and inserted into mammalian expression vector pEB. Other PRK2 and Akt expressing vectors used in the experiments were kindly provided by D. P. J. Coffer (pSG5-Akt and pSG5-Gag), R. A. Roth (pECE-Myr-Akt), T. F. Franke (pCMV6-HA-Akt), and L. A. Quilliam (pCMV2-Flag-PRK2).

In Vivo Binding Assay—HA epitope-tagged Akt was co-transfected with GST-tagged wild type PRK2 or each of various PRK2 deletion mutants in COS cells. Two days after transfection, cells were washed twice with ice-cold STE (150 mM NaCl, 20 mM Tris (pH 7.4), 1 mM EDTA) and lysed in 0.5 ml of lysis buffer (50 mM HEPES (pH 7.2), 1% Nonidet P-40, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were spun at 14,000 rpm in a microcentrifuge for 10 min at 4 °C, and the cleared cell lysates were incubated with 25 μl of 50% slurry glutathione-Sepharose beads for 2 h. After incubation, protein complexes immobilized on glutathione-Sepharose beads were washed twice with lysis buffer and analyzed by immunoblot analyses with 12CA5 anti-HA monoclonal antibody.

Cell Transfection—COS and 293 cells were maintained in DMEM with 10% fetal bovine serum at 37 °C in a 5% CO2-humidified atmosphere. About 70% of the confluent cells were transfected with plasmids by the DEAE-dextran or LipofectAMINE method. Transiently transfected cells were serum-starved or incubated in DMEM with 10% fetal bovine serum at 37 °C in a 5% CO2-humidified atmosphere. About 70% of the confluent cells were transfected with plasmids by the DEAE-dextran or LipofectAMINE method. Transiently transfected cells were serum-starved or incubated in DMEM with 10% fetal bovine serum for 24 h stimulated with various growth factors and chemicals as described in figure legends.

Protein Kinase Assays—Cells were washed twice with ice-cold STE and lysed in 1 ml of lysis buffer. The cell lysates were spun at 14,000 rpm in a microcentrifuge for 10 min at 4 °C, and the cleared cell lysates were immunoprecipitated with anti-HA sera. Akt in vitro kinase assays were performed as described (6) using histone H2B as a substrate. JNK immune complex kinase assays were performed as described (41) using GST-Jun as a substrate.

Immunoblot Analyses—Cell lysates containing equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The transferred membranes were blocked with 2% powdered bovine serum albumin in TBST (Tris-buffered saline with 0.1% Tween 20) for 30 min and further incubated with the same blocking solution in which antibodies, 1:1,000 dilution of anti-HA, a 1:3,000 dilution of anti-AU1, a 1:200 dilution of anti-GST and anti-poly(ADP-ribose) polymerase (PARP), a 1:1,000 dilution of phosphospecific Akt or anti-Akt, or an 1:2,000 BAD antibodies as described previously (42). Antibody detection was completed by a chemiluminescence method using the ECL kit from Amersham Pharmacia Biotech. Anti-HA monoclonal antibody (12CA5) was purchased from Roche Molecular Biochemicals, and anti-AU1 antibody was obtained from Berkeley Antibody Co. Phosphospecific Akt antibodies and anti-Akt antibodies were obtained from New England Biolabs, and phosphospecific antibody was purchased from Upstate Biotechnology. Anti-GST antibodies were a gift from Drs. I. KriBb, Taejon, Korea. Antipoly(ADP-ribose) polymerase antibodies were kindly provided by Dr. J. W. S. Taejon, Korea.

Analyses of Cell Death—Cells were cultured on glass coverslips. Twenty-four hours after transfection, cells were treated with TNF (20 ng/ml) and cycloheximide (10 μg/ml) for 14 h at 37 °C. Stimulation was terminated by aspirating the culture medium and fixing cells with an immediate addition of 4% paraformaldehyde in 100 mM Pipes (pH 7.2), 300 mM NaCl, 1 mM MgCl2, 20 mM β-mercaptoethanol for 15 min at room temperature. The cells were washed once in phosphate-buffered saline (PBS) and then stained with a bisbenzimide (Hoechst 33258; purchased from Sigma) in PBS. Stained coverslips were mounted onto microscope slides with DABCO (1,4-diazabicyclo-[2.2.2]octane) mounting solution (100 mg/ml DABCO in 90% glycerol in PBS) and visualized under a fluorescence microscope. Transfected cells were identified by green fluorescence protein (GFP) fluorescence and scored for apoptosis by change in nuclear morphology.

TdT-mediated dUTP Nick End-labeling (TUNEL) Assay—Transfected cells were isolated by Capture-Tec™ magnetic-activated cell sorting (MACS) system from Invitrogen according to the manufacturer’s instruction. After selection, the transfected cells were subcultured for 12 h, then treated with TNF (20 ng/ml) and cycloheximide (10 μg/ml) for 14 h at 37 °C. Stimulation was terminated by aspirating the culture medium, and cells were fixed with an immediate addition of 4% paraformaldehyde in PBS for 1 h at room temperature. Cells were rinsed with PBS and made permeable by incubating in 0.1 M sodium citrate containing 0.1% Triton X-100 for 15 min on ice. Apoptotic cells were detected using terminal deoxyxynucleotidyltransferase and fluorescein-labeled dUTP from Roche Molecular Biochemicals according to the manufacturer’s instruction. Fluorescein-dUTP fluorescence was detected using a confocal microscope from Carl-Zeiss. The proportion of apoptotic cells was determined by dividing the number of cells with a TUNEL-positive nucleus, measured on 10–20 randomly taken fields, by the total number of cells in the corresponding fields.

RESULTS

Identification of PRK2 as an Akt-binding Protein by Yeast Two-hybrid Screening—We used a yeast two-hybrid screening system (Lex A system developed by Dr. Roger Brent) to identify the proteins that interact with Akt. A bait construct (pEG202-wtAkt) encoding the full-length wild type Akt was used to screen a HeLa cDNA library. Forty-two positive clones were obtained. Their cDNA sequencing analyses showed that the plasmids purified from five independent positive clones encoded C-terminal domains of PRK2 (C2 and C2a-d; Fig. 1A).

Caspase-cleaved C Terminal of PRK2 Associates with Akt—To define the region of PRK2 involved in the protein-protein interaction with Akt, we generated several PRK2 deletion mutants as described in Fig. 1A. Previously, it was reported that PRK2 is proteolyzed by caspases at aspartate 117 and 700, generating a 36-kDa C-terminal fragment (corresponding to amino acid residues 700–984) called C1 (46). Therefore, we constructed plasmids encoding N and C1, which represent these putative cleaved products generated during apoptosis, and tested their interaction with Akt by yeast two-hybrid assay (Fig. 1B). Interestingly, C1 strongly bound to Akt (Fig. 1B), but N failed to interact with Akt (Fig. 1B). This result suggests that the C-terminal structure of PRK2 is sufficient for Akt binding.

To further narrow down the binding motif, we generated C3, which contains amino acids 862 to 984 (Fig. 1A); this fragment was found to bind Akt (Fig. 1B). On the other hand, Aliess and co-workers (47) show that the C-terminal 77 amino acids of PRK2 (amino acids 908–984), called PDK-interacting fragment (PIF), interacts and regulates the Akt kinase domain. The PDK1 is an upstream kinase of Akt. We found that PIF failed to interact with Akt (Fig. 1B, fifth lane). Taken together, these results demonstrate that amino acids 862–984 of the C-terminal region are necessary for its association with Akt.

C1 Fragment of PRK2 Specifically Binds to Akt in Vivo—To investigate whether the association between C1 and Akt occurs...
The black box (45). Numbers domain (43). The experiments. The N-terminal white region indicates a GTPase-binding A

The intensity of the blue color 2 amounts of transformed yeast were inoculated on both X-gal/SD/

completed as described under “Experimental Procedures.” Similar (EGY048) with pEG202-Akt plasmid, and yeast two-hybrid assays were deletion mutants of PRK2 constructed in pJG4–5 were transformed in yeast (EGY048) with pEG202-Akt plasmid, and yeast two-hybrid assays were completed as described under “Experimental Procedures.” Similar amounts of transformed yeast were inoculated on both X-gal/SD/-His/ -Ura/-Trp/Gal (Gal) and X-gal/SD/-His/-Ura/-Trp/Glu (Glu) plates. The intensity of the blue color indicates strength of interaction between Akt and various PRK2 deletion mutants.

in mammalian cells, GST-tagged wild type PRK2 (WT), GST-tagged PRK2 deletion mutants (C0, N, C1, C3, and PIF), or GST alone (Con) was co-expressed with HA-tagged Akt in COS cells. C1 and C3 fragments were specifically co-precipitated with Akt (Fig. 2). However, GST protein, wild type PRK2 and other PRK2 deletion mutants (C0, N, and PIF) were not co-precipitated with Akt (Fig. 2). These results are consistent with the results observed in yeast two-hybrid analyses (Fig. 1B) and confirm that the C1 fragment of PRK2 produced during apoptosis does indeed interact with Akt in the cell. Interestingly, the wild type PRK2 and C0 fragment (46) did not bind Akt despite containing all the sequences of C1 and C3 (Fig. 2). This strongly suggests that the N-terminal region of PRK2 interferes with the interaction between Akt and the C-terminal region.

The PRK2 Cleavage Product C1 Associates with Akt in Apoptotic Cells—To demonstrate that Akt specifically associates with PRK2 in apoptotic cells, PRK2 tagged at its C terminus with AU1 epitope was transiently expressed in 293 cells. After treatment with TNF for various time periods, AU1 tagged-PRK2 was immunoprecipitated by using an anti-AU1 monoclonal antibody, and co-precipitated proteins were analyzed by immunoblot analyses with anti-Akt antibodies. As expected, association between endogenous Akt and PRK2 was only detected from the same time point as the generation of C0 and C1 fragments by PRK2 cleavage, within 4 h of treatment with TNF (top and third panel of Fig. 3). This association was maintained for up to 6 h after TNF treatment. Because only C1 specifically bound to Akt in the prior experiment (Fig. 2), we believe that C1, not C0, binds to Akt in apoptotic cells. Proteolysis of poly-(ADP-ribose) polymerase, a well characterized caspase substrate, confirmed the activation of the caspase pathways in the cells treated with TNF (Fig. 3, bottom panel). These results clearly demonstrate that the C1 fragment specifically associates with Akt in vivo at the early stage of apoptosis. During this apoptotic processes, however, Akt protein itself is not cleaved (Fig. 3, second panel).

C1 Fragment of PRK2 Inhibits the EGF-induced Kinase Activities of Akt—To establish the physiological role for the C1-Akt interaction, we investigated the effect of C1 on the activity of Akt. For these experiments, COS cells were transiently co-transfected with Akt and either wild type PRK2 or a deletion mutant of PRK2 (N, C1 or C3). After EGF stimulation, Akt was immunoprecipitated, and its phosphotransferase activity was measured as described under “Experimental Procedures.” As shown in Fig. 4, co-expression of C1 with Akt significantly inhibited the EGF-induced kinase activity of Akt. However, wild type PRK2 and N did not affect the activation of Akt upon EGF stimulation (Fig. 4). This indicates that the C1 fragment of PRK2 specifically inhibits the EGF-induced phosphotransferase activities of Akt. C3 also inhibited the EGF-induced Akt activity (Fig. 4).
Inhibition of Akt by Apoptotic PRK2 Cleavage

**Fig. 3.** C1 associates with Akt in apoptotic cells. 293 cells expressing PRK2 tagged with AU1 at the C terminus were treated with 20 ng/ml TNF and 10 μg/ml cycloheximide for various time periods as indicated. AU1-tagged PRK2 was immunoprecipitated (IP) by anti-AU1 monoclonal antibody, and co-precipitated proteins were analyzed by anti-Akt (top panel) and anti-AU1 immunoblot (IB) assays (third panel) as described under “Experimental Procedures.” As a control, protein amounts of Akt and poly(ADP-ribose) polymerase (PARP) in whole cell lysates (WCL) were determined by anti-Akt (second panel) and anti-poly(ADP-ribose) polymerase immunoblot analyses (bottom panel) using the same cell lysates used for PRK2 immunoprecipitation experiments. The arrows indicate cleaved PRK2 and anti-poly(ADP-ribose) polymerase.

**Fig. 4.** C1 fragment of PRK2 inhibits the EGF-induced kinase activities of Akt. 1 μg of pCMV6-HA-Akt was transiently co-transfected in COS cells with 4 μg of either an empty expression vector (Con), wild type PRK2 (WT), or a deletion mutant of PRK2 (N, C1, or C3) bearing a GST tag (in pEBG vector). Transfected cells were serum-starved for 24 h and stimulated with EGF (25 ng/ml) for 5 min (+) or untreated (−) as indicated. Akt was immunoprecipitated using 12CA5 anti-HA monoclonal antibody, and the H2B phosphotransferase activities of Akt (top panel) were measured as described under “Experimental Procedures.” Incorporation of 32P into H2B was quantitated by phosphoimaging analyses (third panel). Anti-HA immunoblots for Akt (second panel) and anti-GST immunoblots for PRK2 (bottom panel) were prepared from the same lysates used for the kinase assays.

**Fig. 5.** C1 fragment of PRK2 has no effect on the TNF-induced kinase activity of JNK. 1 μg of pcDNA3-HA-JNK was transiently co-transfected in 293 cells with 4 μg of either an empty expression vector (Con), wild type PRK2 (WT), or a deletion mutant of PRK2 (N, C1, or C3) bearing a GST tag. Transfected cells were incubated in DMEM with 10% FBS for 24 h and treated with TNF (20 ng/ml) and cycloheximide (10 μg/ml) for 30 min (+) or untreated (−) as indicated. JNK was immunoprecipitated using 12CA5 anti-HA monoclonal antibody. The Jun phosphotransferase activities of JNK (top panel) were measured as described under “Experimental Procedures,” and incorporation of 32P into GST-Jun was quantitated by phosphoimaging analyses (third panel). Anti-HA immunoblots for JNK (second panel) and anti-GST immunoblots for PRK2 (bottom panel) were prepared from the same lysates used for the kinase assays.

JNK and either wild type PRK2 or a deletion mutant of PRK2 (N, C1, or C3). After TNF treatment, JNK was immunoprecipitated, and its phosphotransferase activity was measured as described under “Experimental Procedures.” In this experiment, expression of either wild type PRK2, N, C1, or C3 had no effect on the TNF-induced activities of JNK (Fig. 5). These observations support that PRK2 cleavage during apoptosis affects specifically the activity of Akt.

**C1 Fragment of PRK2 Has No Effect on the TNF-induced Kinase Activity of JNK—**To test whether PRK2 cleavage specifically affects the activity of Akt, we examined the effect of PRK2 on the activity of JNK, a stress-activated kinase of the mitogen-activated protein kinase family, which is not related to the phosphatidylinositol 3-kinase/Akt pathway. 293 cells were transiently transfected with JNK alone or co-transfected with JNK and either wild type PRK2 or a deletion mutant of PRK2 (N, C1, or C3). After TNF treatment, JNK was immunoprecipitated, and its phosphotransferase activity was measured as described under “Experimental Procedures.” In this experiment, expression of either wild type PRK2, N, C1, or C3 had no effect on the TNF-induced activities of JNK (Fig. 5). These observations support that PRK2 cleavage during apoptosis affects specifically the activity of Akt.

**C1 Inhibits Akt via the Inhibition of Phosphorylation at Threonine 308 and Serine 473—**As described in the introduction, Akt must be translocated to the plasma membrane and phosphorylated both at threonine 308 and serine 473 to be fully activated (7, 8, 10, 13–15, 17). To determine if the mechanism of the inhibition of Akt by C1 is related to the membrane localization of Akt, we examined the effect of C1 on myristoylated Akt (Myr-Akt; kindly provided by Dr. Roth). Since Myr-Akt has a myristoylation signal at its N terminus, the protein is not only constitutively localized in the plasma membrane but also maintains a constitutively active status in the cell (7). Interestingly, the activity of Myr-Akt was strongly inhibited by co-expression of C1 or C3 in the cell to the same extent as by wortmannin treatment (Fig. 6). This implies that C1 and C3 inactivate Akt by other mechanisms besides affecting its localization in the membrane. Thus, we examined whether C1 or C3 affects the two phosphorylation events required for Akt activation. As expected, wortmannin completely blocked the phosphorylation of both threonine 308 and serine 473 of Akt (Fig. 6). Surprisingly, co-expression of C1 with Akt completely inhibited the phosphorylation at serine 473 and weakly but significantly inhibited the phosphorylation at threonine 308 of Akt (Fig. 6). Co-expression of C3 also inhibited the phosphorylations of Akt
C1 Inhibits Akt by Apoptotic PRK2 Cleavage—Previous studies demonstrate that activation of Akt protects against apoptosis induced by various agents (23-27). Because we found that the C1 fragment of PRK2 was generated during apoptosis and that this fragment strongly inhibited the activity of Akt and its downstream signaling in vivo, the physiological roles of PRK2 cleavage during apoptosis were further investigated. We transiently expressed Gag-Akt in 293 cells transfected with GFP alone, GFP and wild type PRK2, and GFP and a PRK2 deletion mutant (N or C1). In addition, as a control, cells were transfected with Gag and GFP. After inducing apoptosis by TNF treatment, apoptotic cells from each experiment were scored by a change in nuclear morphology among transfected cells, which were identified by GFP expression. Approximately 25% of 293 cells expressing either Gag-Akt alone or co-expressing Gag-Akt and N were apoptotic following TNF treatment (Fig. 8A). In contrast, about 60% of cells transfected with Gag-Akt and C1 underwent TNF-induced apoptosis, which is similar to the percentage of control apoptotic cells expressing Gag alone (Fig. 8A). In addition, expression of wild type PRK2 also significantly inhibited the protection against apoptosis by Gag-Akt (Fig. 8A). To confirm these results, we have completed separate cell death experiments using the MACS system and TUNEL assay as described under “Experimental Procedures.” Transfected cells were selected by magnetic beads and treated with TNF to induce apoptosis. The results obtained in this experiment (Fig. 8B) were very similar to those in Fig. 8A. These two results indicated that inhibition of Akt activity by the C1 fragment blocked Akt-mediated cell survival signaling during apoptosis. Furthermore, the increase in TNF-induced apoptosis caused by expression of wild type PRK2 strongly suggested that PRK2 was proteolyzed during apoptosis and inhibited Akt and its anti-apoptotic activities.

To further study the involvement of C1 in the enhancement of TNF-induced apoptosis, we carried out a similar experiment as described in Fig. 8, except that Gag-Akt was co-expressed with various PRK2 mutants (DA, DE, DADE) containing mutations at the caspase cleavage sites. The aspartate 117 was substituted with alanine in the DA mutant, and the aspartate 700 was mutated to glutamate in DE. In DADE, both sites were mutated to alanine and glutamate, respectively. About 50% of cells co-expressing wild type PRK2 and Gag-Akt were apoptotic...
after TNF treatment, and expression of DA also strongly inhibited the anti-apoptotic activity of Gag-Akt (Fig. 9A). In contrast, co-expression of DE or DADE mutant did not inhibit the anti-apoptotic activities of Gag-Akt (Fig. 9A). We observed consistent results in the experiment using a different assay system for apoptosis (Fig. 9B). Taken together, we conclude that the apoptosis-dependent cleavage of PRK2 at aspartate 700 is critical for protein-protein interaction between Akt and PRK2 C1 and the consequent inhibition of Akt and its in vivo function. In addition, overexpression of wild type or various PRK2 mutants or their co-expression with Gag-Akt did not induce cell death without TNF treatment (Fig. 9C and data not shown).

**DISCUSSION**

In contrast to the recent flood of information concerning the activation mechanisms of Akt, we have a very limited understanding about how cells down-modulate the activities of Akt. Recently, several groups reported that Akt can be inhibited by stresses such as hyperosmotic shock and ceramide treatment (48–51). These observations implicate that the inhibitory signaling mechanisms operate during stress-induced apoptosis to inhibit Akt, and this possibility was further supported by the fact that TNF induces apoptosis despite activating Akt (37). Therefore, we tried to screen for negative regulators for Akt by yeast two-hybrid assay and found PRK2 as an Akt-binding protein. By conducting yeast two-hybrid and in vivo binding assays, we demonstrated that the C-terminal sequences (amino acids 862–908) of PRK2 are necessary for specific binding to Akt in vivo (Figs. 1 and 2). Because the production of C1 fragment (containing amino acids 700 to 984) during apoptosis was previously reported by others (46) and in our Fig. 3, we have focused our study on C1.

C1 does not contain a complete structure required for protein serine/threonine kinase activity, which implies that protein phosphorylation is not involved in the C1-mediated effects on Akt. On the other hand, we believe that the protein-protein interaction between C1 and Akt is critical and that this binding leads to the inhibition of the EGF-induced Akt activity by C1 (Figs. 2 and 4). In addition, C1 expression strongly inhibited the phosphorylation and inactivation of the pro-apoptotic protein BAD by constitutively active Akt (Fig. 7) while having no effect on the activation of JNK (Fig. 5). From these results, we concluded that C1 directly and specifically inhibits Akt and its downstream signalings.

To determine the mechanism by which C1 inhibits the activity of Akt in vivo, we expressed C1 with Myr-Akt, the membrane localized form of Akt, in the cell and measured the activity of Myr-Akt. In this experiment, we found that C1 down-regulates the activity of Myr-Akt (Fig. 6), which confirms that the binding of C1 does not inhibit the activity of Akt by blocking the translocation of Akt to the membrane. Next, we examined whether C1 affects the phosphorylation of threonine 308 and serine 473 in Akt, and surprisingly, we found that C1 completely inhibited the phosphorylation of serine 473 and rather weakly inhibited the phosphorylation of threonine 308 (Fig. 6). Recently, Alessi and co-workers (47) group show that PIF, which encodes amino acids 908–984 of PRK2, acts as a positive regulator for PDK1 in vitro, allowing it to phosphorylate both threonine 308 and serine 473 residues of Akt, and thus, one can raise the possibility that C1 may regulate Akt by affecting PDK1 rather than Akt. However, in contrast to their results, we demonstrated that C1 inhibits rather than enhances the phosphorylation of serine 473 and threonine 308 in Akt (Fig. 6). In addition, in their in vivo experiment, co-expression of PIF had a negligible effect on the activity of Akt and had no effect on the phosphorylation of Akt (52). Therefore, we severely doubt the role of PIF in activating dual Akt kinase activities of PDK1. Moreover, C3, another PRK2 fragment binds to Akt, also specifically inhibits the phosphorylation and activity of Akt (Figs. 4–7). Furthermore, we found that C1 binds to the tail region of Akt containing the serine 473 residue, close to the kinase domain containing the threonine 308 residue. Because of these findings, we believe that C1 directly inhibits Akt, not indirectly through binding with PDK1. However, we still cannot exclude the possibility that these contradicting experimental results may arise from the fact that C1 contains more regions of PRK2 than does PIF. Further studies on the functional differences between C1, C3, and PIF will be needed to elucidate this matter.

Having identified C1 as an Akt inhibitor, we tried to elucidate the physiological roles of C1. C1 cleaved from PRK2, but not full-length PRK2, associates with Akt in apoptotic cells (Fig. 3). The phosphorylation of the pro-apoptotic protein BAD by constitutively active Gag-Akt is almost completely blocked

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2 H. Koh and J. Chung, unpublished results.
by C1 expression (Fig. 7). Furthermore, expression of wild type PRK2 or C1 strongly inhibits the Akt-mediated protection against TNF-induced apoptosis (Fig. 8). In this experiment, wild type PRK2, which did not inhibit the activity of Akt in our previous experiments (Figs. 4, 6, and 7) without apoptotic stimulation, strongly down-regulated the anti-apoptotic activity of Akt. Therefore, we conclude that the C1 fragment of PRK2 generated by caspases potentiates apoptotic signals in vivo by inhibiting Akt.

In previous studies, Akt has been reported to be cleaved during apoptosis (53, 54). Therefore, one may raise the argument that the binding between C1 and Akt may not have actual physiological significance. However, we have repeatedly demonstrated that Akt is not cleaved while C1 binds and inhibits Akt (Figs. 3 and 6) in our experimental conditions. In addition, we have demonstrated the inhibition of the pro-survival activity of Akt by C1 in vivo (Figs. 7 and 8). Therefore, we conclude that the binding between C1 and Akt, by inhibiting the pro-survival activity of Akt, is an important early step in the onset of apoptosis. From our results, the following model is proposed. After receiving a death signal, caspase cascades are activated. Activated caspases quickly cleave PRK2 and generate the C1 fragment. The C1 fragment binds to Akt and inhibits its anti-apoptotic activities. Consequently, apoptotic signalings become prevalent over survival signalings, and apoptosis is allowed to proceed. In support of this model, PRK2 mutated at a caspase cleavage site, aspartate 700 to glutamate, had a significantly diminished pro-apoptotic function compared with wild type PRK2 (Fig. 9).

Although our results suggest a role for PRK2 during apoptosis in the phosphatidylinositol 3-kinase/Akt signaling pathway, PRK2 may also have a regular function in the pathway of normal cells. As described previously, Alessi and co-workers (47) show that the C-terminal 77 amino acids (or PIF) of PRK2 interact with PDK1 (47). Interestingly, our study showed that the sequences directly upstream of this PIF specifically interact with Akt. In addition, PRK2 has been found to contain a mitogen-activated protein kinase kinase in yeast (48), and an SH3-binding domain (44). These results suggest the role of PRK2 as a scaffold protein in the phosphatidylinositol 3-kinase/Akt signaling pathway. We can find a similar example where a protein kinase acts as a scaffold in Pbs2p, a mitogen-activated protein kinase in yeast (55).

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