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Activation of Protein Kinase B Induced by H_2O_2 and Heat Shock through Distinct Mechanisms Dependent and Independent of Phosphatidylinositol 3-Kinase[1]

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Protein kinase B (PKB) is a downstream target of phosphatidylinositol (PI) 3-kinase in the signaling pathway of growth factors, and is activated by cellular stress such as H_2O_2 and heat shock. To study the mechanism of the stress-induced activation of PKB, PI 3-kinase products were measured in stress-stimulated cells. Both PI 3,4-bisphosphate and PI 3,4,5-trisphosphate increased in H_2O_2-treated cells, and the elevation of these phospholipids and activation of PKB were concurrently blocked by wortmannin, a potent inhibitor of PI 3-kinase. In heat-shocked cells, the level of PI 3,4-bisphosphate did not change while that of PI 3,4,5-trisphosphate increased slightly, and an association between PKB molecules was observed. Two active PKB fractions, presumably monomeric and oligomeric forms, were resolved from heat-shocked cells by gel filtration column chromatography. Activation of the former was suppressed by pretreatment with wortmannin, whereas the generation and activation of the latter were not blocked by the PI 3-kinase inhibitor. Only the monomeric form, but not the oligomeric form, was recovered from H_2O_2-treated cells, and its activation was prevented by wortmannin. These results indicate that PKB is activated by two distinct mechanisms that are dependent and independent of PI 3-kinase in stress-stimulated cells.

Key words: heat shock, hydrogen peroxide (H_2O_2), phosphatidylinositol 3-kinase, pleckstrin homology domain, protein kinase B.

Protein kinase B (PKB, also named Akt and RAC-protein kinase) is a serine/threonine protein kinase with a pleckstrin homology (PH) domain in its amino-terminal region and a catalytic domain in its carboxyl-terminal region (*1, 2*). This protein kinase was identified as an enzyme with a catalytic domain closely related to both cAMP-dependent protein kinase and protein kinase C, and also as a cellular counterpart of a rodent viral oncogene *v-akt*. Thus far, three mammalian PKB genes, α, β, and γ, have been isolated. Studies on physiological roles of PKB have revealed that this protein kinase is a downstream target of phosphatidylinositol (PI) 3-kinase in the growth factor signaling pathway (*3-6*). Namely, PKB was first reported to be activated by direct interaction of PI 3,4-bisphosphate (PI 3,4-P_2) with its PH domain (*7-9*), and then the phosphorylation of PKB was shown to be indispensable for its activation (*10-12*). In PKBα, Thr^{308} in the activation loop of its catalytic domain and Ser^{473} in the carboxyl-terminal end region were identified as the phosphorylation sites (*12*). Later, PDK1 (3-phosphoinositide-dependent protein kinase) and related enzymes were isolated that catalyze the phosphorylation of Thr^{308} of PKBα in the presence of PI 3,4,5-trisphosphate (PI 3,4,5-P_3) and PI 3,4-P_2 (*13-16*). Thus, the direct association of the PI 3-kinase products with the PH domain and phosphorylation by upstream protein kinases may be necessary for full activation of PKB upon stimulation of the cells by growth factors.

On the other hand, we have found that PKB is activated by heat shock in COS-7 and NIH 3T3 cells in a manner insensitive to wortmannin, a potent PI 3-kinase inhibitor (*17*). The heat shock-induced activation of PKB has been confirmed using different cell lines (*18, 19*). The mechanism for heat shock-induced activation of the enzyme, however, appears to differ among the cell lines employed. The heat shock-induced activation of PKB is not abrogated by wortmannin in COS-7 and 3T3-L1 cells (*17, 19*), but is

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Abbreviations: PKB, protein kinase B; PH domain, pleckstrin homology domain; PI, phosphatidylinositol; PI 3,4-P_2, phosphatidylinositol 3,4-bisphosphate; PI 3,4,5-P_3, phosphatidylinositol 3,4,5-trisphosphate; HA, hemagglutinin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PI 4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PI 3-P, phosphatidylinositol 3-phosphate; PI 4-P, phosphatidylinositol 4-phosphate.

sensitive to the PI 3-kinase inhibitor in Swiss 3T3 and 293 cells (*18*). Different results are reported in NIH 3T3 cells: the heat shock-induced activation of PKB is not prevented by wortmannin (*17*), whereas heat shock activates PI 3-kinase (*20*), and the heat shock-induced activation of PKB is prevented by the PI 3-kinase inhibitor (*18*). Furthermore, we have shown that PKB is activated by stimulation with H_2O_2 in COS-7 cells (*21*). H_2O_2- and reactive oxygen species-induced activation of PKB has been indicated in 3T3-L1, Swiss 3T3, 293, and vascular smooth muscle cells, which are sensitive to wortmannin (*18, 19, 22*).

In the present study, the PI 3-kinase products (PI 3,4-P_2/PI 3,4,5-P_3) were measured in cultured mammalian cells to determine whether stress-induced PKB activation is dependent on or independent of PI 3-kinase. It was found that heat shock generates a slight increase in PI 3,4,5-P_3, but does not produce PI 3,4-P_2, whereas H_2O_2 induces the formation of both of PI 3,4-P_2 and PI 3,4,5-P_3 in COS-7 and 293T cells. Therefore, the activation mechanism of PKB was analyzed by using various PKBα mutants and monitoring the protein kinase activity of the enzyme recovered from stress-stimulated cells.

MATERIALS AND METHODS

Expression Plasmids—The expression plasmids of FLAG- and HA-epitope tagged rat PKBα, designated FLAG-PKB and HA-PKB, respectively, were constructed as described previously (*17, 23*). The cDNA fragments corresponding to amino acids 1-113 and 149-480 of rat PKBα, which include the PH domain and the protein kinase catalytic domain, respectively, were cloned into pECE vector to make FLAG-epitope tag sequences at their amino-termini. The resulting plasmids were designated FLAG-PH and FLAG-KD, respectively. The expression plasmid of FLAG-epitope tagged rat PKBα in which Arg^{25} is replaced by Cys was constructed by polymerase chain reaction and designated R25C. The cDNAs of human PKBα and its mutated molecules in which the phosphorylation sites Thr^{308} and Ser^{473} are replaced by Ala (*12*) were introduced into pECE vector. These FLAG-epitope tagged constructs were designated hPKB, T308A, and S473A, respectively. The primary amino acid sequences of rat and human PKBα are 98% identical, and conserve these phosphorylation sites (*24, 25*). The DNA sequences were confirmed by the dideoxy chain-termination method using a DNA Sequencer model 373A (Applied Biosystems).

Cells and Transfection—COS-7 and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 incubator. CHO cells were maintained under the conditions described above in the presence of 35 μg/ml proline. COS-7 and CHO cells were transfected with each expression vector by electroporation using a Gene Pulser (Bio-Rad), and 293T cells were transfected by a polyamine transfection reagent *Trans* IT (PanVera). The transfected cells were cultured for 48 h, and treated as described in each experiment. Where indicated, cells were treated with 200 nM wortmannin for 10 min before stimulation.

Immunoprecipitation and Immunoblot—The expressed proteins were immunoprecipitated at 0-4°C essentially as described (*17*). Briefly, cells were washed with phosphate-buffered saline, and lysed in 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, and 50 μg/ml phenylmethylsulfonyl fluoride. The lysate was centrifuged for 10 min at 18,000 × *g*, and the supernatant (500-600 μg of protein) was incubated for 1 h with either an anti-FLAG (Sigma) or an anti-HA (12CA5, Boehringer) monoclonal antibody. Then, protein A-Sepharose (Pharmacia) was added and the mixture was incubated for 30 min. The immunoprecipitates were collected by centrifugation and washed with 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 1% Triton X-100. The immunoprecipitates were boiled in SDS sample buffer, and proteins were separated by SDS-PAGE and transferred onto an Immobilon P membrane (Millipore). Monoclonal antibody against either the FLAG- or HA-epitope was used as the first antibody, and alkaline phosphatase-conjugated anti-mouse antibody (Promega) was employed as the second antibody. The color reaction was carried out using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrates.

Protein Kinase Assay—The enzyme activity of immunoprecipitated PKB was assayed by measuring the incorporation of radioactivity from [γ-^{32}P]ATP into the core histone fraction prepared from calf thymus (*17*). Before assay, the immunoprecipitates collected were washed at 0-4°C with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 150 mM NaCl, and 50 μg/ml phenylmethylsulfonyl fluoride to remove Triton X-100, NaF, and Na_3VO_4. The reaction mixture (25 μl) in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 μM ATP, 15-50 kBq of [γ-^{32}P]ATP, and 200 μg/ml core histone, and the immunoprecipitates were incubated for 30 min at 30°C. After boiling in SDS sample buffer, the phosphorylated proteins were separated by SDS-PAGE, and the radioactivity of the histone band was analyzed with a Bio-imaging Analyzer BAS2000 (Fuji).

Gel Filtration of PKB—Gel filtration analysis was carried out as described by Suzuki *et al.* (*26*) with modifications. COS-7 and CHO cells transfected with FLAG-PKB were disrupted with a Dounce homogenizer in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10 mM $MgCl_2$, 2 mM EGTA, 10 mM 2-mercaptoethanol, 10 mM NaF, 1 mM Na_3VO_4, and 50 μg/ml phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000 × *g* for 30 min, and a 50-μl aliquot of the supernatant (100-150 μg of protein) was subjected to gel filtration chromatography on a prepacked Superdex-200 column (PC3.2/30) equipped with a SMART System (Pharmacia); the column was equilibrated and eluted with homogenization buffer. The chromatographic procedure was repeated three times, and the corresponding column fractions were combined and subjected to immunoprecipitation by the anti-FLAG antibody. The procedures above were carried out at 0-4°C. The protein kinase activity in the immunoprecipitates was assayed by measuring the autophosphorylation of the enzyme under the conditions described above in the absence of exogenously added phosphate acceptor protein.

Analysis of Phosphoinositides—COS-7 and 293T cells were labeled with [^{32}P]orthophosphate (37 MBq/ml) for 4 h in serum- and phosphate-free Dulbecco's modified Eagle's medium. After stimulation, the medium was removed and the reaction was stopped with MeOH:1 N HCl (1:1). The lipid was extracted with $CHCl_3$, deacylated with

methylamine, and the resulting sample was analyzed by HPLC using a Partisphere 5-SAX anion-exchange chromatography column (*27*). The radioactivity in the glycerophosphoinositide peaks was quantitated by scintillation counting and normalized to the radioactivity in the total lipids. For *in vitro* analysis, the cells stimulated with H_2O_2 were suspended in buffer containing 10 mM Tris-HCl, pH 7.5, and 10 mM NaCl for 5 min at 4°C, and then disrupted with a Dounce homogenizer. After removing the cell debris by centrifugation at 500×*g* for 5 min, the sample was further centrifuged at 10,000×*g* for 45 min and the membrane fraction was suspended in 20 mM Hepes-NaOH, pH 7.5, containing 100 mM NaCl. An aliquot of the membrane fraction was incubated at 25°C with 10 μM [γ-^{32}P]ATP (370 kBq) and 10 mM $MgCl_2$. The reaction was stopped by the addition of chloroform:methanol:1 N HCl (10:20:1). The organic phase separated by centrifugation was applied to TLC on a Silica Gel plate (Merck) pretreated with 1% oxalate and developed in chloroform:acetone:methanol:acetic acid:water (40:15:13:12:7). The radioactivity on the dried plate was visualized using a Bio-imaging Analyzer. When the lipid sample was analyzed by HPLC, the extracted lipid was deacylated with methylamine as described above.

RESULTS

PKB Activation and 3-Phosphoinositide Production in Stress-Stimulated Cells—The activity of PKB and the changes in 3-phosphoinositides were measured in stress-stimulated cells (Fig. 1). In transfected COS-7 cells, the PKB activity was elevated by heat shock, and this activation was nearly insensitive to pretreatment of the cells with wortmannin as described previously (*17*) (Fig. 1A). In these cells, PI 3,4-P_2 levels did not change, whereas PI 3,4,5-P_3 levels increased slightly and this rise was blocked by the PI 3-kinase inhibitor (Fig. 1B). H_2O_2 treatment induced PKB activation, consistent with previous reports (*18, 19, 21, 22*), and elevated the levels of both PI 3,4-P_2 and PI 3,4,5-P_3 (Fig. 1, A and B). In contrast to heat shock, the H_2O_2-induced activation of PKB was prevented by wortmannin, and the increases in the levels of PI 3,4-P_2 and PI 3,4,5-P_3 were also abrogated completely by wortmannin.

The H_2O_2-induced generation of 3-phosphoinositides was further studied using 293T cells (Fig. 1C). PI 3,4,5-P_3 levels were elevated transiently by stimulation with either 1 or 10 mM H_2O_2. In cells treated with 10 mM H_2O_2, PI 3,4-P_2 levels increased dramatically after a transient

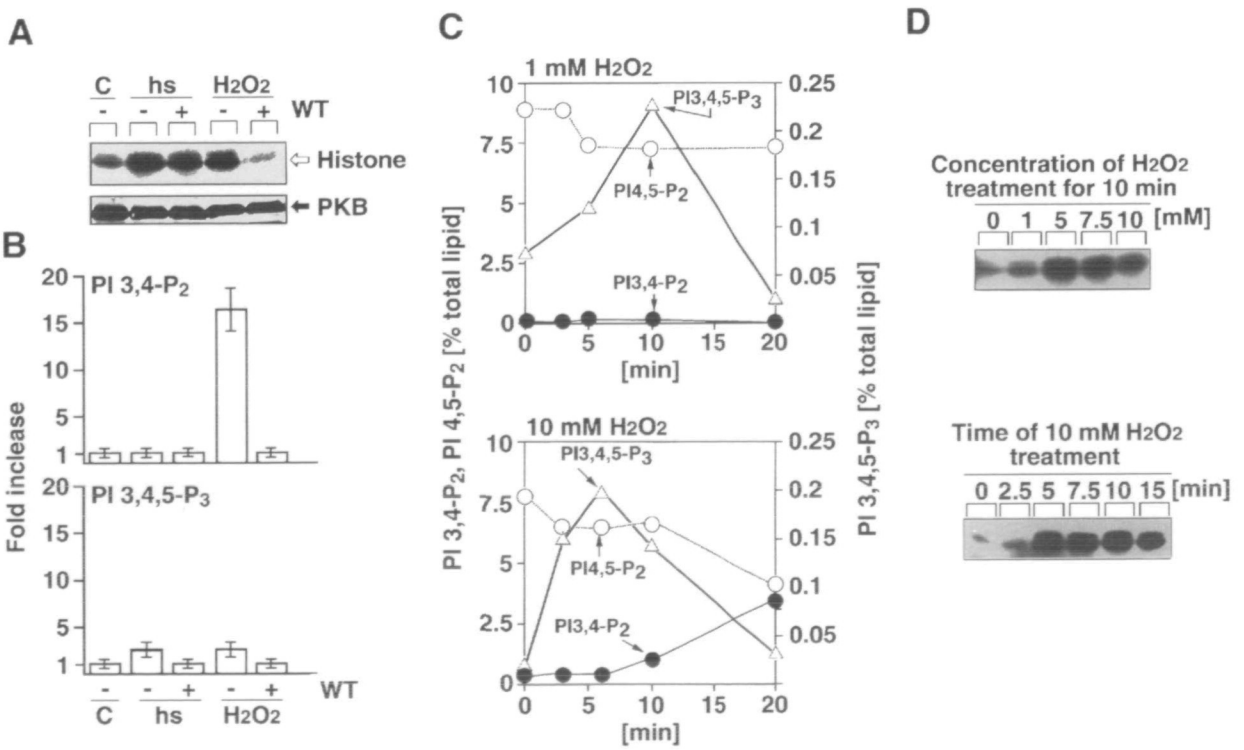

Fig. 1. **PKB activity and 3-phosphoinositide production in stress-treated cells.** (A) PKB activity in COS-7 cells. Cells transfected with FLAG-PKB were treated either at 45°C for 20 min (hs) or with 10 mM H_2O_2 for 10 min (H_2O_2). Untreated control cells are indicated as (C). Cells pretreated with and without wortmannin (WT) are indicated as (+) and (−), respectively. FLAG-PKB was immunoprecipitated with anti-FLAG antibody. Protein kinase activity was measured using core histone as a substrate (upper). Immunoblot analysis was carried out using anti-FLAG antibody (lower). (B) Changes in the amounts of 3-phosphoinositides in COS-7 cells. Cells were metabolically labeled with [^{32}P]orthophosphate, and treated as described in (A). The fold elevations of PI 3,4-P_2 and PI 3,4,5-P_3 were calculated from the normalized radioactivity of each glycerophosphoinositide separated by HPLC. The results are shown as means ± SD for three independent experiments. (C) Changes in the amounts of inositol phospholipids in 293T cells. Cells were metabolically labeled with [^{32}P]orthophosphate, and treated with either 1 mM H_2O_2 (upper) or 10 mM H_2O_2 (lower). The relative levels of PI 3,4-P_2, PI 3,4,5-P_3, and PI 4,5-P_2 calculated from the normalized radioactivity of each glycerophosphoinositide separated by HPLC are shown. (D) PKB activity in 293T cells. Cells transfected with FLAG-PKB were serum starved for 16 h and treated with various concentrations of H_2O_2 for 10 min (upper) or with 10 mM H_2O_2 for various times (lower). Protein kinase activity was measured using core histone as a substrate.

increase in PI 3,4,5-P_3, but did not change in cells treated with 1 mM H_2O_2. In addition, a substantial decrease in the PI 4,5-bisphosphate (PI 4,5-P_2) level was seen in cells treated with the higher concentration of H_2O_2, suggesting that a considerable amount of PI 4,5-P_2 was used in the PI 3-kinase pathway. It seems that the PI 3,4,5-P_3 generated from PI 4,5-P_2 in cells treated with 10 mM H_2O_2 is converted to PI 3,4-P_2. PKB activiation was found in 293T cells stimulated with comparable concentrations of H_2O_2 (Fig. 1D, upper panel) and could be detected 5 min after treatment with 10 mM H_2O_2 (Fig. 1D, lower panel). Therefore, the onset of PKB activation occurred mostly in parallel with the increase in PI 3,4,5-P_3 levels in H_2O_2-stimulated cells, and significant PKB activation and increase in PI 3,4-P_2 were concomitantly observed in the cells treated with higher concentrations of H_2O_2.

Generation of 3-Phosphoinositides in Cell Membranes—The results above suggest that PI-3 kinase may be activated intensely by treatment of the cells with H_2O_2. To confirm this, the generation of 3-phosphoinositides was analyzed using the membrane fraction obtained from 293T cells treated with H_2O_2. After incubation of the membrane fraction with [γ-^{32}P]ATP, the production of PI 3,4,5-P_3 was observed as judged by TLC (Fig. 2A). The increase in PI 3,4,5-P_3 was dependent on the concentration of H_2O_2 employed for the stimulation of the cells. HPLC analysis was performed to examine whether other 3-phosphoinositides were produced in the membrane fraction (Fig. 2B). PI 3,4,5-P_3 was found as by TLC analysis, and PI 3-phosphate (PI 3-P) was constantly produced even in membrane fractions prepared from cells not treated with H_2O_2. PI 3,4-P_2, however, was not detected in the membrane fraction. PI-3 kinase can use PI, PI 4-phosphate (PI 4-P), or PI 4,5-P_2 as a substrate with comparable affinities *in vitro* (*28*). In the membrane, the levels of PI 4-P and PI 4,5-P_2 may be comparable and much lower than the PI level. Despite the contents of these phospholipids, PI 3,4,5-P_3 was preferentially generated in the membrane fraction obtained from cells treated with H_2O_2. Therefore, it is reasonable to assume that PI 4,5-P_2 is recognized specifically by the lipid kinase in the membrane. Presumably, the PI 3,4,5-P_3 phosphatase activity, which dephosphorylates PI 3,4,5-P_3 to produce PI 3,4-P_2 *in vivo* (*29*), may not be available in the membrane. These results confirm the possibility described above that the accumulation of PI 3,4-P_2 in H_2O_2-treated cells is due to the dephosphorylation of PI 3,4,5-P_3 rather than to the direct phosphorylation of PI 4-P by PI 3-kinase.

Distinct Mechanisms of PKB Activation—Both the direct association of PI 3,4-P_2 with the PH domain (*7–9*) and the phosphorylation on Thr^{308} and Ser^{473} of PKBα (*12*) are important for the regulation of PKB activity by growth factors. Therefore, the roles of the PH domain and site-specific phosphorylation in stress-induced PKB activation were examined using the mutant proteins (Fig. 3). Human PKBα employed as a control was activated in a manner

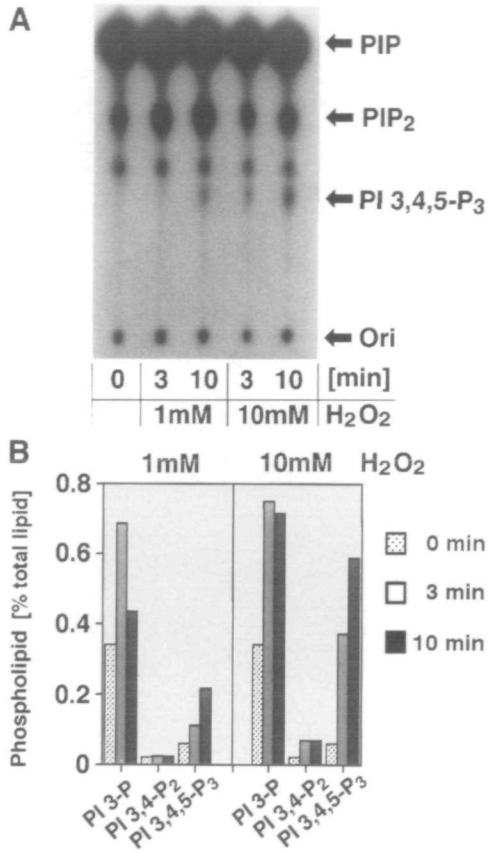

Fig. 2. **Generation of 3-phosphoinositides in the membrane fraction of 293T cells.** (A) TLC of 3-phosphoinositides. After stimulation with H_2O_2 for the indicated time, the membrane fraction was incubated with [γ-^{32}P]ATP and $MgCl_2$, and the lipids were extracted and separated by TLC. PIP and PIP_2 indicate PI-monophosphate and PI-bisphosphate, respectively. (B) HPLC of 3-phosphoinositides. The lipid samples from (A) were deacylated and separated by HPLC. The normalized radioactivities of each glycerophosphoinositide are shown.

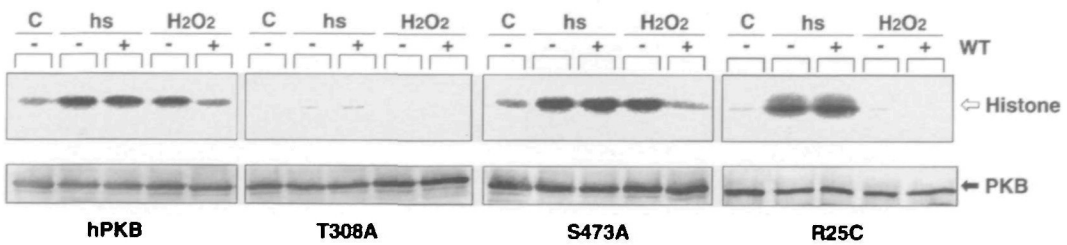

Fig. 3. **The activity of PKB mutants in COS-7 cells.** Cells were transfected with the FLAG-epitope tagged PKB construct of hPKB, T308A, S473A, or R25C, and treated either at 45°C for 20 min (hs) or with 10 mM H_2O_2 for 10 min (H_2O_2). Untreated control cells are indicated as (C). Cells pretreated with and without wortmannin (WT) are indicated as (+) and (−), respectively. FLAG-epitope tagged molecules were immunoprecipitated with anti-FLAG antibody. Protein kinase activity was measured using core histone as a substrate (upper). Immunoblot analysis was carried out using anti-FLAG antibody (lower).

identical to rat PKBα. The R25C mutant, in which Arg^{25} in the PH domain is replaced by Cys, and which does not bind inositol phospholipids (*3*), was not activated in cells stimulated with H_2O_2. The replacement of Thr^{308} with Ala eliminated its basal activity as previously described (*12*), and the T308A mutant showed no protein kinase activity in H_2O_2-treated cells. Therefore, both the direct association of PI 3-kinase products with the PH domain and the phosphorylation of Thr^{308} seem to be necessary for the H_2O_2-induced activation of PKB, as in the case of the regulation of PKB activity by growth factors. The S473A mutant was activated in cells stimulated with H_2O_2 and in heat-shocked cells, suggesting that the phosphorylation of Ser^{473} may not be indispensable for the stress-induced activation of PKBα.

In heat-shocked cells, however, the R25C mutant was efficiently activated, and wortmannin did not block the activation of this mutant. These results suggest that the R25C mutant is activated without the direct binding of PI 3-kinase products to its PH domain. Furthermore, trace but significant enzyme activity was found in the immunoprecipitate of the T308A mutant, although the T308A mutant itself has no catalytic activity (*12*). It has been reported that PKB forms a protein complex by protein-protein interactions between the PH domains, and that PI 3,4-P_2 promotes the dimerization of PKB *in vitro* (*8, 30*). Thus, the possibility of the association of the T308A mutant and PKB expressed endogenously in host cells was examined. To analyze the PKB protein complex, HA-PKB was introduced into COS-7 cells with either FLAG-PH, FLAG-KD, or FLAG-PKB (Fig. 4, A and B). HA-PKB was co-immunoprecipitated constitutively with FLAG-PH (Fig. 4A, left panel), consistent with the previous report (*30*). The association of these two full length molecules was observed in heat-shocked cells, but not in control or H_2O_2-treated cells (Fig. 4A, right panel). HA-PKB did not associate with FLAG-KD (Fig. 4A, middle panel). Therefore, heat shock induces the association of PKB molecules through their PH domains.

Protein Complex Formation—FLAG-PKB was expressed in COS-7 cells, and the supernatant fraction of the cells was subjected to gel filtration column chromatography to analyze the formation of the PKB protein complex. FLAG-PKB was immunoprecipitated from each column fraction, and detected by immunoblot analysis and by monitoring its autophosphorylating activity (Fig. 5A). The recombinant protein from unstimulated control cells was recovered as a single peak in column fractions 11-13 and showed practically no protein kinase activity. When the cells were stimulated by H_2O_2, active PKB was recovered in column fractions 11-13, and the activation is completely blocked by pretreating the cells with wortmannin. Although the appar-

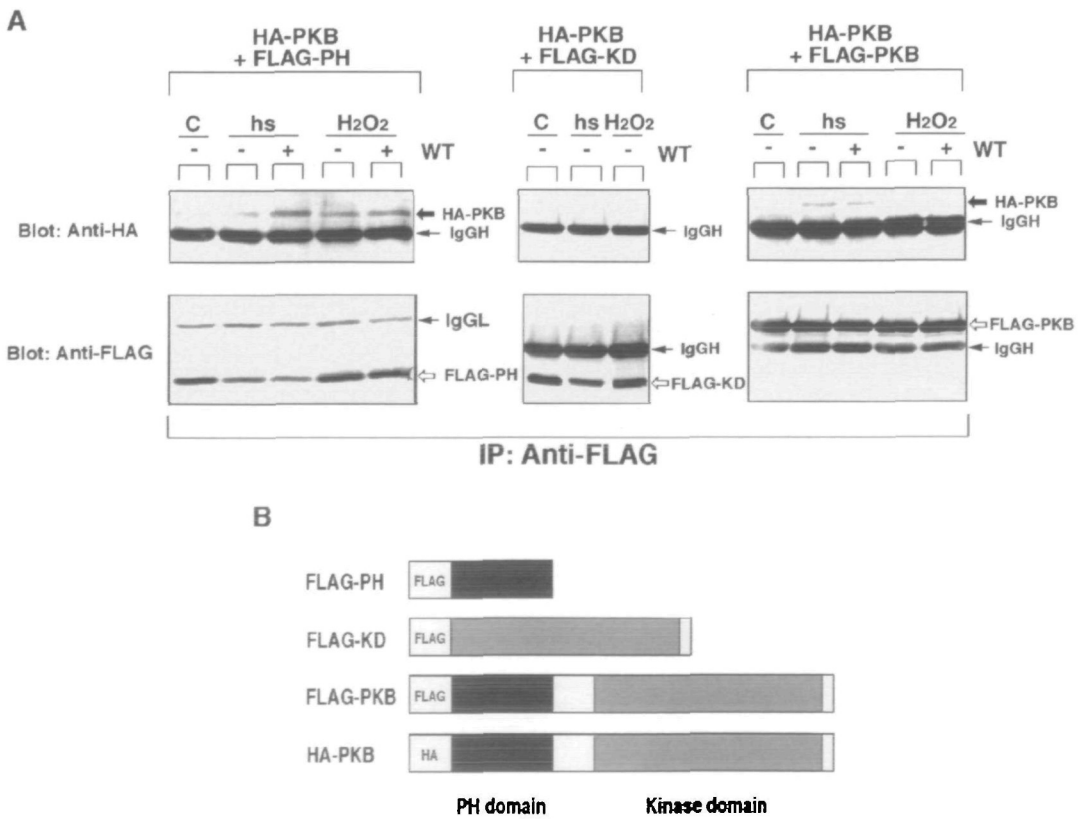

Fig. 4. **Oligomerization of PKB in heat-shocked cells.** (A) Interaction of PKB and deletion mutants in COS-7 cells. Cells were co-transfected with HA-PKB and either FLAG-PH (left), FLAG-KD (center), or FLAG-PKB (right). Cells were treated either at 45°C for 20 min (hs) or with 10 mM H_2O_2 for 10 min (H_2O_2). Untreated control cells are indicated as (C). Cells pretreated with and without wortmannin (WT) are indicated as (+) and (−), respectively. FLAG-epitope tagged molecules were immunoprecipitated with anti-FLAG antibody. Immunoblot analysis was carried out using the anti-HA antibody (upper) or the anti-FLAG antibody (lower). The positions of IgG [heavy (H) and light (L) chains] are indicated. (B) Schematic representation of the epitope-tagged PKB molecules used in (A).

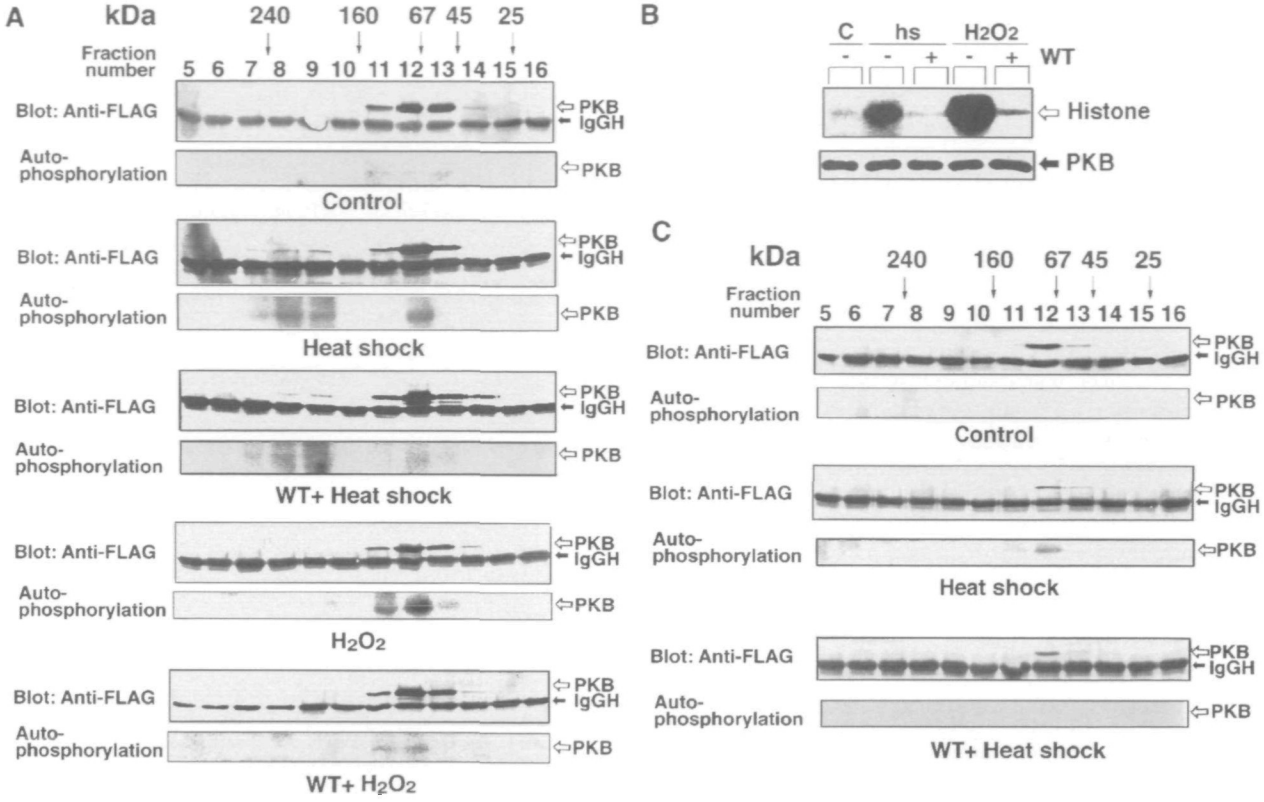

Fig. 5. **Gel filtration analysis of PKB in stress-treated cells.** (A) PKB recovered from COS-7 cells. Cells transfected with FLAG-PKB were treated either at 45°C for 20 min (Heat shock) or with 10 mM H_2O_2 for 10 min (H_2O_2). Untreated control cells are indicated as (Control). Cells pretreated with wortmannin before stimulation are indicated as (WT). The supernatant fractions of cells were applied to a gel filtration column, and FLAG-PKB in the column fractions was immunoprecipitated with anti-FLAG antibody. The immunoprecipitated samples were subjected to the immunoblot analysis using anti-FLAG antibody (upper) and the autophosphorylating activity of PKB was measured (lower). The positions of the IgG heavy chain (H) are indicated. The fraction numbers from column chromatography and the positions of the molecular weight markers are indicated at the top of the control panels. (B) PKB activity in CHO cells. Cells transfected with FLAG-PKB were treated and protein kinase assay and immunoblot analysis were carried out as described in the legend to Fig. 1A. (C) PKB recovered from CHO cells. Cells transfected with FLAG-PKB were treated at 45°C for 20 min with or without pretreatment with wortmannin, and the supernatant fractions were subjected to gel filtration analysis as described in (A).

ent size of the enzyme recovered in fractions 11–13 is 70 kDa, slightly larger than the calculated molecular mass of 57 kDa of the recombinant protein (*24*), the enzyme is presumably a monomeric form as it is found in H_2O_2-treated cells. In contrast, two peaks of protein kinase activity were recovered from heat-shocked cells in column fractions 7–9 and 11–13. The PKB activity that appeared in column fractions 7–9 remained even in cells pretreated with wortmannin. Therefore, the PKB activity obtained in these higher molecular weight fractions seems to be an oligomeric form corresponding to the PKB protein complex observed in Fig. 4A. The enzyme recovered in these higher molecular weight fractions shows an apparent molecular mass of 200 kDa. The activity in heat-shocked cells that eluted in fractions 11–13 disappeared when the cells were pretreated with wortmannin. As heat shock induces a faint increase in the PI 3,4,5-P_3 level as shown in Fig. 1B, it is reasonable to assume that the generation of active PKB activity recovered in fractions 11–13 is regulated by PI 3-kinase. In heat- shocked COS-7 cells, therefore, PKB is activated by two distinct mechanisms: one dependent on PI 3-kinase without the formation of the protein complex, and the other independent of PI 3-kinase accompanying its oligomerization.

In transfected CHO cells, PKB was activated both by H_2O_2 and by heat shock as in the case of COS-7 cells, but wortmannin efficiently prevented the activation of PKB induced not only by H_2O_2 but also by heat shock (Fig. 5B). On gel filtration column chromatography, FLAG-PKB expressed in CHO cells was obtained in a monomeric form from both control and heat-shocked cells, and the PKB protein complex was not found in heat-shocked cells (Fig. 5C). Wortmannin blocked the heat shock-induced activation of the monomeric form of the enzyme as in COS-7 cells. The PI 3-kinase inhibitor also prevented the activation of the monomeric form of PKB in CHO cells treated with H_2O_2 (data not shown). These results indicate that the generation of active PKB that accompanies the formation of the protein complex is not a universal mechanism, but depends on the cell type.

DISCUSSION

PI 3-kinase was shown to be activated by cellular stresses such as H_2O_2 and heat shock. The levels of both PI 3,4-P_2 and PI 3,4,5-P_3 were elevated in cells treated with H_2O_2 as in cells stimulated with growth factors (*28*). H_2O_2 is generated in cells stimulated with growth factors and plays

a role in the signaling pathway (*31*), and thus it is attractive to assume that H_2O_2 takes part in the activation process of PI 3-kinase induced by growth factors. The mechanism for PI 3-kinase activation by H_2O_2 remains unknown at present, but it seems possible that H_2O_2 added to the culture medium activates PKB through PI 3-kinase by mimicking the mechanism of growth factor stimulation. Heat shock also activated PI 3-kinase in COS-7 cells as reported in NIH 3T3 cells (*20*), and a trace increase in the amount of PI 3,4,5-P_3 was observed. Consistent with the increase in the PI 3-kinase product, a small PKB fraction was activated by heat shock in COS-7 cells in a wortmannin-sensitive manner. In CHO cells, however, the heat-shock induced activation of PKB was completely blocked by the PI 3-kinase inhibitor. Thus, this enzyme may be activated by heat shock through PI 3-kinase in Swiss 3T3 and 293 cells, where the heat shock-induced activation of PKB is suppressed by PI 3-kinase inhibitors (*18*). As no apparent increase in the amount of PI 3,4-P_2, which interacts with the PH domain of PKB (*7-9*), was observed in the heat-shocked cells, it is necessary to analyze the role of PI 3,4,5-P_3 in the regulation of PKB activity through the PH domain.

In addition to Thr^{308} in the catalytic domain, Ser^{473} in the carboxyl-terminal region of PKBα is phosphorylated in cells stimulated by growth factors (*12*). Recently, integrin-linked kinase has been shown to phosphorylate Ser^{473}, which is stimulated in a PI 3-kinase-dependent manner (*32*). On the other hand, it has been reported that PDK1, which phosphorylates Thr^{308}, interacts with other proteins and thus shows a different substrate specificity to recognize Ser^{473} (*33*). Even though the S473A mutant was activated in stress-stimulated cells, Ser^{473} was phosphorylated in H_2O_2-treated and heat-shocked cells (data not shown). It seems possible that Ser^{473} is phosphorylated by these PI 3-kinase-dependent protein kinases in stress-stimulated cells, because PI 3-kinase is activated by cellular stress.

PKB was activated not only in a PI 3-kinase-dependent manner, but also in a manner independent of lipid kinase in heat-shocked COS-7 cells. The PI 3-kinase-independent activation of PKB is not a universal mechanism, but may occur in the cellular responses of certain cell types. PKB has also been reported to be activated in a PI 3-kinase-independent manner in rat epididymal fat cells stimulated by β-adrenergic agonists (*34*) and in 293 EBNA and COS-7 cells treated with cAMP-elevating agents (*35*). Although the molecular mechanism is not clear, phosphorylation at a site(s) distinct from Thr^{308} or Ser^{473} in PKBα has been suggested to be important for its cAMP-induced activation (*35*). In this study, a PKB fraction was detected as an oligomeric form of PKB protein complex in heat-shocked cells. It has been reported that PI 3,4-P_2 promotes the dimerization of PKB (*8, 30*), but the PKB protein complex was formed in heat-shocked cells in which PI 3,4-P_2 was not generated. Furthermore, the apparent size of the PKB protein complex was 200 kDa, which is larger than the estimated molecular mass of the PKB dimer. The molecular composition of the PKB protein complex is not clear at present, and needs to be analyzed. We have previously identified a small heat shock protein, Hsp27, that associates specifically with PKB in stress-stimulated cells (*21*). Hsp27 associates with certain proteins as a chaperone (*36*) and may play a role in the complex formation and activation of PKB. PKB has been shown to play a critical role in protection against apoptosis (*2*). A preliminary experiment indicated that H_2O_2-induced apoptosis is suppressed in a CHO cell line that stably overexpresses PKBα. As PKB is activated by H_2O_2-treatment, it is suggested that PKB may take part in the protection of cells from damage caused by cellular stress. Further studies are required to clarify the precise mechanism of PKB activation and the role of the enzyme in the cellular response to stress stimulation.

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