Distinct Activation Mechanisms of Protein Kinase B by Growth-Factor Stimulation and Heat-Shock Treatment†

Hidenori Matsuzaki, Toshiyoshi Yamamoto, and Ushio Kikkawa*

Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

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ABSTRACT: Protein kinase B (PKB) α, having the pleckstrin homology (PH) and catalytic domains in its amino- and carboxyl-terminal regions, respectively, is activated in the signaling pathway of growth factors as a downstream target of phosphatidylinositol 3-kinase and becomes an active form in heat-shocked cells in a manner independent of the lipid kinase. Therefore, the activation mechanisms of PKBα were compared in platelet-derived growth factor (PDGF)-stimulated and heat-shocked cells by monitoring the protein kinase activity and phosphorylation of the mutant molecules expressed in COS-7 cells. In heat-shocked cells, PKBα was activated to a certain level without phosphorylation on Thr-308 in the activation loop and on Thr-450 and Ser-473 in the carboxyl-terminal end region, which is critical for growth-factor-induced activation of PKBα. Metabolic labeling with ^{32}P-orthophosphate in the transfected cells revealed that there is no major phosphorylation site other than the three residues in PKBα. PKBα activated by heat shock was more stable than the enzyme stimulated by PDGF in the cells, and PKBα recovered from heat-shocked cells was resistant to the protein phosphatase treatment, whereas the enzyme obtained from the growth-factor-stimulated cells was inactivated by dephosphorylation. Heat shock also enhanced the association of the PH-domain fragment to the full-length PKBα in the transfected cells. On the other hand, the PH-domain fragment of PKBα, which moves from the cytosol to the plasma membrane upon PDGF stimulation by the interaction with the phosphatidylinositol 3-kinase products, did not translocate but stayed in the cytosol in heat-shocked NIH 3T3 cells. Furthermore, PKBα was associated with the nuclear region in heat-shocked cells, which is not observed in growth-factor-stimulated cells. These results indicate that heat shock induces the conformational change of PKBα that accompanies the protein complex formation and perinuclear/nuclear localization of the enzyme, to generate an active form by a mechanism distinct from that in the growth-factor-signaling pathway.

Protein kinase B (PKB, also named as Akt and RAC-protein kinase)[11] is a serine/threonine protein kinase having the pleckstrin homology (PH) and catalytic domains in its amino- and carboxyl-terminal regions, respectively, and three subtypes of α, *β*, and *γ* have been thus far identified. This protein kinase is revealed to be one of the downstream targets of phosphatidylinositol (PI) 3-kinase in the signaling pathway of growth factors and has roles in various cellular processes such as survival, differentiation, cell cycle, and metabolism (*1*−*4*). Concerning the regulation of the catalytic activity of PKB, the direct association of the PI 3-kinase products to its PH domain and phosphorylation of the enzyme are indispensable for growth-factor-induced activation of PKB. 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, and then phosphorylation of PKB was shown to be indispensable for its activation (*5*−*7*). In PKBα, Ser-124 in the hinge region between the PH and catalytic domains, Thr-308 in the activation loop of its catalytic domain, and Thr-450 and Ser-473 in the carboxyl-terminal end region were identified as the phosphorylation sites (*8*). Among these residues, phosphorylation of Thr-308 and Ser-473 is induced by the stimulation of the cells by growth factors, and especially phosphorylation in the activation loop is revealed to be essential for growth-factor-induced activation of PKB. Later, a protein kinase named PDK1 (3-phosphoinositide-dependent protein kinase 1) was isolated, which catalyzes phosphorylation of Thr-308 of PKBα in the presence of PI 3,4-P_2 and PI 3,4,5-trisphosphate (PI 3,4,5-P_3) (*9*, *10*). Molecular cloning of PDK1 has revealed that this enzyme has the catalytic and PH domains in its amino- and carboxyl-terminal regions, respectively (*11*, *12*). It is hypothesized that the PH domain of PDK1 interacts with the PI 3-kinase products facilitating its translocation to the plasma membrane and that PKB also moves to the membrane to be phosphorylated on the Thr residue in the activation loop by PDK1. The phosphorylation on Ser-473 of PKBα is tightly associated with that on Thr-308, and it has been reported that this site is recognized by integrin-linked kinase (*13*), PDK1 (*14*), and PKB itself (*15*). Thus, the direct association of the PI

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* To whom correspondence should be addressed. Telephone: 81-78-803-5964. Fax: 81-78-803-5972. E-mail: ukikkawa@kobe-u.ac.jp.

[1] Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PBS-T, PBS supplemented with 0.03% Triton X-100; PDK1, 3-phosphoinositide-dependent protein kinase 1; PDGF, platelfet-derived growth factor; PH, pleckstrin homology; PI, phosphatidylinositol; PI 3,4-P_2, PI 3,4-bisphosphate; PI 3,4,5-P_3, PI 3,4,5-trisphosphate; PKB, protein kinase B.

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3-kinase products to the PH domain and phosphorylation in the activation loop and the carboxyl-terminal end region have been regarded to be necessary for full activation of PKB upon stimulation of the cells by growth factors.

On the other hand, PKB has been reported to be regulated by mechanisms independent of PI 3-kinase (*4*, *16*, *17*). We have found that PKB is activated by cellular stress in cultured cell lines (*18*). In heat-shocked cells, PKB is activated at least in part in a manner insensitive to wortmannin, a potent PI 3-kinase inhibitor, without an increase of the PI 3-kinase products of PI 3,4,5-P_3 and PI 3,4-P_2 (*19*). Furthermore, the formation of a PKB protein complex is observed in heat-shocked cells, which is not detected in the control cells (*19*). Therefore, it is important to investigate the activation mechanism of PKB in heat-shocked cells, because the enzyme contributes to cell protection from various damages including cellular stress (*1*−*4*).

In the present paper, the activation mechanisms of PKBα were compared in heat-shocked cells and in the cells stimulated by platelet-derived growth factor (PDGF) by expressing the mutant molecules in COS-7 cells. Phosphorylation of PKB in the stimulated cells was monitored by the phosphorylation site-specific antibodies as well as metabolic labeling of the cells, and the properties of the activated PKB were analyzed in vitro by using a protein phosphatase. Furthermore, the activation of PKBα endogenously expressed in NIH 3T3 cells was studied. The results obtained indicate that heat shock can induce PKB activation without phosphorylation probably by the conformational change of the molecule.

MATERIALS AND METHODS

Expression Plasmids. The expression plasmids of rat PKBα and its mutants were generated as follows. FLAG- and HA-epitope-tagged PKBα, FLAG-epitope-tagged PKBα replacing either Lys-179 by Met or Arg-25 by Cys, and FLAG-epitope-tagged PH domain containing the amino acids 1−113 of PKBα were constructed as described previously (*18*, *19*) and designated FLAG-PKB, HA-PKB, K179M, R25C, and FLAG-PH, respectively. The expression plasmids of FLAG-epitope-tagged PKBα replacing each of Ser-124, Thr-308, Thr-450, and Ser-473 by Ala were constructed by the polymerase chain reaction and designated S124A, T308A, T450A, and S473A, respectively. The mutant PKBα molecules in the FLAG-epitope-tagged expression vector replacing Arg-25 by Cys and Lys-179 by Met, Ser-124, Thr-450, and Ser-473 by Ala, Arg-25 by Cys and Ser-124, Thr-450, and Ser-473 by Ala were generated by successive site-directed mutagenesis and designated R25C/K179M, 3A, and R25C/3A, respectively. The cDNA fragment corresponding to the amino acids 1−113 of R25C was amplified by the polymerase chain reaction, cloned into the FLAG-epitope-tagged expression vector, and designated FLAG-PH/R25C. The full-length PKBα and the cDNA fragment corresponding to the amino acids 1−113 of PKBα were introduced into BS340 vector (*20*), to construct the proteins fused with green fluorescent protein (GFP) and designated PKB-GFP and PH-GFP, respectively. The DNA sequences were confirmed by the dideoxy chain-termination method using DNA Sequencer model 373A (Applied Biosystems).

Cells and Transfection. COS-7 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nacalai tesque) supplemented with 10% fetal calf serum (FCS) and 10% calf serum, respectively, at 37 °C in a 5% CO_2 incubator. COS-7 cells were transfected with each expression plasmid by electroporation using Gene Pulser (Bio-Rad) (*18*), cultured in DMEM supplemented with 10% FCS for 36 h, washed twice with phosphate-buffered saline (PBS), and further cultured in DMEM supplemented with 0.1% bovine serum albumin (BSA) for 16 h. Where indicated, the cells were pretreated with 200 nM wortmannin for 15 min before the experiments. NIH 3T3 cells grown on glass-bottom culture dishes (MatTek) were transfected by using the Fugene 6 transfection reagent (Roche) according to the manufacturer's protocol and subjected to microscopic observation as described below. The temperature of the culture medium was measured directly by using a thermometer model BAT-12 (Physitemp) equipped with a microsensor tip.

Immunoprecipitation. Immunoprecipitation of the expressed proteins was carried out at 0−4 °C essentially as described (*18*). Briefly, the cells were washed with PBS and lysed in 20 mM Tris−HCl at 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 of 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 an anti-FLAG (Sigma) monoclonal antibody, an anti-HA (12CA5, Boehringer) monoclonal antibody, or an anti-PKBα polyclonal antibody (Pharmingen). Then, protein A-Sepharose (Pharmacia) was added to the mixture and incubated for 30 min. The immunoprecipitates were collected by centrifugation and washed with 20 mM Tris−HCl at pH 7.5 containing 150 mM NaCl and 1% Triton X-100.

Immunoblot. The immunoprecipitates were boiled in SDS-sample buffer, and the proteins were separated by SDS−PAGE and transferred onto an Immobilon P membrane (Millipore). The antibodies against the FLAG- and HA-epitopes, PKBα, and the antiphosphopeptide antibodies that selectively recognize phosphorylated Thr-308 (New England Biolabs), Thr-450 (generated in this paper as described below), and Ser-473 (New England Biolabs) of PKBα were used as the first antibodies. These phosphorylation site-specific antibodies were designated as anti-pT308, anti-pT450, and anti-pS473, respectively. The alkaline phosphatase-conjugated antimouse and antirabbit antibodies (Chemicon) were employed as the second antibodies. The color reaction was carried out using 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium as the substrates. All results were representative from three independent experiments. The intensity of the color reaction catalyzed by the anti-FLAG antibody was quantitated by using the Image Gauge computer program (Fujix). FLAG-PKB from transfected COS-7 cells (*21*) was employed as a standard. The polyclonal antibody directed against PKBα phosphorylated on Thr-450 was raised essentially as described (*22*) by immunizing rabbits with a phosphopeptide CQMITIpTPPDQD coupled to keyhole limpet hemocyanin, which has an additional cysteine residue at the amino-terminal end of the amino acid sequence of 444−455 of rat PKBα, where pT corresponds to phosphothreonine. The antibody was purified by successive column chromatographies on the resins coupled with the phosphopeptide and the nonphosphopeptide, respectively.

Protein Kinase Assay. The enzyme activity of the immunoprecipitated PKB was assayed by measuring the incorporation of radioactivity from [γ-^{32}P]ATP to the core histone fraction prepared from calf thymus (*18*). Before assay, the immunoprecipitates collected were washed at 0−4 °C with 20 mM Tris−HCl at pH 7.5 containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 150 mM NaCl, and 50 μg/mL of phenylmethylsulfonyl fluoride to remove Triton X-100, NaF, and Na_3VO_4. The reaction mixture (25 μL) containing 20 mM Tris−HCl at pH 7.5, 10 mM $MgCl_2$, 20 μM ATP, 15−50 kBq of [γ-^{32}P]ATP, and 200 μg/mL of core histone was added to the immunoprecipitates and incubated for 30 min at 30 °C. After boiling in SDS sample buffer, phosphorylated proteins were separated by SDS−PAGE, and the radioactivity of histone band was analyzed by Bio-imaging Analyzer BAS2500 (Fujix). All results were representative from three independent experiments. The specific activity of the wild-type and mutant proteins was determined by calibrating each protein kinase activity by its protein amount quantitated as described above. In some experiments, GSK-3 Fusion Protein (Cell Signaling), a synthetic substrate specific to PKB, was employed as a phosphate acceptor of PKB (*23*, *24*).

Metabolic Labeling. COS-7 cells transfected with each expression plasmid were cultured in phosphate-free DMEM supplemented with 0.1% BSA for 1 h and then metabolically labeled with [^{32}P]orthophosphate (18 MBq/6 cm dish) for 4 h. After the treatment at 42 °C for 15 min, the cells were lysed. FLAG-PKB and its mutants were immunoprecipitated, and immunoblot analysis was carried out using the anti-FLAG antibody as described above. The ^{32}P-radioactivity incorporated into the immunoprecipitated proteins was determined by Bio-Imaging Analyzer. The data were representative from three independent experiments.

In Vitro Dephosphorylation. FLAG-PKB and R25C immunoprecipitated from the transfected cells were washed twice with the reaction buffer containing 50 mM Tris−HCl at pH 7.5, 5 mM dithiothreitol, 2 mM $MnCl_2$, and 100 μg/mL of BSA. Then, immunoprecipitates were incubated with 100 units (1 unit is defined as the amount of enzyme that hydrolyzes 1 nmol of p-nitrophenolphosphate/min) of protein phosphatase λ (Calbiochem) in the reaction buffer (50 μL) for 10 min at 30 °C.

Observation of GFP−Fusion Protein. Transfected NIH 3T3 cells were cultured in DMEM supplemented 10% calf serum for 24 h and then in a phenol red-free DMEM supplemented with 0.1% BSA for 24 h. After stimulation of the cells, the fluorescence of GFP and GFP−fusion proteins was monitored with a confocal laser-scanning fluorescent microscope (LSM 510 invert, Carl Zeiss) at 488-nm argon excitation using a 515−525-nm band-pass barrier filter (*25*). All results were represntative from three independent experiments.

Immunocytochemical Staining. NIH 3T3 cells grown on glass-bottom culture dishes were directly fixed with 4% parafolmaldehyde in 0.2 M sodium phosphate at pH 7.3 and permeabelized with 0.3% Triton X-100. Where indicated, the cells were subjected to the detergent extraction with 0.1% Triton X-100 in 80 mM Pipes at pH 6.9, 5 mM EDTA, and 1 mM $MgCl_2$ at room temperature for 2 min and then fixed with cold methyl alcohol for 3 min (*26*). After blocking with 5% normal goat serum in PBS supplemented with 0.03% Triton X-100 (PBS-T), the cells were incubated with the anti-PKBα antibody in PBS-T containing 1% normal goat serum and then with an Alexa 488-conjugated antirabbit antibody (Molecular Probe) in PBS-T. The fluorescence of Alexa 488 was observed under a confocal laser-scanning fluorescent microscope at 488-nm argon excitation using a 510−525-nm band-pass barrier filter. All results were representative from three independent experiments.

RESULTS

*Growth-Factor- and Heat-Shock-Induced Activation of PKB*α. Activation of PKBα was examined in COS-7 cells transfected with FLAG-PKB (Figure 1). PDGF stimulated the protein kinase activity as repeatedly reported, which reached to the maximal level 10 min after the treatment of the cells (Figure 1A). In the cells treated at 42 °C, the PKB activity reached a plateau at 20 min, which is less than that in PDGF-stimulated cells, whereas the temperature of the culture medium became 42 °C within 2 min (Figure 1B). Activation of PKBα was found in the cells treated at 40 °C, and the treatment of the cells at temperatures higher than 42 °C did nothing to further enhance the enzymatic activity (Figure 1C). These results indicate that both the growth factor and heat shock induce activation of PKBα, but the former stimulates the protein kinase activity more rapidly and efficiently than the latter. In the following heat-shock experiments, the cells were treated at 42 °C.

*Mutation Analysis of PKB*α. Both the direct association of the PI 3-kinase products to the PH domain and phosphorylation on the threonine residue in the activation loop are indispensable for growth-factor-induced activation of PKBα (*8*). Namely, it was shown that R25C mutating Arg-25 by Cys in the PH domain that does not bind inositol phospholipids (*27*) was not activated in the cells stimulated by growth factors (*28*) and that the mutant molecule replacing Thr-308 in the activation loop by Ala had no protein kinase activity (*8*). In addition to Thr-308, three residues such as Ser-124 in the hinge region between the PH and catalytic domains and Thr-450 and Ser-473, the turn and hydrophobic motif sites, respectively, have been identified as the phosphorylation sites in PKBα (*8*). Therefore, the mechanism of heat-shock-induced activation of PKBα was analyzed by using the mutant molecules replacing these residues (Figure 2, Table 1). Wortmannin, a potent inhibitor of PI 3-kinase, abolished the PDGF-induced stimulation of the wild-type PKB. The PI 3-kinase inhibitor attenuated heat-shock-induced activation of the enzyme, but still a significant activity of the wild-type enzyme was observed in heat-shocked cells as reported in the previous papers (*18*, *19*). Therefore, heat shock induces PKB activation through a PI 3-kinase-dependent pathway; however, the PKB activity is regulated at least in part in an independent manner from PI 3-kinase. Moreover, R25C was not activated by PDGF but was stimulated by heat shock. Further replacement of R25C at Lys-179 in the ATP-binding site, namely, R25C/K179M, made the product inactive as in the case of K179M, the kinase-negative mutant (*18*). Similar protein kinase activities were observed by using GSK-3 Fusion Protein instead of core histone as a specific substrate of PKB (data not shown). These results confirm that the protein kinase activity of R25C measured in these experiments represents R25C itself but not other protein kinase that might contaminate the immu-

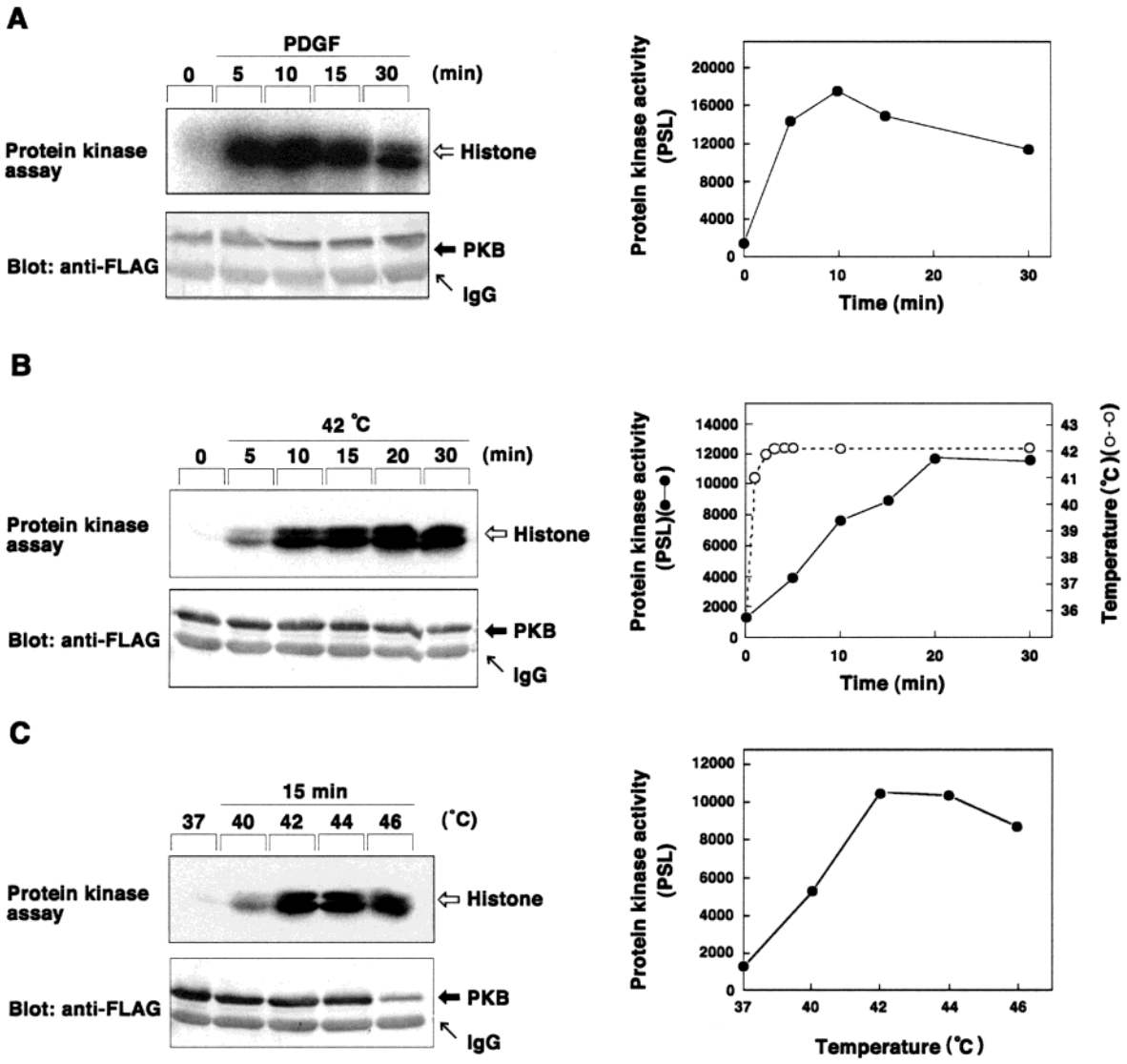

FIGURE 1: Heat-shock-induced activation of PKB in COS-7 cells. The cells transfected with FLAG-PKB were stimulated with 20 ng/mL of PDGF for the indicated time (A), treated at 42 °C for the indicated time (B), or treated at the indicated temperature for 15 min (C). FLAG-PKB were immunoprecipitated by anti-FLAG antibody, the protein kinase activity was measured by using core histone as a substrate (left upper panel), and immunoblot analysis was carried out by using anti-FLAG antibody (left lower panel). The results in the left panels are quantitated and shown in the right panels.

noprecipitate. Concerning the mutant molecules replacing the phosphorylation sites, T308A introducing a mutation in the activation loop showed no protein kinase activity even in the heat-shocked cells as reported (*19*), but the mutant molecules replacing other phosphorylation sites were still enhanced by PDGF and heat shock. Namely, S124A was activated as efficiently as the wild-type enzyme, indicating that the phosphorylation of this residue in the hinge region of the enzyme is not required for its activation. The enhancement of the protein kinase activity was observed in T450A and S473A in both the PDGF-stimulated and heat-shocked cells, even though PDGF-induced activation was less significant than that of the wild-type enzyme. Thus, the phosphorylation on Thr-450 and Ser-473 is not essential for its enzymatic activity but may be necessary for full activation of PKB. It is worth noting that heat-shock-induced activation of these two mutants, as well as that of R25C, was not suppressed by the pretreatment of the cells with wortmannin. The triple mutant 3A replacing Ser-124, Thr-450, and Ser-473 showed properties similar to those of T450A and S473A, and the mutant R25C/3A was not stimulated by PDGF but was still activated by heat shock as in the case of R25C.

*Phosphorylation of PKB*α. The role of phosphorylation in PKB activation was further studied by using the antibodies that selectively recognize phosphorylated Thr-308, Thr-450, and Ser-473 residues (Figure 3A). In the wild-type PKBα, PDGF-induced activation and phosphorylation on Thr-308 were both blocked by wortmannin. Heat shock induced a slight increase of phosphorylation on Thr-308 in the wild-type enzyme, which was prevented by the PI 3-kinase inhibitor. The wild-type PKBα was, however, still activated without detectable phosphorylation on Thr-308 in the heat-shocked cells pretreated with wortmannin. Phosphorylation on Thr-308 was not found in R25C, but the mutant was activated by heat shock. To confirm that PKB is activated without phosphorylation on Thr-308, the immunoreactions of the wild-type PKB and R25C detected by the anti-pT308 antibody were compared with their protein kinase activities (Figure 3B). Phosphorylation on Thr-308 was not detected in the wild-type PKB recovered from heat-shocked cells in

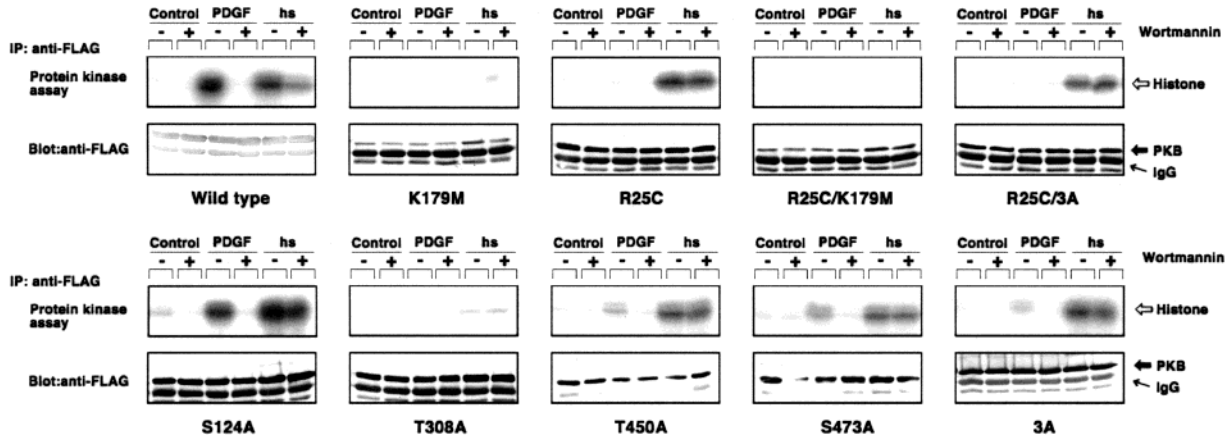

FIGURE 2: Protein kinase activity of the PKB mutants in COS-7 cells. The cells transfected with FLAG-PKB (wild type) for each PKB mutant were treated with 20 ng/mL of PDGF for 10 min or at 42 °C for 15 min [heat shocked (hs)]. The cells pretreated with and without wortmannin are indicated as (+) and (−), respectively. The expressed proteins were immunoprecipitated by anti-FLAG antibody, the protein kinase activity was measured by using core histone as a substrate (upper panel), and immunoblot analysis was carried out by using anti-FLAG antibody (lower panel).

Table 1: Quantitation of the Protein Kinase Activity of the PKB Mutants in COS-7 Cells[a]

	control		PDGF		heat shock	
PKB	(−)	(+)	(−)	(+)	(−)	(+)
wild type	11 ± 1	6 ± 1	100	7 ± 1	67 ± 25	32 ± 10
K179M	5 ± 3	5 ± 3	4 ± 2	5 ± 4	10 ± 2	9 ± 5
R25C	3 ± 2	2 ± 1	3 ± 2	3 ± 2	29 ± 18	27 ± 12
R25C/K179M	5 ± 1	4 ± 1	4 ± 2	6 ± 1	9 ± 6	11 ± 2
T308A	2 ± 1	2 ± 1	2 ± 1	2 ± 1	11 ± 6	9 ± 3
T450A	3 ± 2	3 ± 2	17 ± 2	3 ± 2	37 ± 10	37 ± 9
S473A	5 ± 1	5 ± 1	29 ± 10	6 ± 1	42 ± 6	38 ± 9
3A	7 ± 2	4 ± 3	16 ± 1	8 ± 3	31 ± 12	26 ± 6
R25C/3A	5 ± 2	5 ± 4	4 ± 2	5 ± 2	34 ± 5	31 ± 11

[a] The specific protein kinase activity of the wild-type enzyme in PDGF-stimulated cells is shown as 100%. The cells pretreated with and without wortmannin are indicated as (+) and (−), respectively. Data are the average of the three independent experiments ± standard deviation.

the presence of wortmannin, whereas the comparable amount of PKB recovered from PDGF-stimulated cells was clearly phosphorylated. Phosohorylation on Thr-308 was, of course, not found in R25C. These results indicate that heat shock can induce activation of PKB without phosphorylation on Thr-308. The anti-pS473 antibody employed in this paper weakly recognized the nonphosphorylated form, because it reacted with R25C/3A (Figure 3A). In NIH 3T3 cells, endogenously expressed PKB was not detected by this antibody after serum starvation, as described later. The wild-type enzyme was recognized by the antibody in control cells, but the signal was significantly enhanced by PDGF and heat shock and prevented by wortmannin. It indicates that the stimulation of the cells by either PDGF or heat shock can induce the phosphorylation on this residue in the wild-type PKBα, which is prevented by wortmannin. On the other hand, the phosphorylation on Ser-473 was increased slightly in R25C after stimulation by PDGF but not altered by heat shock. In contrast, Thr-450 was constitutively phosphorylated in the wild-type enzyme as well as in R25C, and the level of phosphorylation on this site was not effected by the stimulation of the cells. It seems that phosphorylation on Thr-450 and Ser-473 has different roles in the regulation of the PKB activity; the former is constitutive, whereas the latter is inducible, although the mutants T450A and S473A showed similar properties. Phosphorylation of PKBα was analyzed in the cells metabolically labeled with [^{32}P]orthophosphate (Figure 3C). The wild-type PKBα was recovered as a phosphoprotein from the control cells. The phosphorylation of the wild-type protein was increased slightly by heat shock, which is prevented by the pretreatment of the cells with wortmannin. R25C was also found as a phosphoprotein, but its phosphorylation was not enhanced by heat shock. These results agree with those in Figure 3 that heat-shock-induced phosphorylation on Thr-308 and Ser-473 in the wild-type PKBα but not in R25C. Phosphorylation of the proteins detected in the unstimulated cells reflect its constitutive modification presumably on the sites such as Ser-124 and Thr-450. In contrast, almost no phosphorylation was detected in R25C/3A, although this mutant is activated in the heat-shocked cells as shown in Figure 3. These results indicate that there is no major phosphorylation site other than the four residues analyzed in PKBα. Furthermore, it is concluded that heat shock can activate PKBα in part without phosphorylation of this enzyme.

*Inactivation of PKB*α. After the cells expressing FLAG-PKB were stimulated, the enzymatic activity and phosphorylation on Thr-308 were monitored, while the transfected cells were incubated in the absence of growth factors at 37°C (Figure 4). In the cells once stimulated by PDGF, the PKB activity was reduced rapidly accompanying the concomitant decrease of phosphorylation on Thr-308. Inactivation and dephosphorylation on Thr-308 were slower in the heat-shocked cells than those in the growth-factor-stimulated cells. The PKB activity was sustained during the observation in the heat-shocked cells pretreated with wortmannin. Of course,

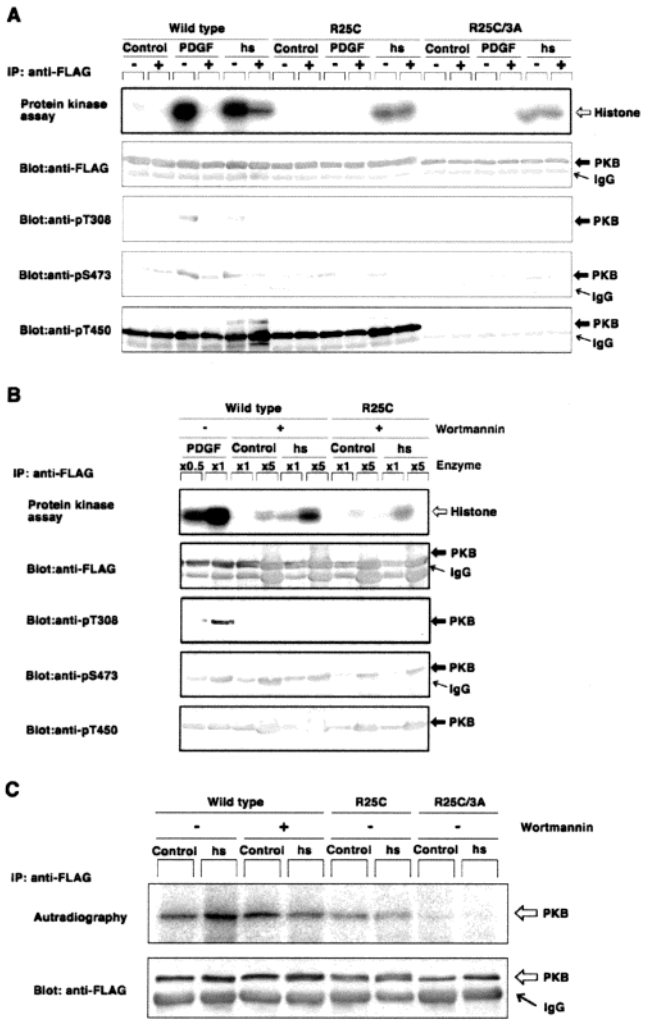

FIGURE 3: Phosphorylation of PKB in COS-7 cells. (A) The cells transfected with FLAG-PKB (wild type), R25C, or R25C/3A were treated with 20 ng/mL of PDGF for 10 min or at 42 °C for 15 min (hs). The cells pretreated with and without wortmannin are indicated as (+) and (−), respectively. The expressed proteins were immunoprecipitated by anti-FLAG antibody, the protein kinase activity was measured by using core histone as a substrate (top panel), and immunoblot analysis was carried out by using either anti-FLAG, anti-pT308, anti-pS473, or anti-pT450 antibody (lower four panels). (B) The cells transfected with FLAG-PKB or R25C were treated with 20 ng/mL of PDGF for 10 min or at 42 °C for 15 min (hs). The cells pretreated with and without wortmannin are indicated as (+) and (−), respectively. The amounts of the lysate and antibody were changed to 0.5 and 5 times the standard protocol. The protein kinase activity was measured by using core histone as a substrate (top panel), and immunoblot analysis was carried out by using either anti-FLAG, anti-pT308, anti-pS473, or anti-pT450 antibody (lower four panels). (C) The cells transfected with either FLAG-PKB (wild type), R25C, or R25C/3A labeled with [^{32}P]orthophosphate were treated at 42 °C for 15 min (hs). The cells pretreated with and without wortmannin are indicated as (+) and (−), respectively. The expressed proteins were immunoprecipitated, immunoblot analysis was carried out using anti-FLAG antibody (lower panel), and the [^{32}P] radioactivity incorporated into the proteins was visualized (upper panel).

phosphorylation on Thr-308 was not detected in the cells pretreated with the PI 3-kinase inhibitor. When taken together, PKB activated in heat-shocked cells without phosphorylation on Thr-308, which is generated in an independent manner from PI 3-kinase, is more stable than the enzyme stimulated by PDGF in the PI 3-kinase-dependent pathway.

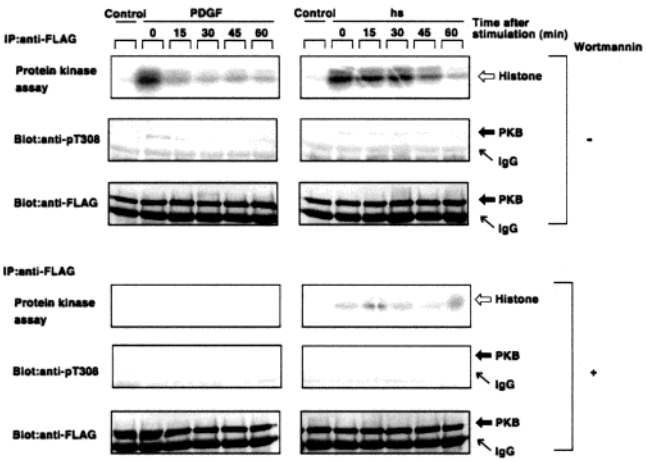

FIGURE 4: Dephosphorylation and inactivation of PKB in COS-7 cells. The cells transfected with FLAG-PKB were treated with 20 ng/mL of PDGF for 10 min or at 42 °C for 15 min (hs). The cells pretreated with and without wortmannin are indicated as (+) and (−), respectively. Then, the cells were further cultured at 37 °C in a fresh medium containing 0.1% BSA for the indicated time. FLAG-PKB was immunoprecipitated by anti-FLAG antibody, the protein kinase activity was measured by using core histone as a substrate (top panel), and immunoblot analysis was carried out by using either anti-pT308 or anti-FLAG antibody (lower two panels).

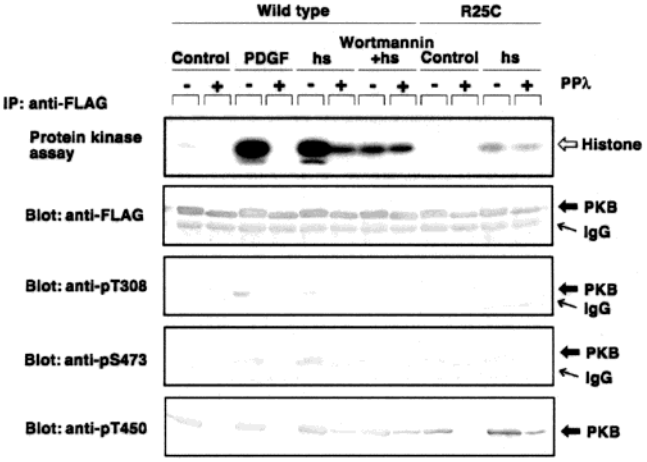

FIGURE 5: Dephosphorylation of PKB in vitro. COS-7 cells transfected with either FLAG-PKB or R25C were treated with 20 ng/mL of PDGF for 10 min or at 42 °C for 15 min (hs). Where indicated, the cells were pretreated with wortmannin. The expressed proteins were immunoprecipitated by anti-FLAG antibody and incubated with or without protein phosphatase λ (PPλ). Then, the protein kinase activity was measured by using core histone as a substrate (top panel), and immunoblot analysis was carried out by using either anti-FLAG, anti-pT308, anti-pS473, or anti-pT450 antibody (lower four panels).

Next, the properties of PKBα recovered from the stimulated cells were studied in vitro (Figure 5). When the wild-type PKBα immunoprecipitated from the PDGF-stimulated cells was treated with protein phosphatase λ, the enzymatic activity was abolished. Phosphorylation on Thr-308 and Thr-450 disappeared, and that on Ser-473 was heavily reduced by the phosphatase treatment. A significant enzymatic activity remained for the wild-type PKBα recovered from the heat-shocked cells even after the phosphatase treatment, which removes phosphate from Thr-308. The wild-type enzyme recovered from the heat-shocked cells pretreated with wortmannin was resistant to protein phosphatase λ, and R25C immunoprecipitated from the heat-shocked cells was insensi-

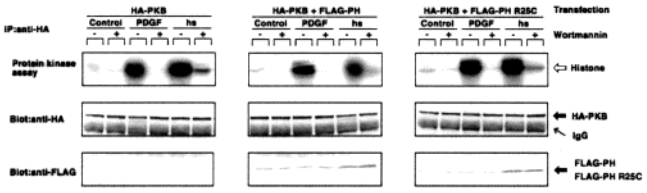

FIGURE 6: Association of PKB and PH-domain fragments in COS-7 cells. The cells transfected with HA-PKB alone and with either FLAG-PH or FLAG-PH R25C were treated with 20 ng/mL of PDGF for 10 min or at 42 °C for 15 min (hs). The cells pretreated with and without wortmannin are indicated as (+) and (−), respectively. HA-PKB was immunoprecipitated by anti-HA antibody, the protein kinase activity was measured by using core histone as a substrate (top panel), and immunoblot analysis was carried out by using either anti-HA (middle panel) or anti-FLAG antibody (lower panel).

tive to protein phosphatase λ. On the other hand, the phosphatase treatment efficiently removed phosphate from Ser-473 of the enzyme recovered from the heat-shocked cells. The phosphate on Thr-450 in the enzyme obtained from the heat-shocked cells, however, still remained in part after the treatment with protein phosphatase λ, whereas the enzyme recovered from the other cells was completely dephosphorylated by the phosphatase treatment. These results are consistent with the properties of PKBα observed in vivo and suggest that heat shock induces a structural change to make the enzyme resistant to the phosphatase.

Oligomerization of PKB. In the heat-shocked cells, the oligomerization of PKB has been observed (*19*). To analyze the oligomerization, HA-PKB was cotransfected with either FLAG-PH or FLAG-PH R25C in the cells (Figure 6). These two fragments associated with the full-length PKBα even in the control cells, and the complex formation was enhanced by heat shock but not by the growth factor. The association was not suppressed by wortmannin. Therefore, the intact structure of the PH domain for the association with the PI 3-kinase products is not required for the heat-shock-induced complex formation. The PH domain of PKBα seems to have a role to associate between the domains in addition to the binding of inositol phospholipids. It has been reported that the PH-domain fragment works as a dominant negative of PKB (*29*, *30*), but the PH-domain fragment associated with PKB did not block the enzymatic activity.

Activation and Localization of Endogenously Expressed PKB. Heat-shock-induced activation of PKB has been observed, which is expressed endogenously in NIH 3T3 cells (*18*). Therefore, phosphorylation of the endogenous enzyme in NIH 3T3 cells was analyzed by using the phosphorylation site-specific antibodies (Figure 7). Analysis of the enzyme immunoprecipitated by the PKBα-specific antibody revealed that PDGF induces activation and phosphorylation on Thr-308 and Ser-473, which are blocked by wortmannin. In contrast, heat-shock-induced activation of PKB and phosphorylation on these motif sites. Wortmannin blocked phosphorylation on these sites, but the protein kinase activity was still detected in the presence of wortmannin. Heat shock seems to activate PKB endogenously expressed in the cells sufficiently in an apparently independent manner from PI 3-kinase. Phosphorylation of Thr-450, in contrast, was found in the control as well as stimulated cells consistent to the analysis in COS-7 cells. In addition, the PI 3-kinase-independent pathway for PKB activation is more significant in NIH 3T3 cells than in COS-7 cells.

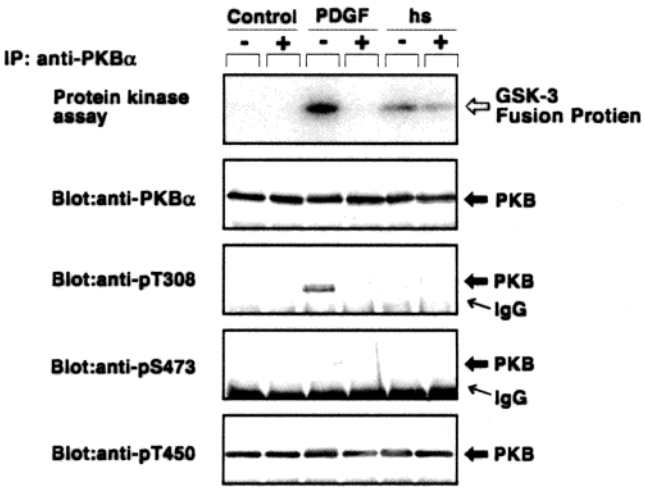

FIGURE 7: Activation of PKB endogenously expressed in NIH 3T3 cells. After the cells were cultured in DMEM supplemented with 0.1% BSA, the cells were treated with 20 ng/mL of PDGF for 10 min or at 42 °C for 15 min (hs). The cells pretreated with and without wortmannin are indicated as (+) and (−), respectively. PKB was immunoprecipitated by anti-PKBα antibody, the protein kinase activity was measured by using GSK-3 Fusion Protein as a substrate (top panel), and immunoblot analysis was carried out by using either anti-PKBα, anti-pT308, anti-pS473, or anti-pT450 antibody (lower three panels).

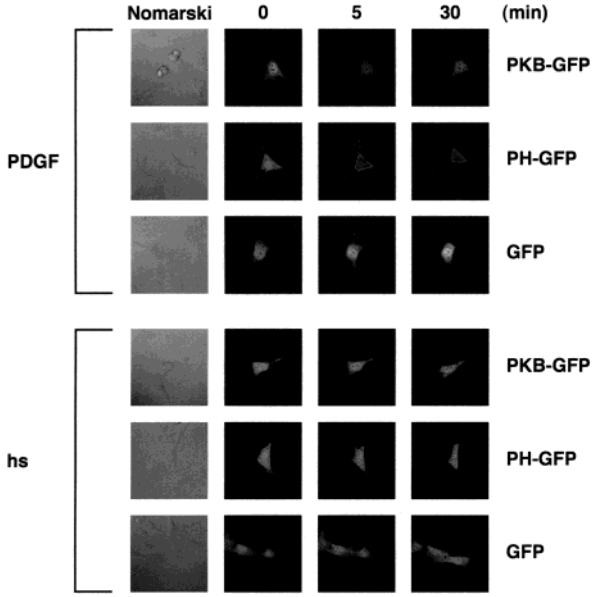

FIGURE 8: Localization of PKB-GFP and PH-GFP in NIH 3T3 cells. The cells transfected with either PKB-GFP, PH-GFP, or GFP were treated with 20 ng/mL of PDGF or at 42 °C (hs) for the indicated time. The fluorescence of the proteins was monitored with a confocal laser-scanning fluorescent microscope. Morphology of the cells observed under a Nomarski interfering microscope at 0 min is shown (left panels).

On the other hand, the PH domain of PKBα is revealed to interact with the PI 3-kinase products, and the fluorescence of its fusion protein with GFP, PH-GFP, has been employed to monitor the phospholipid kinase activity in intact cells (*31*−*34*). PDGF consistently induced translocation of PH-GFP from the cytosol to the plasma membrane in transfected NIH 3T3 cells, whereas the fusion protein stayed in the cytosol in heat-shocked cells (Figure 8). These results indicate that heat shock does not induce the elevation of the PI 3-kinase activity enough to translocate PH-GFP. Mem-

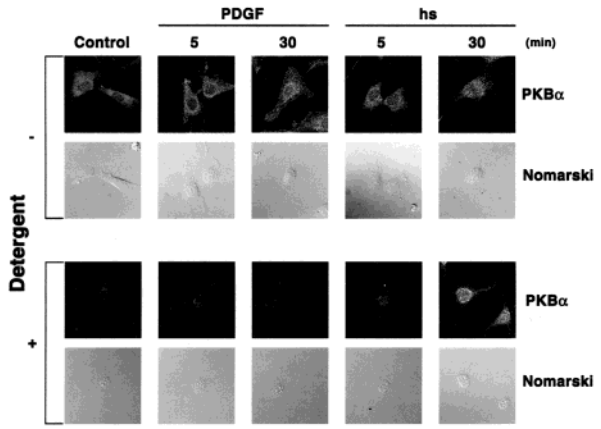

FIGURE 9: Localization of PKB in NIH 3T3 cells. The cells were treated with 20 ng/mL of PDGF or at 42 °C (hs) for the indicated time, fixed, and stained with anti-PKBa antibody (upper panels). Morphology of the cells was observed under a Nomarski interfering microscope (lower panels). The cells extracted with detergent before fixation and the cells without the extraction procedure are indicated as (+) and (−), respectively.

brane translocation of PKB-GFP was not induced by either growth-factor stimulation or heat-shock treatment. Last, the localization of the endogenous PKB was studied in NIH 3T3 cells by immunostaining using the PKBα-specific antibody and the observations under a fluorescent microscope (Figure 9). The cells were stained by the PKBα-specific antibody and observed under a fluorescent microscope. PDGF activates PI 3-kinase, and the products bind to the PH domain of PKB; however, no obvious association of the protein with the plasma membrane was observed after stimulation of the cells, consistent with the observations of PKB-GFP. In heat-shocked cells, the immunofluorescence was concentrated slightly in the nuclear region. For the further analysis, the cells were extracted with a detergent, Triton X-100, before fixation to visualize proteins associated with intracellular structures. In heat-shocked cells, PKBα was found in perinuclear and nuclear areas, whereas most PKBα was removed by the extraction in the control and PDGF-stimulated cells. Wortmannin treatment did not change the localization of PKBα in the heat-shocked cells (data not shown). Namely, PKBα shows localization in heat-shocked cells, which is distinct from that in the growth-factor-stimulated cells.

DISCUSSION

PKB is one of the downstream targets of PI 3-kinase in the signaling pathway of the growth factors (*1*−*4*), and we have found that PKB is activated in stress-stimulated cells especially by heat shock (*18*, *19*). In the previous papers, we had monitored the temperature of the water bath employed to warm the culture plates and reported that PKBα is activated significantly at temperatures higher than 44 °C (*18*, *19*). Later, the temperature of the culture medium was measured directly by using a microsensor tip, and it was shown that the temperature of the culture medium rises gradually and is lower than that of the water bath even after incubation for several minutes under the conditions employed. Therefore, we improved the conditions to warm the culture medium and revealed that PKBα is activated effectively in the cells treated at 42 °C and that the enzymatic activity is not enhanced further at temperatures higher than 42 °C (Figure 1). PDGF and heat-shock-induced PKB activation still indicates by different time courses and extents that PKBα is regulated by distinct mechanisms in growth-factor-stimulated and heat-shocked cells. Thus, activation of PKBα needs to be analyzed in detail.

Concerning the molecular mechanisms for heat-shock-induced activation of PKBα, we had shown that PKBα recovered from the heat-shocked cells is resolved into two active fractions on a gel filtration column chromatography, presumably the monomeric enzyme and the protein complex (*19*). Activation of the former is suppressed by the pretreatment of the cells with wortmannin, whereas generation and activation of the latter are not blocked by the PI 3-kinase inhibitor, supporting that PKBα is activated in manners dependent on and independent of PI 3-kinase. In this paper, the wild-type PKBα was shown to be activated in part by heat shock without the phosphorylation on Thr-308 in the presence of wortmannin (Figures 2 and 3). R25C was stimulated by heat shock even in the cells pretreated with wortmannin, which shows no protein kinase activity in growth-factor-stimulated cells because it lacks the Arg residue essential for the binding of inositol phospholipids in the PH domain. Consistently, the mutant molecule was not phosphorylated on Thr-308. The wild-type PKBα and R25C recovered from heat-shocked cells were resistant to protein phosphatase λ in vitro, whereas the wild-type enzyme obtained from growth-factor-stimulated cells was inactivated by the phosphatase treatment (Figure 5). These results indicate that heat shock can generate such an active enzyme apparently without phosphorylation in the activation loop. However, the nonphosphorylated mutant T308A did not have protein kinase activity even in heat-shocked cells (Figure 2). The intact structure of the activation loop may be necessary for protein kinase activity. In addition, phosphorylation on other residues including the sites since detected such as Ser-124, Thr-450, and Ser-473 was not indispensable for activation of PKBα (Figures 2 and 3). These findings, of course, do not necessarily eliminate the role of phosphorylation for the catalytic activity of PKBα, but its protein kinase activity can be elevated to a certain level without phosphorylation of the molecule. PKBα is recovered as a protein complex from heat-shocked cells as shown here (Figure 6) and in a previous paper (*19*). This enzyme could have an active conformation, which is different from that ordered by phosphorylation, especially in the activation loop. If so, a trace enzymatic activity detected in the immunoprecipitates of K179M, R25C/K179M, and T308A recovered from heat-shocked cells may derive from the endogenous PKB included in the protein complex, because these mutant molecules have no protein kinase activity.

It has been shown that the PH-domain fragment of PKB associates with the full-length enzyme and has a dominant-negative action to the enzyme (*29*, *30*). In this paper, the association of FLAG-PH to the full-length PKBα was detected even in the nonstimulated cells, which is enhanced by heat shock (Figure 6). Heat-shock-induced activation of PKB was, however, not prevented by the association of the fragment. Furthermore, FLAG-PH R25C, which lacks the intact structure of the PH domain for the association with the PI 3-kinase products, also associated with the full-length PKB. It is not clear why the PH-domain fragment of PKB has been shown to have a dominant-negative action. The

results obtained indicate that the active enzyme generated in heat-shocked cells forms an oligomer through the association between the PH domain, but the protein complex formation is not essential for its activation. TCL1, a protooncogene product expressed in lymphocytes, has been shown to bind to the PH domain of PKB (*35*−*38*). It is reported that TCL1 induces oligomerization and transphosphorylation of PKB to enhance its protein kinase activity. It seems to be possible that the binding of TCL1 induces the conformational change of PKB as induced by heat shock and that the kinase domain is thus liberated. Several proteins such as CTMP, Hsp90, Hsp27, and periplakin are proposed to interact with PKB (*3*, *39*, *40*). Specific binding of these proteins may be an integral part in regulating PKB as in the case of TCL1.

The structural analysis of protein kinases has been carried out, and conformational plasticity induced by phosphorylation as well as interactions with regulatory domains or proteins is regarded to be a central feature of the regulatory mechanisms of the enzyme family (*41*−*43*). Recently, the three-dimensional structure of the kinase domain of PKBβ is presented, which has a primary amino acid sequence only subtly different from that of PKBα (*44*). The crystal structure of the phosphorylated state in the activation loop adopts the conformation as the unphosphorylated inactive enzyme, and further phosphorylation on the hydrophobic motif is proposed to induce the reconfiguration of the kinase domain by the interaction of the hydrophobic motif in the carboxyl-terminal end with the hydrophobic groove in the amino-terminal region. The three-dimensional structure of the whole molecule and the analysis of the interaction with the binding proteins are required for understanding the complex mechanisms of the regulation of the PKB activity.

Inactivation of PKBα was slower in heat-shocked cells than those in PDGF-stimulated cells, and the enzyme remained active in the cells pretreated with wortmannin for the period observed (Figure 4). Furthermore, the study of the intracellular localization of the endogenously expressed PKBα in NIH 3T3 cells revealed that heat shock induces association of the enzyme with intracellular structures to keep it in perinuclear and nuclear areas (Figure 9). In addition, the analysis using the fusion proteins with GFP showed that PDGF induces translocation of the PH domain of PKBα from the cytosol to the plasma membrane through the association of the PI 3-kinase products, whereas heat shock does not effect the intracellular localization of the fusion protein (Figure 8). It seems possible that the long acting enzyme with distinct intracellular localization plays a role distinct from the enzyme activated in the receptor-mediated pathway. It is interesting to assume, for example, that the active enzyme generated by heat shock takes part in a feedback mechanism to protect cells from damage of cellular stress. Further analysis is required to clarify the practical roles of PKB in the signaling of stress responses.

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