Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27

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Abstract Protein kinase B (PKB, also named as Akt or RAC-protein kinase), that is activated by cellular stress such as heat shock and hyperosmotic treatment, was revealed to be activated by oxidative stress and by chemical stressors of $CdCl_2$ and $NaAsO_2$ by measuring the activity of the enzyme immunoprecipitated from the transfected COS-7 cells. Upon stress treatment, a 30-kDa phosphoprotein was co-immunoprecipitated with PKB from the cells metabolic labeled with [^{32}P]orthophosphate. The phosphoprotein was identified as Hsp27, a small heat shock protein, by immunoblot analysis and co-immunoprecipitation. The association of Hsp27 was specific to PKB as the heat shock protein was not co-immunoprecipitated with other protein kinases such as protein kinase C and PKN. When the cells were treated with H_2O_2, PKB was activated gradually and the association of Hsp27 with PKB increased concurrently with the enhancement of PKB activity. In heat-shocked cells, activation of PKB and the association of Hsp27 were detected immediately after the treatment, and the association of the heat shock protein decreased while PKB kept stimulated activity when the cells were further incubated at 37°C. These results suggest that Hsp27 is involved in the activation process of PKB in the signal transduction pathway of various forms of stress.

Key words: Protein kinase B; Akt or RAC-protein kinase; Oxidative stress; Heat shock; Heat shock protein; Hsp27

1. Introduction

Protein kinase B (PKB, also named Akt or RAC-protein kinase) is a family of serine/threonine protein kinase having a pleckstrin homology (PH) domain and a catalytic domain closely related to cAMP-dependent protein kinase and protein kinase C (PKC) in its amino- and carboxyl-terminal regions, respectively, and three subtypes of α, β and γ, have thus far been identified [1–7]. PKB has been indicated to be activated by various growth factors as a direct downstream target of phosphatidylinositol 3-kinase (PI 3-kinase), and it has also been reported that phosphorylation of PKB by a putative PKB kinase is necessary for activation of the enzyme [for reviews, [8,9]]. Thus, the precise mechanism of activation of PKB by growth factors is not yet fully understood. In the meantime, PKB has revealed to be activated by cellular stress such as heat shock and hyperosmolarity [10]. As activation of PKB by cellular stress is insensitive to wortmannin, a potent inhibitor of PI 3-kinase, it is plausible that PKB is activated by the growth factors and by cellular stress alternatively in manners dependent on and independent of PI 3-kinase, respectively, and that the enzyme is located in the different pathways of the intracellular signal transduction. On the other hand, it is well known that heat shock and other environmental stress stimulate synthesis of several heat shock proteins including Hsp27 [for a review, [11]]. In this study, we report that PKB is activated by oxidative stress and by chemical stressors of $CdCl_2$ and $NaAsO_2$, and this enzyme associates with a small heat shock protein, Hsp27, in the cells stimulated by various extracellular stress.

2. Materials and methods

2.1. Cells and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 incubator. Expression vectors of PKBα [6] and FLAG-epitope tagged PKBα, -β, -γ [10], PKCα [12] and PKN [13] were employed. Cells were transfected with each expression vector by electroporation using Gene Pulser (Bio-Rad), cultured for 48 h, and treated as described in each experiment. Where indicated, transfected COS-7 cells were further cultured for 1 h in phosphate-free DMEM supplemented with dialyzed fetal calf serum, and then incubated for 3 h with [^{32}P]orthophosphate (18 MBq/6 cm dish) before treatment.

2.2. Immunoprecipitation and immunoblot analysis

Expressed proteins were immunoprecipitated at 0–4°C essentially as described [10]. Briefly, cells were washed with phosphate-buffered saline, 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 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 monoclonal antibody (Kodak Scientific Imaging Systems), a monoclonal antibody against human Hsp27 (Stressgen Biotechnologies) or each polyclonal antibody against PKB (anti-N-terminal and anti-C-terminal, Upstate Biotechnology), PKCα (Transduction Laboratories) and PKN [14]. Then, protein A-Sepharose beads (Pharmacia) were added to the mixture and incubated for 30 min. The immunoprecipitates were collected by centrifugation and washed 4 times with 20 mM Tris-HCl at 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 [15] and transferred onto an Immobilon P membrane (Millipore). The monoclonal antibodies against FLAG epitope and human Hsp27 and the polyclonal antibodies against PKB and mouse Hsp25 (Stressgen Biotechnologies) were used as the first antibodies. The alkaline phosphatase-conjugated anti-rabbit and anti-mouse antibodies (Promega) were employed as the second antibodies and the color reaction was carried out using 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium as substrates.

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Abbreviations: Hsp, heat shock or stress protein; PH domain, pleckstrin homology domain; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C

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2.3. Protein kinase assay

The enzyme activity of PKB was assayed by measuring the incorporation of radioactivity from [γ-^{32}P]ATP to the core histone fraction prepared from calf thymus [10]. 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 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, 200 μg/ml core histone and the immunoprecipitates were incubated for 30 min at 30°C. After boiling in SDS sample buffer, phosphorylated proteins were separated by SDS-PAGE, and the radioactivity of each protein band was analyzed by Bio-imaging Analyzer BAS2000 (Fuji).

3. Results

3.1. Activation of PKB subtypes by extracellular stimuli

In the previous report [10], we found that cellular stress such as heat shock and hyperosmolarity induces PKB activation, whereas protein synthesis inhibitors such as anisomycin and cycleheximide, that activate the stress-activated protein kinase/c-Jun amino-terminal kinase family [16], could not enhance the PKB activity. Here, we studied the effects of other cellular stress on the PKB activity by using COS-7 cells transiently expressing each FLAG-epitope tagged PKB subtype (Fig. 1). PKB activation was observed in the cells treated with H_2O_2, $CdCl_2$ and $NaAsO_2$. The responses of each PKB subtype to extracellular stimuli were slightly different one another. For example, PKBα and -β were activated by H_2O_2 in a dose-dependent manner among the concentrations employed, whereas PKBγ showed the highest activity in the presence of lower concentrations of H_2O_2. PKBα was activated by NaCl at 700 mM, however PKBβ and -γ did not show evident increase in the presence of high concentrations of NaCl. Among the subtypes, PKBβ was less sensitive to cellular stress than PKBα and -γ. It has been reported that tumor necrosis factor, an inflammatory cytokine, activates stress-activated protein kinase/c-Jun amino-terminal kinase [16], although the inflammatory cytokine did not increase the activity of PKB expressed in COS-7 cells (data not shown).

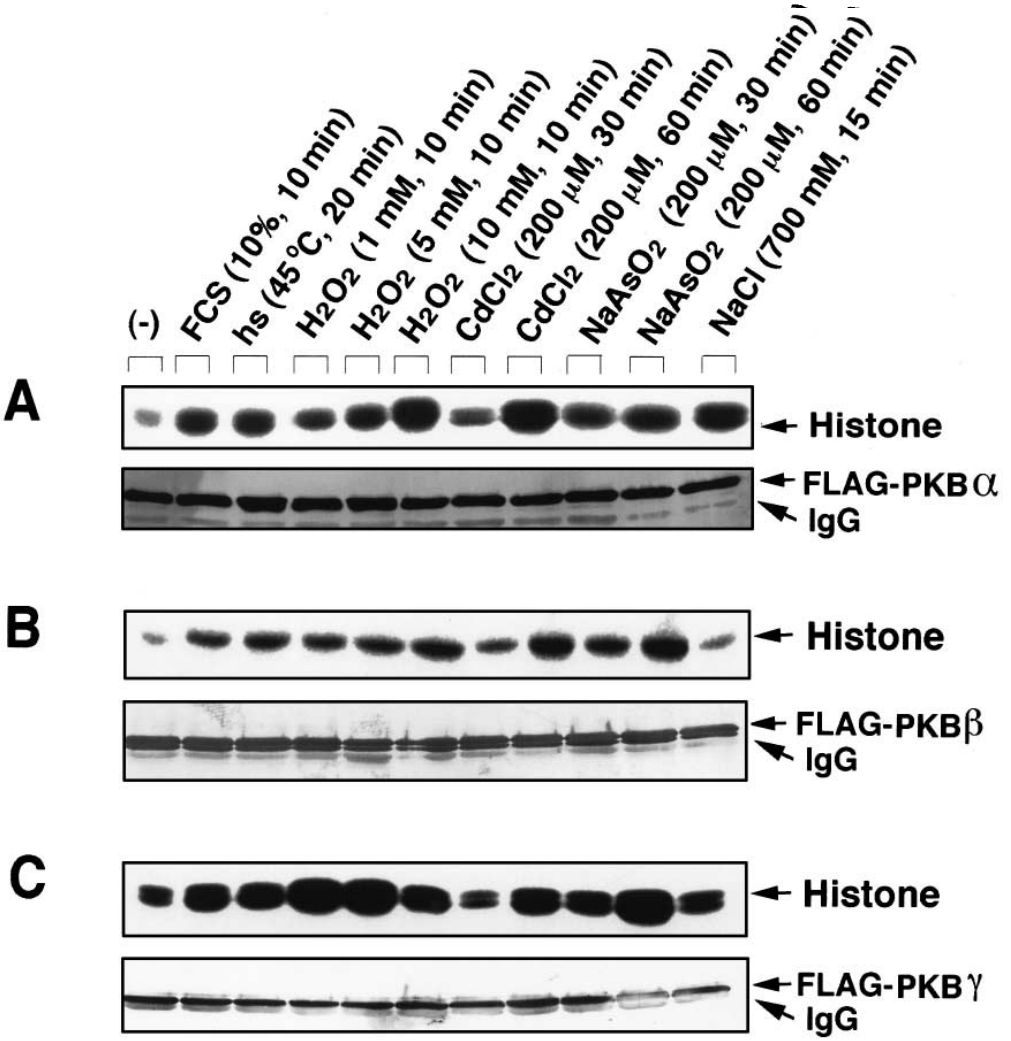

Fig. 1. Activation of PKBα, -β and -γ in transfected COS-7 cells. COS-7 cells transfected with the expression plasmid of either FLAG-epitope tagged PKBα (A), PKBβ (B) or PKBγ (C) were serum-starved for 24 h, treated as indicated, and each FLAG-epitope tagged PKB was immunoprecipitated by the anti-FLAG antibody. Cells without treatment are indicated as (−). Protein kinase activity in the immunoprecipitates was measured by using core histone as substrate (upper panels). The amounts of FLAG-epitope tagged PKB in the immunoprecipitates are shown by immunoblot analysis using the anti-FLAG antibody (lower panels). The positions of phosphorylated histone, FLAG-epitope tagged PKB and IgG (heavy chain) are indicated by arrows. FCS and hs indicate fetal calf serum and heat shock, respectively.

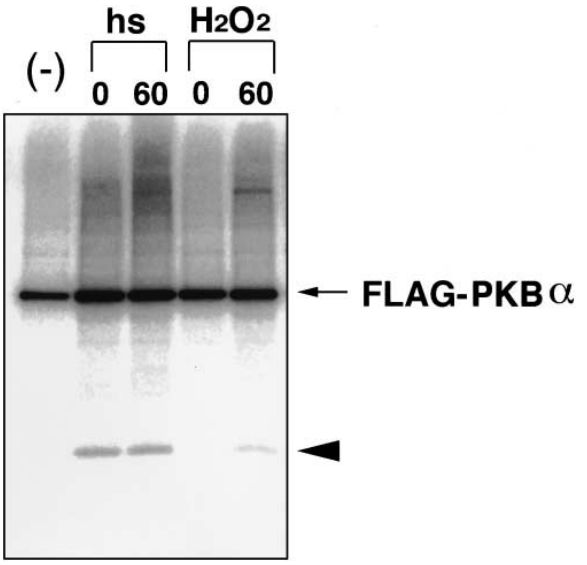

Fig. 2. Phosphorylation of PKB in vivo and its binding protein. COS-7 cells transfected with FLAG-epitope tagged PKBα expression plasmid were metabolically labeled with [^{32}P]orthophosphate, treated either at 45°C for 20 min (hs) or with 10 mM H_2O_2 for 10 min (H_2O_2), and FLAG-epitope tagged PKBα was immunoprecipitated by the anti-FLAG antibody immediately after treatment (0) or after further incubation at 37°C for 60 min (60) in DMEM supplemented with 10% fetal calf serum. Cells without treatment are indicated as (−). Phosphorylated proteins were visualized after SDS-PAGE. The position of phosphorylated FLAG-epitope tagged PKBα is indicated by an arrow, and the position of the 30-kDa protein associated with FLAG-epitope tagged PKBα is indicated by an arrowhead.

3.2. Phosphorylation of PKB in vivo

It has been reported that the phosphorylation reaction of PKB is essential for its activation [8]. To analyze the mechanism of PKB activation by cellular stress, COS-7 cells expressing FLAG-epitope tagged PKBα were metabolically labeled with [^{32}P]orthophosphate and FLAG-epitope tagged PKBα was immunoprecipitated. PKBα was phosphorylated during the incubation of the cells for 3 h with [^{32}P]orthophosphate in the presence of fetal calf serum, and the amount of the phosphorylation increased 2–3-fold by stress stimulation such as heat shock and H_2O_2 (Fig. 2). The phosphorylation of PKB did not decrease when the cells were further incubated for 60 min at 37°C or in the absence of H_2O_2. On the other hand, a phosphoprotein with an approximate M_r of 30 kDa was found to be co-immunoprecipitated with PKB in heat-treated cells. The phosphoprotein was still detected after incubation for 60 min at 37°C following the heat shock. In H_2O_2-treated cells, the 30-kDa protein was not detected immediately after treatment of the cells, and observed after incubation for 60 min in the absence of H_2O_2.

3.3. Identification of the 30-kDa protein as Hsp27

The 30-kDa phosphoprotein was characterized since it associates with PKB activated by cellular stress. It has been

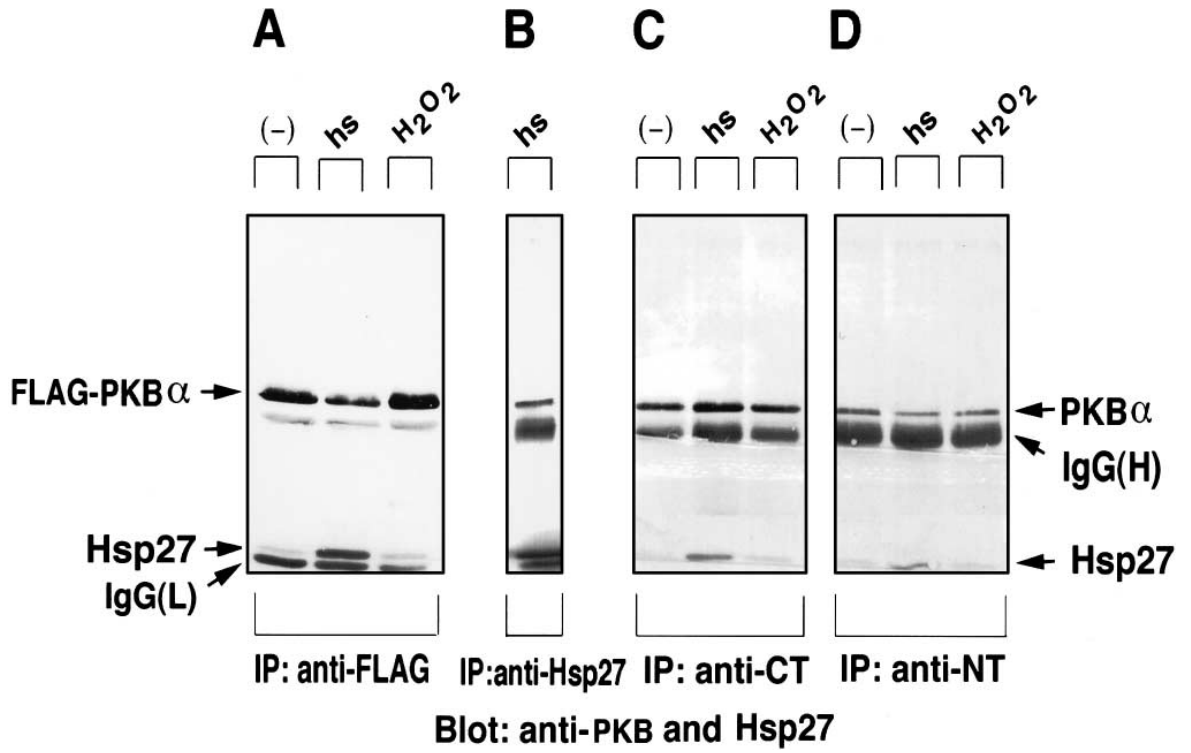

Fig. 3. Identification of the 30-kDa PKB binding protein as Hsp27. COS-7 cells transfected with the expression plasmid of FLAG-epitope tagged PKBα (A,B) and PKBα (C,D) were treated either at 45°C for 20 min (hs) or with 10 mM H_2O_2 for 10 min (H_2O_2). Cells without treatment are indicated as (−). FLAG-epitope tagged PKBα was immunoprecipitated by the anti-FLAG antibody (A), Hsp27 was immunoprecipitated by the anti-human Hsp27 antibody (B), and PKBα was immunoprecipitated by either the anti-PKB C-terminal antibody (anti-CT) (C) or the anti-PKB N-terminal antibody (anti-NT) (D). Immunoblot analysis was carried out by using a mixture of the antibodies against PKB and human Hsp27 as the first antibodies and the anti-rabbit and anti-mouse antibodies were employed as the second antibodies. The positions of FLAG-epitope tagged PKBα, PKBα, Hsp27 and IgG (heavy and light chains) are indicated by arrows.

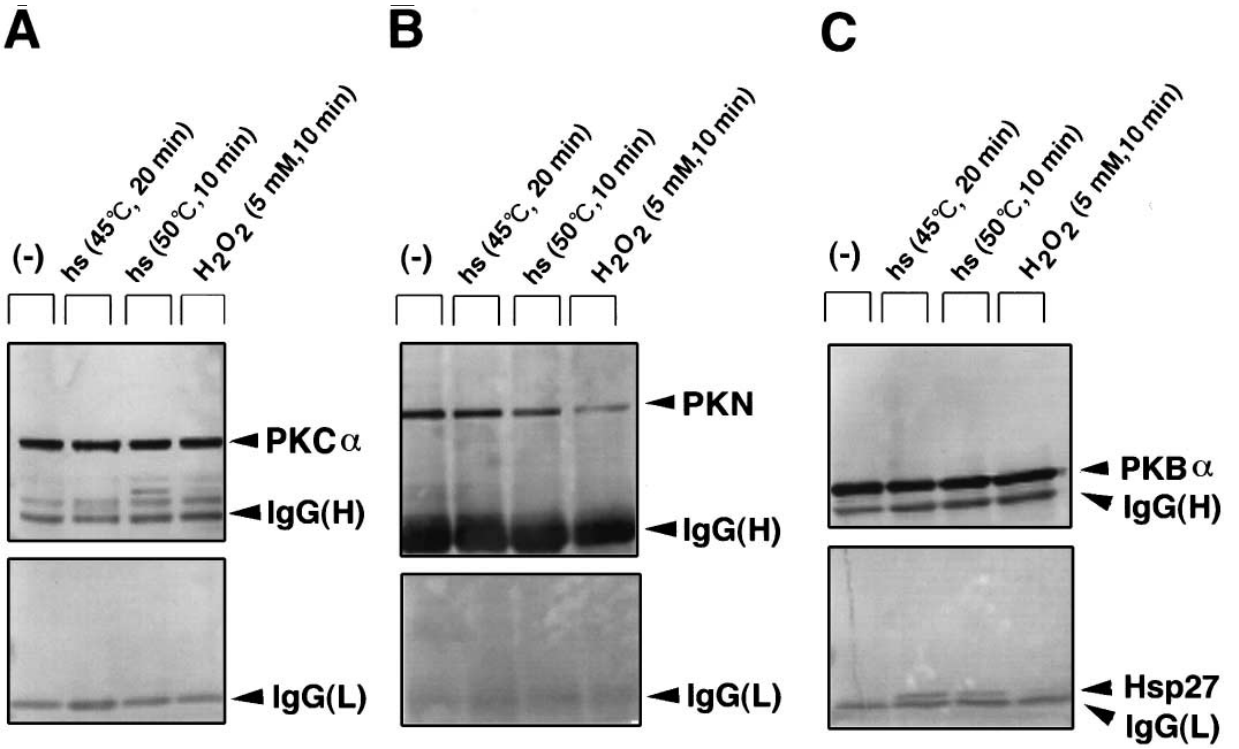

Fig. 4. The specificity of the association of Hsp27. COS-7 cells transfected with the FLAG-epitope tagged expression plasmid of either PKCα (A), PKN (B) or PKBα (C). Cells were treated at 45°C for 20 min, 50°C for 20 min or with 5 mM H_2O_2 for 10 min, and each protein kinase was immunoprecipitated with the anti-PKCα, anti-PKN and anti-PKB antibodies, respectively. Cells without treatment are indicated as (−). Immunoblot analysis was carried out by using the anti-FLAG antibody (upper panels) and the anti-human Hsp27 antibody (lower panels), respectively. The positions of FLAG-epitope tagged protein kinases, Hsp27 and IgG (heavy and light chains) are indicated by arrows.

demonstrated that Hsp27, a small heat shock protein with an apparent M_r of 27 kDa, is phosphorylated upon the treatment of the cells by various stress stimuli such as heat shock, H_2O_2, $CdCl_2$ and $NaAsO_2$ [11,17–21]. As the conditions for Hsp27 phosphorylation are similar to those for PKB activation, immunoblot analysis using the antibody against human Hsp27 was carried out to examine whether the 30-kDa protein is Hsp27 (Fig. 3A). Hsp27 was detected as an endogenous protein of 30 kDa of COS-7 cells that associates with FLAG-epitope tagged RAC-PKα in the heat-shocked cells. Hsp27 was found faintly in the immune complex in H_2O_2-treated cells and was hardly detected in the control cells. The 30-kDa protein was recognized also by another antibody against Hsp25, a mouse homologue of Hsp27 (data not shown). When Hsp27 was immunoprecipitated from the heat-treated transfected cells, FLAG-epitope tagged PKBα was detected in the immune complex vice versa (Fig. 3B). The FLAG-epitope tag is not involved in the association with Hsp27, because the association was observed when PKBα without the epitope tag was expressed and immunoprecipitated by the antibodies against the amino- and carboxyl-terminal sequences of PKB (Fig. 3C,D). Other PKB subtypes such as β and γ also associated with Hsp27 in heat-treated cells (data not shown).

3.4. Specificity of the association of Hsp27

As PKB has a catalytic domain closely related to PKC [1–7] and PKN [13], the association of Hsp27 to these protein kinases was studied. Hsp27 was not co-immunoprecipitated with either PKCα or PKN, whereas the small heat shock protein associates with PKB under the conditions employed (Fig. 4). The binding site of Hsp27 was not narrowed down in the protein kinase molecule, as Hsp27 was shown to associate with both the PH domain and the catalytic domain in PKB by expressing each domain in COS-7 cells (data not shown).

3.5. Relation of PKB activation and association with Hsp27

The time courses of PKB activation and the association with Hsp27 were compared in heat-shocked and H_2O_2-treated cells (Fig. 5). When the cells were treated with 10 mM H_2O_2 for 10 min, PKB was activated and its activity was further enhanced during the incubation of the cells without H_2O_2. The association with PKB increased in a manner similar to the enhancement of PKB activity. In heat-shocked cells, PKB was activated and kept its high activity even 90 min after incubation at 37°C, although the association of Hsp27, that is detected immediately after the treatment, decreased gradually during the incubation at 37°C.

4. Discussion

PKB has been demonstrated to be a downstream target of PI 3-kinase that is activated upon stimulation of the cells by various growth factors [8,9]. Products of PI 3-kinase such as phosphatidylinositol 3,4-bisphosphate and/or phosphatidylinositol 3,4,5-trisphosphate have been suggested to interact with the PH domain of PKB to activate the enzyme. On the other hand, it has been reported that the phosphorylation of PKB by a putative PKB kinase is important for its activation. In this study, PKB was revealed to be stimulated by various stress such as H_2O_2, $CdCl_2$ and $NaAsO_2$, in addition to heat

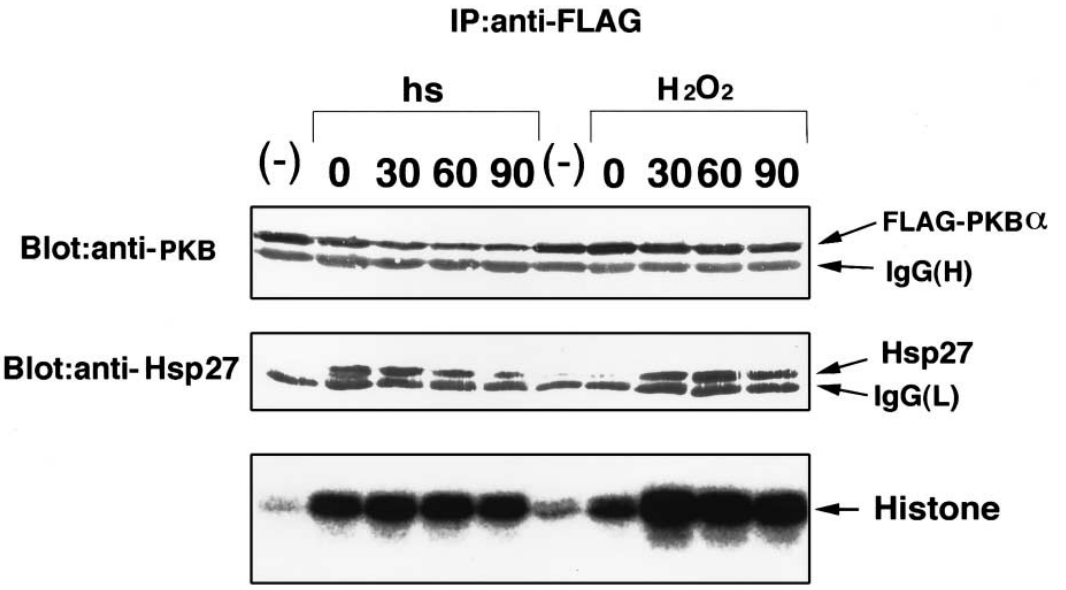

Fig. 5. Stress-dependent association of PKB and Hsp27. COS-7 cells transfected with FLAG-epitope tagged PKBα expression plasmid were treated at 45°C for 20 min (hs) or with 10 mM H_2O_2 for 10 min (H_2O_2). Cells without treatment are indicated as (−). After changing the culture medium, the cells were further incubated at 37°C for 30 min (30), 60 min (60) and 90 min (90), and FLAG-epitope tagged PKBα was immunoprecipitated by the anti-FLAG antibody. Where indicated (0), FLAG-epitope tagged PKBα was immunoprecipitated immediately after treatment without further incubation. Immunoblot analysis was carried out by using the anti-FLAG antibody (top panel) and the anti-human Hsp27 antibody (middle panel), respectively. Protein kinase activity in the immunoprecipitates was measured by using core histone as substrate (bottom panel). The positions of FLAG-epitope tagged PKBα, Hsp27, histone and IgG (light chain) are indicated by arrows.

shock and hyperosmolarity as reported in the previous study [10]. It is attractive if each PKB subtype responds to different stimuli, since the response of PKB to cellular stress differed slightly among the family. It was also shown that PKB is phosphorylated during the incubation in the presence of serum, and the phosphorylation of PKB was enhanced by stress treatment. Thus, the phosphorylation seems to be important for the regulation of the PKB activity by the growth factors and cellular stress, even though activation by the former is mediated through PI 3-kinase and the latter is not. It is necessary to study the precise nature of PKB phosphorylation.

During the analysis of the phosphorylation of PKB, we found that Hsp27 is associated with PKB in stress-stimulated cells. The association with Hsp27 was specific to PKB, as Hsp27 does not interact with other protein kinases such as PKC and PKN that have a kinase domain closely related to PKB. It is worth noting that PKN is translocated from the cytosol to the cell nucleus by heat shock [22], however, this protein kinase does not interact with the heat shock protein. Hsp27 is speculated to be a molecular chaperone, because this protein exists in different intracellular components, associates with prosomal particles and actin filaments, and promotes folding and refolding of model substrate proteins [11]. The physiological substrates for the chaperoning function of the small heat shock protein are not yet clear. The amounts of the binding of Hsp27 with PKB correlated with its protein kinase activity in the cells treated with H_2O_2, whereas the association of Hsp27 decreased while the enzyme keeps a high level of activity in heat-shocked cells. In $CdCl_2$- or $NaAsO_2$-treated cells, activation and Hsp27 binding were also correlated as in the case of H_2O_2-treated cells, however, Hsp27 did not associate with PKB in the cells stimulated with serum (data not shown). These results suggest that Hsp27 might function as a chaperon to change the conformation of the enzyme for its activation upon stress stimulation, rather than an indispensable factor for activation of PKB in the cells. If so, Hsp27 may dissociate from PKB that is once fully activated in heat-shocked cells. It seems to be also possible that PKB is activated by different mechanisms in heat-shocked and H_2O_2-treated cells, as the association of Hsp27 with PKB is evident in the former cells, but not clear in the latter cells immediately after the treatment. It is necessary to study the phosphorylation of PKB and the activity of PI 3-kinase in stress-treated cells.

It has been shown that Hsp27 is phosphorylated in response to various stress [11], and MAPKAP kinase-2, the protein kinase activated by the phosphorylation with p38 MAP kinase, has been identified as an enzyme responsible for the phosphorylation of Hsp27 [23–25]. The phosphorylation of Thr-308 and Ser-473 of PKBα is reported to be critical to generate a high level of the enzyme activity, and that Ser-473 is phosphorylated by MAPKAP kinase-2 at least in vitro [8,26]. The precise relationship between activation of PKB and the association of Hsp27 is not yet clear; however, both PKB and Hsp27 might be phosphorylated by MAPKAP kinase-2 in cells stimulated by growth factors or cellular stress. Hsp27 might have some role in the regulation of cell survival, because PKB has been demonstrated to prevent apoptosis [8,9]. Analysis of the interaction of PKB and Hsp27 will be helpful to understand the mechanism of PKB activation and the role of this protein kinase in the intracellular signal transduction.

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