Isolation of the active form of RAC-protein kinase (PKB/Akt) from transfected COS-7 cells treated with heat shock stress and effects of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate on its enzyme activity

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Abstract RAC-protein kinase (PKB/Akt) has been shown to be activated by growth factor stimulation as a downstream target of phosphatidylinositol 3-kinase and also by heat shock through a pathway independent of phosphatidylinositol 3-kinase. RAC-protein kinase was purified by antibody affinity chromatography from COS-7 cells transfected with the epitope-tagged expression plasmid. The protein kinase activity of RAC-protein kinase purified from heat-treated cells was 9-fold higher than the enzyme isolated from untreated control cells. Phosphatidylinositol 3,4,5-trisphosphate did not enhance the activity of RAC-protein kinase purified from either heat-treated cells or control cells, whereas phosphatidylinositol 4,5-bisphosphate suppressed the enzyme isolated from heat-treated cells. These results indicate that RAC-protein kinase may interact with phosphoinositides, however, it could not be activated by simple association with the product of phosphatidylinositol 3-kinase reaction.

Key words: RAC-protein kinase; Phosphatidylinositol 3-kinase; Stress; Phosphatidylinositol 3,4,5-trisphosphate; Phosphatidylinositol 4,5-bisphosphate; COS-7 cell

1. Introduction

Stimulation of the intrinsic protein tyrosine kinase activity of growth factor receptors by ligand-binding initiates several signaling pathways, and one of these signaling pathways involves PI 3-kinase, which phosphorylates the hydroxyl group at position 3 of the inositol ring of phosphoinositides, generating PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 [1,2]. These phospholipids have been considered to be second messengers since any known phospholipase type C can not cleave the inositol group with 3-phosphate. In fact, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 have been reported to activate some PKC subspecies in vitro [3–7]. Recently, RAC-protein kinase (RAC-PK, also named as PKB or Akt), a serine/threonine protein kinase having a PH domain and a catalytic domain closely related to both cAMP-dependent protein kinase and PKC in its amino- and carboxyl-terminal regions, respectively [8–14], has been indicated to be a downstream target of PI 3-kinase [15–19]. Namely, it has been shown that RAC-PK is activated by several growth factors, and that growth factor-induced activation of RAC-PK is blocked by PI 3-kinase inhibitors and by the expression of a dominant-negative mutant of PI 3-kinase, and platelet derived growth factor-receptor mutants which fail to activate PI 3-kinase are incapable of activation of RAC-PK. PtdIns(3)P, one of the products of the PI 3-kinase reaction, has been shown to activate directly RAC-PK in vitro and has been suggested to bind to the PH domain of RAC-PK [15], as PtdIns(4,5)P2 recognizes the hydrophobic pocket of the PH domain [20]. Very recently, RAC-PK has been revealed to be activated by cellular stress such as heat shock and hyperosmolarity in a manner independent of PI 3-kinase [21]. Thus, RAC-PK seems to be regulated by distinct mechanisms in signal transducing pathways. In this study, the active and inactive forms of RAC-PK were purified from transfected COS-7 cells to study the mechanism of activation of this enzyme.

2. Materials and methods

2.1. Cells and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. The expression plasmid of FLAG-epitope tagged RAC-PKα which contains the cDNA insert of rat RAC-PKα in the pECE vector was transfected to COS-7 cells by electroporation using GENE PULSAR (Bio-Rad). The expression plasmid of dominant negative RAC-PKα (K179M) was constructed as described [21]. A CHO cell line stably overproducing FLAG epitope tagged RAC-PKα was constructed by using a pRc/CMV vector system (Invitrogen), and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and proline (35 μg/ml) at 37°C in a 5% CO_2 incubator.

2.2. Immunoprecipitation

The following procedures were carried out at 0–4°C. 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 (lysis buffer). After centrifugation for 10 min at 18000×*g*, the supernatant (500–600 μg of protein) was incubated for 1 h with an anti-FLAG

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Abbreviations: PI 3-kinase, phosphatidylinositol 3-kinase; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; RAC-PK, RAC-protein kinase; PH domain, pleckstrin homology domain; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate

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monoclonal antibody (1 μg of protein, Kodak Scientific Imaging Systems), and then protein A-Sepharose beads (Pharmacia) were added to the mixture and incubated for 30 min. The immunoprecipitates were collected by centrifugation and washed four times with 20 mM Tris-HCl at pH 7.5 containing 150 mM NaCl and 1% Triton X-100.

2.3. Purification of RAC-PKα

COS-7 cells were transfected with the expression plasmid of FLAG-epitope tagged RAC-PKα and cultured for 48 h with 10% fetal bovine serum at 37°C. FLAG-epitope tagged RAC-PKα was purified either from cells treated at 45°C for 20 min or from cells without treatment. The purification procedures were carried out at 0–4°C. The extract from COS-7 cells (1×10^7 cells) was applied to a FLAG M2 affinity gel column (0.8×2 cm, Kodak Scientific Imaging Systems) equilibrated with lysis buffer without Triton X-100. After extensive washing, FLAG-RAC-PK was eluted with 1 ml of 0.1 M glycine-HCl at pH 3.0. The eluate was neutralized immediately by adding 20 μl of 1 M Tris-HCl at pH 8.0, and used for the protein kinase assay. About 1 μg of purified FLAG RAC-PKα per 10 cm dish of confluent COS-7 cells was obtained by these purification methods. The enzyme obtained was stable for at least 1 week when stored at 0–4°C.

2.4. Protein kinase assay

The core histone fraction (a mixture of H2A, H2B, H3, and H4 histones) prepared from calf thymus was employed for the routine assay for RAC-PK. The reaction mixture (25 μl) containing 20 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 20 mM ATP, 15–50 kBq of [γ-^{32}P]ATP, 200 μg/ml core histone and the enzyme fraction was incubated for 30 min at 30°C. After boiling in SDS sample buffer, phosphorylated proteins were separated by SDS-PAGE, and the radioactivity of protein bands was determined using a Bio-imaging Analyzer BAS2000 (Fuji). When immunoprecipitated enzyme was employed, the immunoprecipitates were washed before the protein kinase assay 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 at 0–4°C to remove Triton X-100, NaF and Na_3VO_4. Autophosphorylation of RAC-PKα was monitored by the phosphorylation reaction without exogenous phosphate acceptor proteins. Where indicated, PtdIns(4,5)P2 (bovine brain, Boehringer Mannheim) and PtdIns(3,4,5)P3 (fatty acid moieties are palmitate, C16:0) [22] dissolved in water by vigorous mixing were added to the reaction mixture.

2.5. Immunoblot analysis

After boiling in SDS sample buffer, and proteins were separated by SDS-PAGE and transferred onto an Immobilon P membrane (Millipore). Immunoblot analysis was carried out using the polyclonal antibody against RAC-PKα as the first antibody and the alkaline phosphatase-conjugated anti-rabbit second antibody (Promega) as described [13].

3. Results and discussion

The effects of growth factor signal and heat shock on RAC-PK were studied in serum-starved cells such as COS-7 cells transiently expressing the FLAG-epitope tagged RAC-PKα and CHO cells stably overproducing the enzyme (Fig. 1). In the previous study using CHO cells expressing the wild type of RAC-PKα, RAC-PKα was activated by heat shock but not by serum [21]. After prolonged serum starvation, treatment with fetal bovine serum induced activation of RAC-PK in CHO cells overproducing the enzyme (Fig. 1B). It is not clear why CHO cells expressing the wild-type and epitope-tagged enzymes show different responses, however, serum-induced activation of RAC-PK might depend on the amounts of enzyme expressed in CHO cells, since the expression of the epitope-tagged RAC-PKα is lower than that of the wild-type enzyme. Thus, the effects of serum and heat treatment were compared employing COS-7 cells and CHO cells. Either serum or heat shock activated RAC-PK, and the serum stimulation and heat shock were not additive for activation of RAC-PK (Fig. 1A,B). Although serum and heat treatment activate RAC-PK in different manners, it is difficult to distinguish the effects of serum stimulation and heat shock since heat shock induces activation of RAC-PK in cells cultured in the presence of serum [21]. Furthermore, activation of RAC-PK has been demonstrated using immunoprecipitated enzymes that might contain other materials that could regulate the enzyme activity [15–20]. Thus, RAC-PK was purified to study the molecular mechanism of activation of RAC-PK. The epitope-tagged RAC-PKα was isolated from COS-7 cells transfected with the expression plasmid and cultured in the presence of fetal bovine serum by using affinity chromatography on the resin coupled with a monoclonal antibody to the FLAG epitope (Fig. 2). RAC-PK was purified to apparent homogeneity from the heat-treated cells and control cells (Fig. 2A). The enzyme purified from heat-treated cells without any obvious associating proteins showed a much higher enzyme activity than that purified from untreated control cells (Fig. 2B). The kinase-negative RAC-PK (K179M) was also purified using the same procedures, and the enzyme preparations isolated from both the heat-treated and control cells did

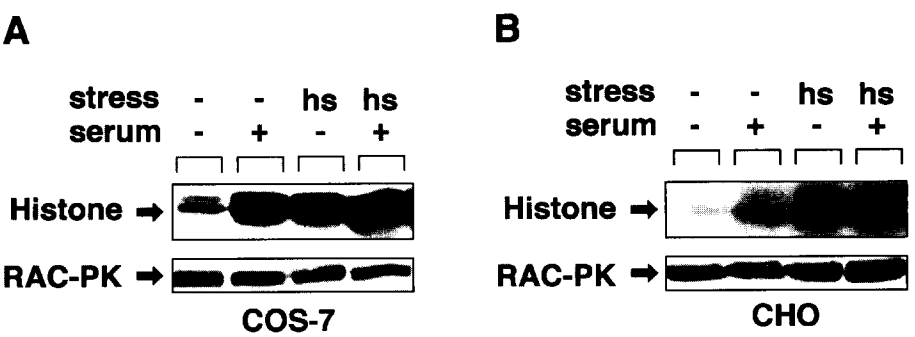

Fig. 1. Activation of RAC-PKα in COS-7 and CHO cells. Cells were treated as indicated and RAC-PKα was immunoprecipitated by the anti-FLAG antibody. Protein kinase activity measured by using core histone as substrate is shown in the upper panel. The amounts of RAC-PKα in the immunoprecipitates are shown by immunoblot analysis in the lower panels. Cells treated at 45°C for 20 min, with 10% fetal bovine serum for 20 min, and without treatment are indicated as (hs), (+), and (−), respectively, above the upper panel. The positions of RAC-PKα and phosphorylated histone are indicated by arrows. (A) COS-7 cells. Cells were transfected with the expression plasmid of FLAG-epitoped tagged RAC-PKα, cultured for 24 h, serum-starved for 12 h, and treated as indicated. (B) CHO cells. Cells overproducing FLAG-epitope tagged RAC-PKα were serum-starved for 24 h, and treated as indicated.

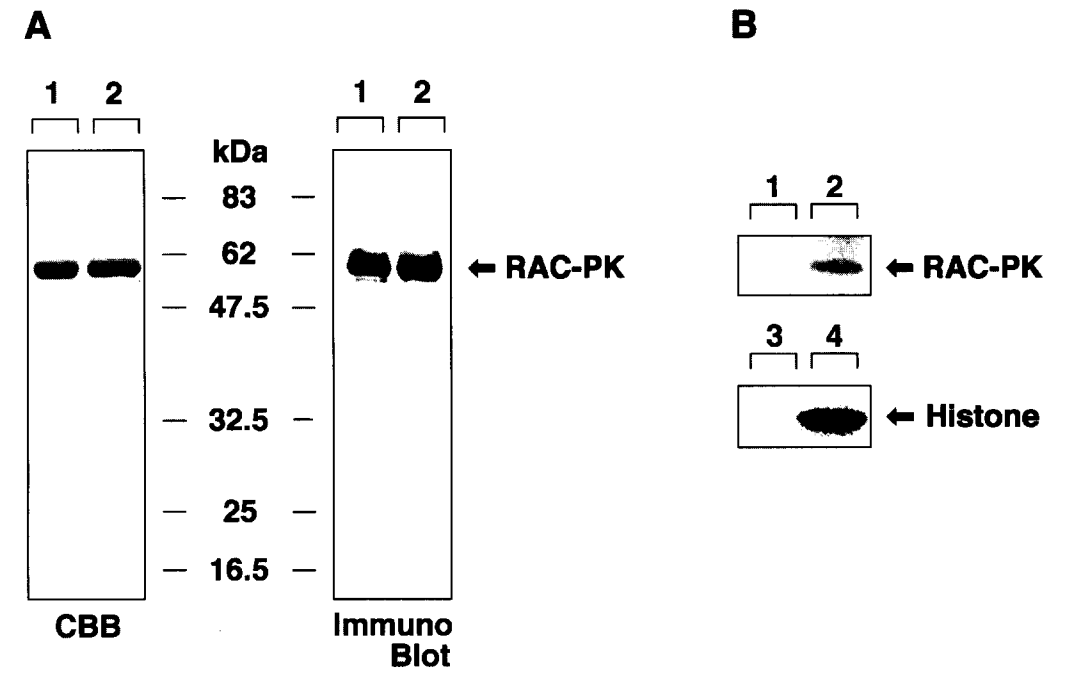

Fig. 2. RAC-PKα purified from transfected COS-7 cells. FLAG-epitope tagged RAC-PKα was purified from cells without treatment or from cells treated at 45°C for 20 min. (A) Purification of RAC-PKα. Purified RAC-PKα (0.2 μg) was applied on SDS-PAGE and stained with Coomassie brilliant blue (left panel) and immunoblotted with the anti-RAC-PK antibody (right panel). Lanes 1,2: RAC-PKα purified from untreated and heat-treated COS-7 cells, respectively. The positions of size markers are indicated in kDa, and the position of RAC-PKα is indicated by an arrow. (B) Protein kinase activity of RAC-PKα. Purified RAC-PKα (0.1 μg) was employed to measure protein kinase activity by autophosphorylation (lanes 1,2) and by using core histone as substrate (lanes 3,4). Lanes 1,3: RAC-PKα purified from untreated cells; lanes 2,4, RAC-PKα purified from heat-treated cells. The positions of RAC-PKα and phosphorylated protein are indicated by arrows.

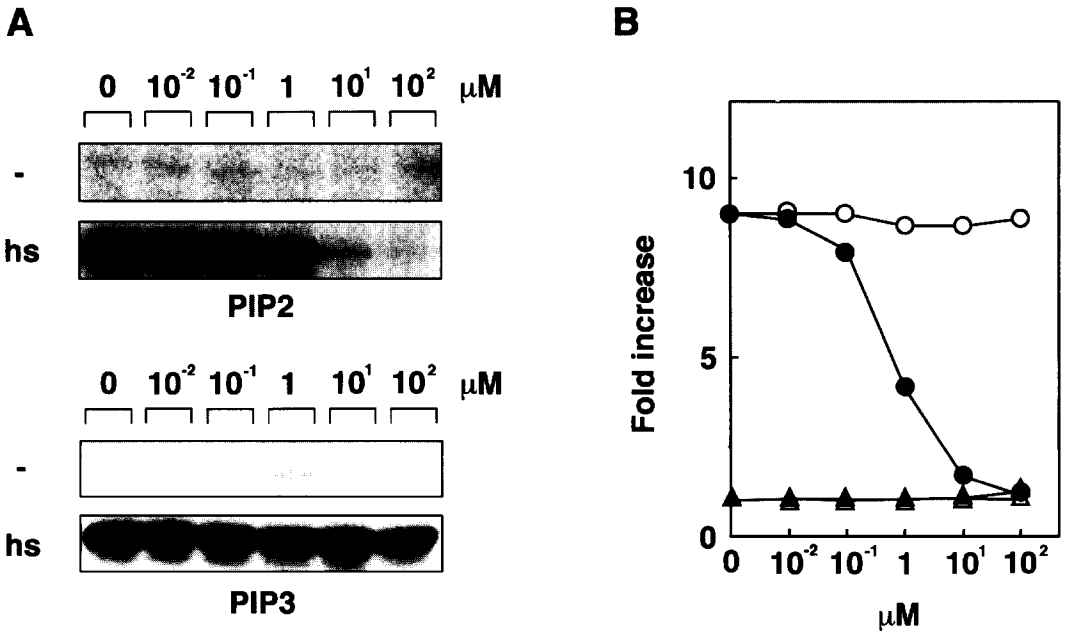

Fig. 3. Effects of PtdIns(4,5)P2 and PtdIns(3,4,5)P3 on purified RAC-PKα. RAC-PKα purified from COS-7 cells without treatment and from heat-treated cells was assayed by using core histone as substrate in the presence of various concentrations of PtdIns(4,5)P2 and PtdIns(3,4,5)P3. (A) Autoradiography of the phosphorylation of core histone. RAC-PKα was assayed in the presence of PtdIns(4,5)P2 (upper half) and PtdIns(3,4,5)P3 (lower half). PtdIns(4,5)P2 and PtdIns(3,4,5)P3 are shown as PIP2 and PIP3, respectively. RAC-PKα purified from cells without treatment and from heat-treated cells are denoted (−) and (hs), respectively. The positions of phosphorylated core histone are indicated by arrows. (B) Quantitation of the results of A. Circles and triangles indicate the enzyme purified from cells with and without heat-treatment, respectively. Open and closed symbols correspond to the activity in the presence of PtdIns(3,4,5)P3 and PtdIns(4,5)P2, respectively. The -fold stimulation of the RAC-PKα activity relative to the enzyme purified enzyme from untreated cells is shown.

not show detectable enzyme activity, confirming that the enzyme preparations obtained in these experiments are free from other protein kinases (data not shown). It has been reported that RAC-PK activated by serum in Swiss 3T3 cells is phosphorylated on serine and threonine residues and shows decreased mobility on SDS-PAGE, and phosphatase treatment of the immunoprecipitated RAC-PK from stimulated cells changes electrophoretic mobility and results in the reduction of kinase activity to the basal level [19]. RAC-PK purified from heat-treated and control cells cultured in the presence of serum showed the same mobility on SDS-PAGE as judged by protein staining and immunoblot analysis (Fig. 2A). It is not clear why the active enzyme obtained from heat-shocked cells in this study does not show a different mobility from the inactive enzyme. It might be possible that serum enhances the phosphorylation of RAC-PK whereas heat shock activates the enzyme without phosphorylation of RAC-PK.

RAC-PK has been indicated to be a downstream target of PI 3-kinase, and PtdIns(3)P, a product of the PI 3-kinase reaction, has been shown to activate the enzyme, presumably by associating with the PH domain of RAC-PK [15]. On the other hand, it has been reported that RAC-PK binds to PtdIns(3,4,5)P3 and PtdIns(3,4)P2, however, PtdIns(3)P is inert for the binding, and furthermore, the interaction of RAC-PK with PtdIns(3,4,5)P3 and PtdIns(3,4)P2 does not activate the kinase activity [23]. Thus, the effect of PtdIns(3,4,5)P3 was studied using RAC-PK purified from transfected COS-7 cells (Fig. 3). PtdIns(3,4,5)P3 did not activate the enzyme isolated from control cells. RAC-PK, activated approx. 9-fold by heat shock through a pathway independent of PI 3-kinase, was not enhanced by PtdIns(3,4,5)P3, either, and rather inhibited by PtdIns(4,5)P2 at higher concentrations. PtdIns(4,5)P2 had no effect on the enzyme activity isolated from control cells. These results indicate that RAC-PK is not activated by the simple association with PtdIns(3,4,5)P3, however, PtdIns(4,5)P2 could associate with the PH domain of RAC-PK. It is still not known how RAC-PK is activated by stress. Further studies are necessary to elucidate the molecular mechanisms of activation of RAC-PK through different signaling pathways.

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