Identification of Protein Kinase B (PKB) as a Phosphatidylinositol 3,4,5-Trisphosphate Binding Protein in *Dictyostelium discoideum*

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We have searched for phosphatidylinositol (PI)-3,4,5-trisphosphate (PIP₃) binding proteins in *Dictyostelium discoideum* using beads bearing a PIP₃ analogue, PIP₃-APB. One of the binding proteins with a molecular mass of 55 kDa was purified and its amino acid sequence was partially analyzed. Database searches showed that the analyzed sequence was identical to that of protein kinase B (PKB) of *D. discoideum*. The specific activity of *D. discoideum* PKB, when expressed together with constitutively active PI-3 kinase in mammalian cells, was elevated by about three-fold, suggesting that PKB could also act downstream of PI-3 kinase in *Dictyostelium* cells.

**Key words:** phosphatidylinositol 3,4,5-trisphosphate; PI-3 kinase; protein kinase B; *Dictyostelium discoideum*; phosphatidylinositol 3,4,5-trisphosphate binding protein

Phosphatidylinositol (PI)-3 kinase is important in various cell responses, such as signal transduction from the growth factor receptor to the nucleus, cytoskeleton reorganization, and vesicle transport.³ PI-3 kinase is rapidly activated upon growth factor stimulation to elevate the cellular levels of its specific product, phosphatidylinositol 3,4,5-trisphosphate (PIP₃).²-⁴ PIP₃ is subsequently dephosphorylated by a specific phosphatase to yield PI-3,4-P₂.⁵-⁷ PIP₂ and PI-3,4-P₂ seem to act as second messengers. PIP₂ and PI-3,4-P₂ activate certain subtypes of atypical protein kinase C (aPKC) and protein kinase B (PKB)/Akt, respectively.⁶-⁸ However, there are so many cell responses that could be regulated by PI-3 kinase that it is difficult to explain specific actions of PI-3 kinase by the activation of a few protein kinases downstream of PI-3 kinase. To address this problem, we have searched for PIP₃ binding proteins in mammalian cells using PIP₃-APB beads. By this method, we have already identified PKB, Tec tyrosine kinase,⁹ and PIP₂BP which contains a region with sequence similarity to Arf-GTPase activating protein (GAP),¹⁰ as PIP₂-binding proteins.

A cellular slime mold, *Dictyostelium discoideum*, is a simple eucaryote that is a good system for studying signal transduction pathways regarding cytoskeletal reorganization, vesicle transport, differentiation, and morphogenesis at a molecular level. In *Dictyostelium*, proteins involved in such phenomena can be identified by specific gene targeting or tagging mutagenesis. Thus, once PIP₂ binding proteins could be identified, their physiological roles could be examined by examining phenotypes of the mutant cells disrupted in each protein. Five PI kinase-like genes have been cloned in *D. discoideum*. Three of them, DdPIK-1, -2, and -3 are most closely related to the mammalian p110 PI 3-kinase.¹¹ Their functions may be redundant, because disruption of any single gene had no effect on *D. discoideum* growth or development. However, disruption of both DdPIK-1 and -2 caused in multiple defects in growth, vesicle transport, cytoskeletal reorganization, and development, while double knockouts of DdPIK-1 and -3 and DdPIK-2 and -3 were lethal.¹² These findings suggest that in *Dictyostelium* cells, signal transduction pathways involving PI-3 kinase are similar to those of mammalian cells.

In this paper, we examine the physiological role of each downstream target of PI-3 kinase, we first screened for the PIP₂ binding proteins from *D. discoideum* cells, and found that PKB seems to be downstream of PI-3 kinase in *Dictyostelium* cells, like mammalian cells.

**Materials and Methods**

*Dictyostelium strain and cell culture.* The *D. discoideum* strain AX²¹ was used in this study. The cells were

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* Abbreviations: PKB, protein kinase B; PI-3 kinase, phosphatidylinositol 3 kinase; PI-3,4-P₂, phosphatidylinositol 3,4-bisphosphate; PIP₂, phosphatidylinositol 3,4,5-bisphosphate; GAP, GTPase activating protein; PH domain, pleckstrin homology domain; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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grown in HL5 medium containing Proteose peptone No. 2 or No. 3 (Difco Laboratories, Inc, Detroit, MI). After the cells were cultured at 22°C in suspension so that the cell density did not exceed 6 x 10⁶ cells/ml, they were harvested by centrifugation.

**Purification and PIP₃ binding assay for PIP₃ binding proteins using the PIP₃ analogue (PIP₃-ABP) beads.** The structure and synthesis of PIP₃-ABP is described elsewhere. The affinity beads were prepared as described before. The cells were homogenized in a buffer containing 20 mM Tris-Cl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF. After the removal of cell debris by centrifugation at 10,000 x g for 1 h, 170 μl of the PIP₃-ABP beads was added and incubated for 2 h at 4°C. The beads were washed extensively with the same buffer and the proteins bound to the beads were eluted with 2% SDS sample buffer. In some cases, free PIP₃ (4 μM or 0.4 μM) was mixed with the sample before the addition of PIP₃-ABP beads in a small scale experiment. The protein was analyzed by SDS-polyacrylamide gel electrophoresis.

**Amino acid sequencing.** Several bands eluted were blotted onto a polyvinylidene difluoride membrane. Then the proteins were reduced and S-carboxymethylated as described before. After digestion with *Achromobacter* protease I (Lys-C), the resulting fragments were separated by reverse phase HPLC and the amino acid sequence were sequenced.

**The protein kinase assay for protein kinase B.** The full length *D. discoideum* PKB cDNA was amplified by polymerase chain reaction using two oligonucleotides, 5'...'CCAGATCTAATGTCACACGCACAAATGATCTC and 5'...'CATCTCAGTTATCTTAAATGTTTCCAGTTCGC-3' according to the EMBL database (accession number, U15210). The cDNA was cloned into an expression vector, pMIKNeo at the downstream of the myc-tag sequence joined to the initiation codon ATG. The activated PI-3 kinase gene was expressed by the expression vector based on pMIKNeo. pHisBD110 codes for the activated PI-3 kinase with a his-tag at the amino-terminal end. COS7 cells were transfected with the expression vectors by DEAE-dextran or electroporation methods. The cells were lysed in a buffer containing 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X100, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₄, 1 mM NaF, and 1 mM PMSF. After removal of the cell debris, the *D. discoideum* PKB was immunoprecipitated with a monoclonal anti-myc antibody, 9E10 (hybridoma purchased from ATCC). The activity of PKB in the immunoprecipitate was analyzed as described before. The *D. discoideum* PKB in the immunoprecipitate was measured by western blotting with 9E10. In some cases, cells were treated with 10 mM H₂O₂ two days after the transfection with the expression vectors.

**Results and Discussion**

**Screening of the PIP₃ binding proteins in *D. discoideum* by the PIP₃ analogue beads**

The lysate of *D. discoideum* cells was mixed with the PIP₃-APB beads, and the proteins bound to the beads were analyzed by SDS-PAGE. As shown in Fig. 1, several bands were detected (lane 1). To see whether the binding was specific to PIP₃, free PIP₃ was added in the binding solution to compete in the binding (Fig. 1, lane 2). The bands with molecular weights of 180, 110, 60, 55, 50, and 45 kDa disappeared in the competition experiment, suggesting that they recognize the PIP₃ analogue for the binding. One band with a molecular mass of 55 kDa crossreacted with a anti-PIP₃BP polyclonal antibody (data not shown). Since the PIP₃BP antibodies recognize the PH domains of PIP₃BP, it was likely that the 55-kDa protein also contained PH domains. Therefore, the partial amino acid sequence of the 55 kDa protein was selected for further analysis. The p55 protein was eluted from the beads and digested with lysyl endopeptidase, and the amino acid sequence of several peptide fragments was determined. The sequences were identical to the sequence of *D. discoideum* PKB by database searches. The molecular mass of *D. discoideum* PKB calculated from the deduced amino acid sequence is about 51 kDa which is close to 55 kDa obtained from the mobility of the protein in SDS-PAGE.
The data suggest that *D. discoideum* PKB binds PIP$_3$, and works downstream of PI-3 kinase in *D. discoideum* as well as in mammalian cells. To test this possibility, we cloned the *D. discoideum* PKB gene into a mammalian expression vector, pMIKNeo, with a myc-tag at the amino terminus, using the reverse transcriptase mediated polymerase chain reaction. After transfection of COS7 cells with the construct, cell lysates were prepared and the binding of *D. discoideum* PKB to PIP$_3$ was tested. As shown in Fig. 2, the recombinant *D. discoideum* PKB expressed in COS7 cells bound to the PIP$_3$ analogue beads, and the binding was inhibited by the free PIP$_3$, confirming that *D. discoideum* PKB specifically binds PIP$_3$. In addition, it was shown that the binding of the recombinant *D. discoideum* PKB to the PIP$_3$ analogue beads was also inhibited by the free PI-3,4-P$_2$.

**Fig. 2.** Binding of *D. discoideum* PKB Expressed in COS7 Cells to the PIP$_3$ Analogue Resin.

Cell lysates expressing myc-tagged *D. discoideum* PKB were mixed with various free competitors at the concentration indicated in the figure before mixing with the PIP$_3$ analogue resin. Cell lysates transfected with an empty vector were mixed with the PIP$_3$ analogue resin (control). The protein bound to the resin were separated by SDS-PAGE with a 10% polyacrylamide gel, and the recombinant PKB was detected by western blotting with 9E10, a monoclonal antibody to myc.

**D. discoideum PKB is regulated by PI-3 kinase in vivo.**

It has been shown that mammalian PKB binds PI-3,4-P$_2$ and PIP$_3$, and works downstream of PI-3 kinase. We, therefore, tested whether the activity of *D. discoideum* PKB is also regulated by the PI-3 kinase products in vivo. COS7 cells were co-transfected with an expression vector carrying *D. discoideum* PKB together with an expression vector for a catalytic subunit of PI-3 kinase, p110 protein, or a constitutively active PI-3 kinase, the BD110 protein. As shown in Fig. 3, the activity of *D. discoideum* PKB was elevated by the expression of PI-3 kinases. The expression level of the *D. discoideum* PKB was not affected by the PI-3 kinase activity, suggesting that the specific activity of the PKB was elevated. It has been shown that mammalian PKB was regulated by stresses. Activation of the *D. discoideum* PKB by heat shock or the treatment with H$_2$O$_2$ was tested. The *D. discoideum* PKB was unstable in mammalian cells to a heat treatment, which made us unable to test the activity of the PKB (data not shown). However, the H$_2$O$_2$ treatment did increase the PKB activity, although somewhat weaker than the activation of the mammalian PKB under similar conditions (Konishi et al., submitted).

It has been shown that the activity of PKB is regulated in various ways including phosphorylation by other kinases and binding of the PI-3 kinase products. We found that the activation of PKB after the heat treatment was insensitive to wortmannin, a selective inhibitor of PI-3 kinase, but that after H$_2$O$_2$ treatment was clearly inhibited by wortmannin, suggesting that there may be at least two activation mechanisms, one dependent on PI-3 kinase and another independent of it. Among the 3’-phosphorylated phosphoinositides, not PIP$_3$ but PI-3,4-P$_2$ has been suggested to be responsible for direct activation of PKB, although both phos-
phoinositides binds PKB. Our recent experiments showed that the levels of PIP2 were elevated after H2O2 or heat treatment, but that PI-3,4-P2 is greatly elevated only after H2O2 treatment, not after heat treatment (Konishi et al., submitted). These results support our previous finding that there may be PI-3 kinase-dependent and independent mechanisms for the PKB activation, and suggest that the elevation of PI-3,4-P2 is required for the activation of PKB.

As shown in this study, D. discoideum PKB was also activated by the constitutively active form of PI-3 kinase and by the H2O2 treatment in mammalian cells, and it also bound to both PI3P and PI-3,4-P2. These similarities between mammalian and D. discoideum PKBs suggest that PKB could also be downstream of PI-3 kinases in D. discoideum cells. If this is the case, it is likely that PI-3,4-P2 could be responsible for the direct activation of D. discoideum PKB. Further, the level of PIP2 was elevated 3 min after H2O2 treatment and then declined to the basal level within a few min. In contrast, the level of PI-3,4-P2 was elevated within 3 min after H2O2 treatment and PI-3,4-P2 was accumulated to give an extremely high level 10 min after H2O2 treatment (Konishi et al., submitted). As shown in Fig. 3, D. discoideum PKB was activated at 10 minutes after H2O2 treatment as well as at 3 minutes after the treatment, suggesting that D. discoideum PKB was also activated by PI-3,4-P2. As in mammalian cells, D. discoideum PI-3 kinases are involved in various cellular functions including growth, actin reorganization, vesicle transport, and development. The PI-3K/PKB pathway might be specifically involved in one of these functions in D. discoideum, although this is yet to be examined by the mutant analysis.

References