Different properties of monomer and heterodimer forms of phosphatidylinositol 3-kinases

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Phosphatidylinositol (PI) 3-kinase plays an important role in the signalling of cell growth. We previously purified two types of PI 3-kinase from bovine thymus, a monomer from (PI 3-kinase I) and a heterodimer form (PI 3-kinase II) [Shibasaki, Homma and Takenawa (1991) *J. Biol. Chem.* **266**, 8108–8114]. Here we examine the properties of these purified PI 3-kinases. Both PI 3-kinases were inhibited strongly by quercetin and isoquercetin. The inhibition of PI 3-kinase I and PI 3-kinase II by quercetin appears to be non-competitive, with apparent K_i values of 4 μ M and 2.5 μ M respectively. PI 3-kinase II, but not

PI 3-kinase I, co-immunoprecipitates with pp60^{v-src} and polyoma middle T (mT)/pp60^{c-src}, even under conditions where the PI 3-kinases are not phosphorylated, suggesting that non-phosphorylated PI 3-kinase recognizes autophosphorylated pp60^{v-src}. PI 3-kinase II is phosphorylated by pp60^{v-src} and binds to it. Anti-p85 (85 kDa subunit of PI 3-kinase II) antibody precipitates not only PI 3-kinase II but also co-immunoprecipitates pp60^{v-src} in *src*-transformed cells, suggesting that PI 3-kinase II binds to pp60^{v-src} *in vivo*. These data suggest that the two PI 3-kinases may be regulated independently.

INTRODUCTION

Phosphatidylinositol (PI) 3-kinase was first shown to be present as a complex with polyoma mT/pp60^{c-src} [1]. PI 3-kinase catalyses the phosphorylation of the 3-position of the inositol ring to produce a new lipid, 3-phosphate. This is in contrast with classical PI kinase, which phosphorylates the 4-position of the inositol ring in PI. Moreover, PI 3-kinase can phosphorylate not only PI but also PI 4-phosphate (PIP) and PI 4,5-bisphosphate (PIP₂) to form PI 3,4-bisphosphate and PI 3,4,5-trisphosphate respectively [2–4]. These novel lipids are not hydrolysed by phospholipase C, and are apparently not part of the pathway for the production of inositol phosphates. The PI 3-kinase associates physically with tyrosine kinases such as pp60^{v-src}, mT/pp60^{c-src}, the platelet-derived growth factor (PDGF) receptor, the colony stimulating factor 1 receptor and the insulin receptor, and its activity is correlated with the appearance of the 85 kDa protein (p85) that is tyrosine-phosphorylated by these kinases [5–11]. These findings strongly suggest that PI 3-kinase containing p85 plays an important role in oncogenic signalling. Recently we succeeded in purifying two forms of PI 3-kinase, PI 3-kinase I and PI 3-kinase II, from bovine thymus [12]. Carpenter et al. [13] have also purified PI 3-kinase II from rat liver. PI 3-kinases have both monomeric (molecular mass 110 kDa) and heterodimeric (110 kDa and 85 kDa subunit) forms. On peptide mapping, these two 110 kDa polypeptides appear to be the same protein (p110), suggesting that p110, but not the p85 subunit, has PI 3-kinase activity.

More recently, cDNAs of p85 have been isolated from bovine, murine, and human cells by three independent groups [14–16]. Amino acid sequence analysis shows that p85 contains both the SH2 and SH3 domains previously found in a variety of proteins involved in signal transduction, such as phospholipase C, *ras*-

GTPase activating protein, non-receptor tyrosine kinases, tyrosine phosphatase 1C, and *akt* serine/threonine kinase [17–21]. Studies have revealed that the SH2 domain recognizes a specific peptide sequence containing phosphotyrosine and forms a complex with tyrosine-phosphorylated proteins [22,23]. On the other hand, the role of the SH3 domain remains unclear, although an association with the cytoskeleton is anticipated [24]. Therefore it is thought that p110 catalyses PI 3-kinase activity and p85 regulates the activity through an association with tyrosine-phosphorylated proteins and the cytoskeleton. In this paper we investigate the properties of PI 3-kinase I and PI 3-kinase II, and also examine their association with pp60^{v-src} using purified PI 3-kinases.

MATERIALS AND METHODS

Materials

PI, phosphatidylserine (PS), phosphatidylcholine (dioleoyl), phosphatidylethanolamine, phosphatidic acid (PA) (all from porcine liver) and cardiolipin (from beef heart) were purchased from Serdary Research Laboratories (London, Ontario, Canada). PIP and PIP₂ were purified from bovine spinal cords as described previously [25]. Various nucleotides and nucleosides were from Sigma. [γ -³²P]ATP (6000 Ci/mmol) was from New England Nuclear. Anti-*src* antibody was from Oncogene Science (Manhasset, N.Y., U.S.A.), and anti-phosphotyrosine antibody (PY20) was from ICN Biochemicals Inc. Dodecylsucrose (sucrose monolaurate) was from Mitsubishi–Kasei Food Co. (Tokyo, Japan).

Preparation of pp60^{v-src}

Rous sarcoma virus-transformed rat 3Y1 cells were lysed in

Abbreviations used: PI, phosphatidylinositol; PIP₂, PI 4,5-bisphosphate; PIP, PI 4-phosphate; PA, phosphatidic acid; PS, phosphatidylserine; GST, glutathione S-transferase; PDGF, platelet-derived growth factor, GTP[S], guanosine 5'-[γ -thio]triphosphate.

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500 μ l of 40 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, 1 % Triton X-100, 1 mM sodium vanadate, 0.1 mM phenylmethanesulphonyl fluoride and 0.1 mM diisopropylfluorophosphate. After centrifugation for 20 min at 12000 g , the supernatant was used for cell lysates. From the lysates, pp60^{v-src} was immunoprecipitated using anti-src antibody or anti-phosphotyrosine antibody. These immuno precipitates were used as pp60^{v-src} tyrosine kinase.

PI 3-kinase activity

PI 3-kinase was assayed in a reaction mixture of 50 μ l containing 50 mM Tris/HCl buffer (pH 7.5), 10 mM MgCl₂, 0.5 mM EGTA, 50 mM NaCl, 1 mM PI, 0.4 mM PS, 100 μ M ATP, [γ -³²P]ATP (0.5–1 μ Ci), and enzyme protein. Other details were as described previously [12]. The reaction products of PI 3-kinase were spotted on to thin layer plates (silica gel 60, 10 cm $\times$ 20 cm; Merck) and developed in methanol/chloroform/ammonia/water (20:14:3:5, by vol.). The plates were visualized by autoradiography.

Preparation of two forms of PI 3-kinase

Two forms of PI 3-kinase were purified from bovine thymus cytosol as described previously [12]. Briefly, both PI 3-kinase I (monomer type) and PI 3-kinase II (heterodimer type) were purified by seven successive chromatographic steps, including DEAE-cellulose, cellulose phosphate, Mono Q, Superdex 26/60, Heparin-5 PW, Mono S and Superose 12. The molecular masses of PI 3-kinases I and II were 110 kDa and 190 kDa respectively in the native form. PI 3-kinase I was a monomer, but PI 3-kinase II consisted of two subunits, of 110 kDa and 85 kDa. With PI as a substrate, the specific activities of PI 3-kinase I and PI 3-kinase II were 250 and 50 nmol/min per mg of protein respectively, as described previously [12].

Phosphorylation of PI 3-kinases I and II

Phosphorylation assays were performed by incubating PI 3-kinase I or II with 70 μ l of phosphorylation buffer containing 20 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 5 mM MnCl₂ and 10 μ Ci of [γ -³²P]ATP (20 μ M) for 10 min at 30 °C in the presence or absence of pp60^{v-src} immunoprecipitates. Assays were terminated by the addition of SDS sample buffer prior to analysis by SDS/PAGE.

Expression and purification of p85

A full-length cDNA encoding α -type p85 of Pi 3-kinase was cloned from HeLa cells using the reverse transcriptase-mediated PCR technique. For synthetic oligomers, the following primers were used: CAGATTTGCAAACATGAGTGCTGAG, CAGA-GAAGCCATATTTCCCATCTCG, ACTAAAATGCATGG-TAATTATACTC and AGTAACTCGCTTCATCGCCTCTG-CTGTGC. The products were cloned into the *Hinc*II site of vector pUC9. The cDNA sequences were found to have two differences from the sequence published by Skolnik et al. [15]: at nucleotide positions 1031 and 1599, A was substituted by G. Since several independent clones obtained from HeLa cells had the same changes, we conclude that this difference is due to polymorphism. The cDNA was fused in-frame to the glutathione S-transferase

(GST) gene of pGEX-2T (Pharmacia). Plasmic-bearing *Escherichia coli* (JM109) were induced with 100 μ M isopropylthiogalactoside for 2 h at 37 °C after a 3 h preincubation, collected by centrifugation, and lysed with RIPA buffer containing 50 mM Tris (pH 7.4) 150 mM NaCl, 1 % Triton X-100, 1 % deoxycholate and 0.1 % SDS. The lysates were sonicated for 5 $\times$ 1 min, and centrifuged at 105000 g for 30 min at 2 °C. The expressed GST fusion protein was purified from the centrifugation supernatant by affinity chromatography with glutathione-Sepharose beads (Pharmacia).

Production of anti-p85 antibody

A protein eluted with 100 μ g/ml thrombin (human thrombin; Sigma) from glutathione-Sepharose beads was applied to a HiTrap Heparin column (Pharmacia) pre-equilibrated with 20 mM Tris (pH 7.6), 1 mM EGTA and 10 % glycerol. The column was developed with a 30 ml linear gradient of 0–0.5 M NaCl in the same buffer. Each fraction was checked by SDS/PAGE. New Zealand White female rabbits were immunized with the purified recombinant p85 produced in *E. coli* emulsified with Freund's complete adjuvant according to standard procedures. The rabbits received booster injections every 2 weeks. After 2 months, the antiserum was collected and the immunoglobulin fraction was obtained by 40 % saturated ammonium sulphate fractionation.

RESULTS

Effect of phospholipids on PI 3-kinase activity

Various phospholipids, such as phosphatidylcholine, phosphatidylethanolamine and cardiolipin, when added to dispersed PI, inhibit the activities of PI 3-kinase I and PI 3-kinase II (Figure 1). Of these phospholipids, cardiolipin is the strongest inhibitor. Phosphatidylcholine inhibits PI 3-kinase II strongly but PI 3-kinase I only weakly. On the other hand, PA enhances activity, more markedly for PI 3-kinase II than for PI 3-kinase I. These data suggest different properties for the monomer and heterodimer forms.

Effects of nucleotides and inhibitors on PI 3-kinase activity

The effects of various kinds of nucleotides and flavonoids on PI 3-kinases I and II were examined. Guanosine 5'-[γ -thio]-triphosphate (GTP[S]), UTP, CTP, GTP and GDP were not strong inhibitors. ADP, adenosine and AMP inhibited the activities of both PI 3-kinase I and PI 3-kinase II, but only at concentrations higher than 100 μ M. Generally, PI 3-kinase II is more sensitive to these inhibitors than PI 3-kinase I. The inhibition of PI 3-kinases I and II by adenosine appears to be a mixture of competitive and non-competitive inhibition (results not shown). These values are comparable with those determined using crude enzymes [26].

Among flavonoids, quercetin has a strong inhibitory effect on both PI 3-kinase I and PI 3-kinase II. The addition of 10 μ M quercetin resulted in more than 50 % inhibition of both PI 3-kinase activities. We also examined the inhibitory effects of other flavonoids (Figure 2). Isoquercetin, along with quercetin, was the strongest inhibitor, while other flavonoids including phloretin, catechin, genistein, apigenin and hesperetin appeared not to have any inhibitory effect. The inhibition of PI 3-kinases I and II by quercetin appears to be non-competitive, with apparent K_i values of 4 μ M and 2.5 μ M for PI 3-kinase I and PI 3-kinase II respectively (Figure 3).

Figure 1 Effect of various phospholipids on PI 3-kinase I and PI 3-kinase II activities

Various phospholipids dispersed by sonication were added to reaction mixtures containing 0.1 μg of PI 3-kinase I (a) or 0.2 μg of PI 3-kinase II (b). The enzyme activities are expressed as percentages of control values (without phospholipids). Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CAR, cardiolipin. Data are shown as means $\pm$ S.E.M. in triplicate determinations.

Figure 2 Effect of flavonoids on PI 3-kinase I and PI 3-kinase II activities

Flavonoids solubilized in dimethyl sulphoxide (DMSO) were added to reaction mixtures containing 0.1 μg of PI 3-kinase I or 0.2 μg of PI 3-kinase II at a final DMSO concentration of 2%. This concentration of DMSO does not affect the activity. PI 3-kinase activities were measured at two concentrations of flavonoids, 10 μM (a) and 100 μM (b). The enzyme activities are expressed as percentages of control values (without flavonoid).

Figure 3 Inhibition of PI 3-kinase I (a) and PI 3-kinase II (b) by quercetin

PI 3-kinase activities were measured with several concentrations of ATP in the presence of various concentrations of quercetin. Lineweaver-Burk plots are shown.

Binding of PI 3-kinase to pp60^{src}

To examine the association between purified PI 3-kinase and pp60^{src}, cell lysates from src/3Y1, src/CEF and polyoma

mT/3Y1 cells were incubated in phosphorylation buffer for 10 min at 30 $^{\circ}\text{C}$. The reaction was then stopped with 5 mM EDTA, and further incubation was carried out with purified PI

Figure 4 Association of purified PI 3-kinase II with pp60^{v-src}

Purified PI 3-kinase II was incubated for 2 h at 4 °C with immunoprecipitates of pp60^{v-src} or mT/pp60^{c-src} in binding buffer consisting of 20 mM Tris/HCl (pH 7.6), 2 mM EDTA, 0.5 mM sodium vanadate and 10% glycerol. The beads were washed and then PI 3-kinase activities were assayed. The products were developed by t.l.c. as described in the Materials and methods section and exposed on X-Omat film (Kodak) overnight. pp60^{v-src}, pp60^{c-src} and mT/pp60^{c-src} were immunoprecipitated with anti-src antibody from src/3Y1 (lanes 1 and 4), src/CEF (lanes 2 and 5) and polyoma mT/3Y1 (lanes 3 and 6) cells respectively. PI-3P, PI 3-phosphate.

Figure 5 pp60^{v-src} phosphorylates and binds to PI 3-kinase II

pp60^{v-src} and pp60^{c-src} were immunoprecipitated with anti-src antibody from src/CEF (lanes 1 and 4), src/3Y1 (lanes 2 and 5) or 3Y1 (lanes 3 and 6) cells. Purified PI 3-kinase II was added to immunoprecipitated pp60^{v-src} or pp60^{c-src} in phosphorylation buffer and then incubated at 30 °C for 10 min. After the phosphorylation was stopped by the addition of 10 mM EDTA, the immunoprecipitates were washed once with 0.5 M LiCl and 20 mM Tris/HCl buffer (pH 7.4), once with water, twice with phosphate-buffered saline, and once with 20 mM Tris/HCl (pH 7.4) containing 150 mM NaCl and 2 mM EDTA. Immunoprecipitates were electrophoresed in SDS/polyacrylamide gels and subjected to autoradiography.

3-kinase I or II. The PI 3-kinase activity co-immunoprecipitating with pp60^{v-src} was measured (Figure 4) under conditions of low specific radioactivity of [γ -³²P]ATP (100 μ M). Under these conditions, endogenous PI 3-kinase activity was not detected. Only exogenously added PI 3-kinase II, but not PI 3-kinase I (results not shown), co-precipitated with pp60^{v-src} and mT/pp60^{c-src}. Immunoprecipitated pp60^{v-src} from src/3Y1, src/CEF and 3Y1 cells was incubated with purified PI 3-kinase II in phosphorylation buffer. SDS/PAGE showed that exogenously added PI 3-kinase II was phosphorylated by immunoprecipitated pp60^{v-src} but not by pp60^{c-src} (Figure 5), and that the phosphorylation occurs in the p85 subunit. These data

Figure 6 Anti-p85 antibody immunoprecipitates PI 3-kinase II

(a) Purified PI 3-kinase II (lanes 1 and 2) or the anti-p85 antibody immunoprecipitate from 3Y1 cell lysates (lanes 3 and 4) were electrophoresed in SDS/7.5%-polyacrylamide gels. A sample was stained with Coomassie Brilliant Blue (lanes 1 and 3), and the remainder was transferred to nylon membranes and stained with anti-p85 antibody (lanes 2 and 4). (b) Cell lysates of 3Y1 cells were immunoprecipitated with pre-immune serum (lane 1) or anti-p85 antibody (lane 2). The precipitates were used to measure PI 3-kinase activity. The products were developed in t.l.c. and exposed to X-Omat film overnight. PI-3P, PI 3-phosphate.

Figure 7 pp60^{v-src} binds to the p85 subunit of PI 3-kinase II

(a) Cell lysates from src/3Y1 (lane 1) or 3Y1 (lane 2) cells were immunoprecipitated with anti-p85 antibody. The immunoprecipitates were electrophoresed in SDS/polyacrylamide gels and then transferred to nylon membranes. The membranes were stained with anti-pp60^{v-src} antibody. (b) The anti-p85 antibody (lanes 1 and 2) or anti-pp60^{v-src} antibody (lanes 3 and 4) immunoprecipitates from src/3Y1 (lanes 1 and 3) or 3Y1 (lanes 2 and 4) cells were phosphorylated *in vitro* in phosphorylation buffer at 30 °C for 10 min, and then analysed by SDS/PAGE. Gels were exposed to X-Omat film overnight.

confirm the previous indirect evidence that a putative PI 3-kinase, p85, is phosphorylated by pp60^{v-src}.

Next, cell lysates from src/3Y1 and 3Y1 cells were immunoprecipitated with anti-p85 antibody. This antibody immunoprecipitates not only p85 but also p110, suggesting that the heterodimeric form, PI 3-kinase II, is being immunoprecipitated (Figure 6a). Moreover, this antibody could precipitate PI 3-kinase activity from 3Y1 cell lysates (Figure 6b). The immunoprecipitates were electrophoresed in SDS/polyacrylamide gels and transferred to the membranes, and then stained with anti-src antibody (Figure 7a). However, only trace amounts of pp60 were detected in the immunoprecipitates. This result suggests that only small amounts of pp60 are associated with p85. Therefore, *in vitro* phosphorylation was carried out to increase the sensitivity. As shown in Figure 7(b), anti-p85 antibody precipitates caused the phosphorylation of not only p85 but also pp60^{v-src} in src/3Y1 cells. Even in non-transformed 3Y1 cells, p85 was phosphorylated while pp60^{c-src} was not. This result suggests that another protein kinase may be associated with p85 in 3Y1 cells. On the other hand, several proteins, including pp60^{v-src} and p85, were phosphorylated in immunoprecipitates with anti-pp60^{v-src} anti-

body, while anti-*src* antibody immunoprecipitates from 3Y1 cell lysates did not cause the phosphorylation of p85 (Figure 7b).

DISCUSSION

We previously purified two types of PI 3-kinase from bovine thymus [12], PI 3-kinase I, a monomer of molecular mass 110 kDa, and PI 3-kinase II, a heterodimer consisting of 110 kDa and 85 kDa proteins. These data suggest that the 110 kDa polypeptide has the PI 3-kinase activity. Indeed, molecular cloning of a cDNA encoding the 85 kDa subunit clearly demonstrates that this protein is a regulatory subunit with SH2 and SH3 domains. So far, we have not succeeded in separating the two subunits in the heterodimeric form, since PI 3-kinase II is resistant to detergent treatments such as Triton X-100, octyl glucoside, and sodium deoxycholate. Urea treatment also fails to separate the subunits. However, monomeric PI 3-kinase has a higher activity than the heterodimeric form; thus it is possible that the activation of PI 3-kinase is caused by the conversion of the heterodimer to the monomer.

It is well known that p85 is phosphorylated on a tyrosine residue in *v-src*-transformed cells, polyoma-transformed cells and in cells stimulated with PDGF, i.e. in which PI 3-kinase appears to be activated [2,27]. However *in vitro* phosphorylation of PI 3-kinase II by pp60^{*v-src*} does not result in an increase in activity or induce the dissociation of the two peptides. These data suggest that another factor(s) is necessary to activate PI 3-kinase II.

The biochemical properties of PI 3-kinase I and PI 3-kinase II, such as lipid dependency and nucleotide effects, are not very different, as described in this paper. Both enzymes are fairly resistant to nucleotides such as GTP[S], UTP, CTP, GTP and GDP. ADP, adenosine and cyclic AMP inhibited the activities of PI 3-kinases I and II at concentrations higher than 100 μ M. However, quercetin was found to be a very strong inhibitor of both enzymes. Among various flavonoids examined, isoquercetin was also a strong inhibitor. In addition, we have shown that the p85 subunit of PI 3-kinase II plays an important role in binding to pp60^{*v-src*}, as PI 3-kinase I does not co-precipitate with pp60^{*v-src*}. This binding seems to occur through the SH2 domain, since this domain is known to be important in recognizing and binding tyrosine-phosphorylated sites [28]. It can be seen in Figure 4 that purified PI 3-kinase II, which is not phosphorylated, can bind to pp60^{*v-src*}; this suggests that the SH2 domain in p85 recognizes the phosphorylation site on pp60^{*v-src*}. The fact that the SH2 domain of pp60^{*v-src*} recognizes the phosphorylated p85 has been reported to be important [5]. These results suggest that these two SH2-containing proteins can recognize each other in different conditions. However, the problem of how PI 3-kinase is activated

in response to the activation of tyrosine kinase remains to be solved.

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