Characterization of the bovine brain cytosolic phosphatidylinositol 3-kinase complex

Fernanda RUIZ-LARREA,*|| Patricia VICENDO,* Pnina YAISH,* Peter END,* George PANAYOTOU,* Michael J. FRY,* Sarah J. MORGAN,* Andrew THOMPSON,* Peter J. PARKER‡ and Michael D. WATERFIELD*§

*Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, ‡Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, and §Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

Receptor-linked phosphatidylinositol (PtdIns) 3-kinase may generate a second-messenger signal. Here a large-scale purification of the bovine brain enzyme, based on methods developed by Morgan, Smith and Parker [(1990) *Eur. J. Biochem.* **191**, 761–767] and Fry, Panayotou, Dhand, Ruiz-Larrea, Gout, Nguyen, Courtneidge and Waterfield [(1992) *Biochem. J.* **288**, 383–393] is described. The purified enzyme is shown to be a heterodimer of 85 kDa and 110 kDa protein subunits (p85 and p110). Labelling

with 5'-*p*-fluorosulphonylbenzoyl-adenosine shows that p110 contains an ATP-binding site and confers catalytic activity to the complex. The purified complex is known to be highly phosphorylated on both p85 α and p110 subunits, and dephosphorylation generates a deactivated complex, indicating that phosphorylation is an important covalent modification of the complex and may modulate PtdIns 3-kinase activity.

INTRODUCTION

Several growth-factor receptors, including those for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, insulin-like growth factor 1 (IGF-1) and colony-stimulating factor 1 (CSF-1), possess a ligand-stimulated intrinsic protein-tyrosine kinase. Members of the src family also possess an intrinsic tyrosine kinase, which in some cases may be linked to receptor activation (reviewed by Ullrich and Schlessinger, 1990). In 1984 the first reports appeared (Sugimoto et al., 1984; Macara et al., 1984) showing not only that proteins might be phosphorylated by oncogene-product-associated kinases, but that lipids may also be substrates for phosphorylation. The phospholipid PtdIns is an important component of cell membranes and has been found in all eukaryotic cells examined. A PtdIns kinase associated with oncogene products was first discovered in pp60^{v-src} immunoprecipitates from transformed cells and later was shown to be strongly associated with the middle-T antigen–pp60^{c-src} complex from polyoma-virus-transformed cells (Whitman et al., 1985; Talmage et al., 1989), with products of the oncogenes *fms*, *yes*, *crk* and *abl* and with several growth-factor receptors [PDGF, EGF, insulin, CSF-1, IGF and nerve growth factor (NGF) receptors] (reviewed by Cantley et al., 1991).

The PtdIns kinase associated with protein-tyrosine kinases phosphorylates the inositol ring at the D-3 position, and herein it is named PtdIns 3-kinase. Its activity is inhibited by neutral detergents and is classified as a type I PtdIns kinase (Whitman et al., 1987); this is the first step in a new pathway of PtdIns metabolism generating novel potential signalling molecules. This pathway is distinct from the classical pathway that leads from PtdIns to PtdIns(4,5)P₂ and then to the second messengers Ins(1,4,5)P₃ and diacylglycerol via phospholipase C (PLC). Interestingly, the phosphoinositides derived from the action of

PtdIns 3-kinase do not appear to be substrates for any of the known PLC isoforms (Serunian et al., 1989).

PtdIns 3-kinase seems to be involved not only in mitogenic responses, but also in signalling within terminally differentiated cells such as platelets or neutrophils. For example, platelets stimulated with thrombin (Huang et al., 1991) show an increase in PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ levels; this response is also observed when GTP analogues are introduced by permeabilization of the plasma membrane, suggesting the involvement of a G-protein in the transmission of the signal. Similarly, neutrophils stimulated by *N*-formylmethionyl-leucyl-phenylalanine (Stephens et al., 1991) show increased levels of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂. All the evidence suggests that PtdIns 3-kinase produces a crucial second messenger that is involved in an as-yet uncharted signal-transduction pathway.

Previously it has been shown that PtdIns 3-kinase activity appears to be associated with a heterodimeric complex of 85 kDa and 110 kDa subunits (p85 and p110) (Morgan et al., 1990; Carpenter et al., 1990; Shibasaki et al., 1991). The presence of tyrosine-phosphorylated 85 kDa protein in activated tyrosine kinase immunoprecipitates correlates with the presence of PtdIns 3-kinase activity in those immunoprecipitates. However, our previous studies have indicated that p85 does not itself possess any PtdIns kinase activity, but binds and is a substrate for tyrosine phosphorylation by the PDGF-, EGF- and CSF-1-activated receptors, the polyoma mT:pp60^{c-src} complex and *erbB2*, pp60^{c-src}/p59^{c-fyn} protein-tyrosine kinases (Otsu et al., 1991; Gout et al., 1992).

Here we have investigated the nature of the catalytic subunit of the PtdIns 3-kinase complex from bovine brain cytosol and provide direct evidence that the p110 subunit contains an ATP-binding site and confers catalytic activity to the complex. It is also shown that phosphorylation of the proteins that constitute the PtdIns 3-kinase complex modulates its activity.

Abbreviations used: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor 1; CSF-1, colony-stimulating factor 1; NGF, nerve growth factor; PLC, phospholipase C; RU, resonance units; bcr, product of the *bcr* gene (breakpoint cluster region); FSBA, 5'-*p*-fluorosulphonylbenzoyl-adenosine; PVDF, polyvinylidene difluoride.

|| To whom correspondence should be addressed.

EXPERIMENTAL

Purification of the enzyme

In a standard preparation, 12 bovine brains were the starting material for the purification. The first three steps of the purification procedure [precipitation with $(\text{NH}_4)_2\text{SO}_4$, DEAE-cellulose and phosphocellulose chromatographies] were carried out as described previously (Morgan et al., 1990). The next step in the purification was chromatography on a 50 ml Pharmacia Q-Sepharose column, eluted with a 250 ml linear gradient of 0–0.2 M NaCl in buffer A, which consisted of 10 mM sodium β -glycerophosphate, pH 7, 0.2 mM sodium orthovanadate, 20 mM NaF, 2 mM EDTA, 10 mM benzamidine and 0.3% β -mercaptoethanol. The active fractions were pooled, and Triton X-100 was added to a final concentration of 0.1% to disrupt protein–protein interactions. After 15 min under continuous agitation at 4 °C the sample was diluted with 3 vol. of buffer A, and ATP/MgCl₂ was added to a final concentration of 1 mM. The sample was loaded on a Mono Q HR 10/10 column pre-equilibrated in buffer A containing 1 mM ATP/MgCl₂. The sample was eluted with a 60 ml linear gradient of 0–0.5 M NaCl in buffer A containing 1 mM ATP/MgCl₂ and 0.02% Triton X-100. The active fractions were pooled and concentrated by dialysis against buffer A in 50% glycerol. The last step of the purification was affinity chromatography with phosphopeptide affinity beads prepared as described elsewhere (Fry et al., 1992); 20 μ l of packed beads was incubated with 50 μ l of the active pool in 300 μ l of affinity buffer B, which consisted of 10 mM sodium β -glycerophosphate, pH 7.5, 2 M NaCl, 0.5% Triton X-100, 0.2 mM sodium orthovanadate, 20 mM NaF, 2 mM EDTA, 10 mM benzamidine and 0.1 mM dithiothreitol. After incubation for 2 h at 4 °C with rotation, the supernatant was removed and the beads were washed three times with buffer B. Bound proteins were released from the beads in SDS-sample buffer for SDS/PAGE analysis, or in 3 M urea, yielding an inactive preparation. Proteins were released in an active state with an eluent consisting of 150 mM NaCl and 2% sodium cholate in 10 mM phosphate buffer, pH 7, and 80% glycerol.

PtdIns 3-kinase activity

Enzyme activity was assayed in a reaction mixture (60 μ l) containing 10 μ l of sample, 2.5 mM MgCl₂, 75 μ M ATP (approx. 15000 c.p.m./ μ l), 10 μ l of substrate prepared as described below, and assay buffer, consisting of 20 mM β -glycerophosphate, 0.2 mM sodium orthovanadate, 20 mM NaF, 5 mM pyrophosphate, 10 mM benzamidine, 0.3% β -mercaptoethanol, pH 7.5. PtdIns ($\text{L-}\alpha$ -phosphatidylinositol from soybean; Sigma; 99% pure) was prepared as a stock at 3 mg/ml in 1% cholate in assay buffer and sonicated with a Contes sonicator at 4 °C for 60 s at 10 s intervals. For PtdIns 4-kinase assays, 1% (v/v) Nonidet 40 was included in the PtdIns buffer to inhibit PtdIns 3-kinase activity. Assays were initiated by addition of ATP and stopped after incubation for 10 min at 37 °C by addition of 100 μ l of 1 M HCl. PtdIns 3-kinase bound to the phosphopeptide affinity beads remained fully active and could be used as a bioreactor of immobilized enzyme. Kinetics of PtdIns 3-kinase activity with immobilized enzyme were studied in 500 μ l of the reaction mixture described above, and 50 μ l samples were taken at different time intervals from the supernatant after pelleting the solid beads containing the enzyme by pulse centrifugation in a table centrifuge. The products of PtdIns 3-kinase activity were extracted and quantified by liquid-scintillation counting or t.l.c. as previously described (Morgan et al., 1990). Deacylation, deglyceration and h.p.l.c. analysis of the products of the PtdIns

3-kinase reaction were done as previously described (Morgan et al., 1990).

Binding of substrate lipid vesicles monitored by BIAcore

BIAcore (Pharmacia) was used for characterization of the binding of different phosphoinositides to the PtdIns 3-kinase complex from bovine brain. The instrument is described in detail by Jönson et al. (1991), Jönson and Malmqvist (1992) and Fägerstam (1991), as well as in the application notes issued by Pharmacia. The detection principle is based on surface plasmon resonance (SPR), a quantum-mechanical effect which can be observed in metals such as gold and silver. The interaction between proteins or proteins and lipids is measured in real time as a function of changes in refractive index. These changes occur when proteins or lipids bind to others already immobilized in a carboxymethylated dextran matrix which is mounted on the supporting gold film (Löfås and Johnson, 1990). Lipid vesicles to be analysed for binding to PtdIns 3-kinase complex are delivered from a micro-fluidic cartridge by a pump system. Thus the successive binding of different proteins and of phosphoinositide-containing lipid vesicles can be monitored in real time. The precise experimental set-up is given in Figure 3 legend. The binding is shown in real-time plots called sensorgrams, where resonance units (RU), which reflect the change in refractive index, are plotted against time. A signal of 1000 RU corresponds to a change in SPR angle of about 0.1, or a change (binding or dissociation) in protein concentration of approx. 1 ng/mm², respectively. The standard vesicle composition used in most experiments was 200 μ g of phosphatidylcholine, 50–100 μ g of phosphoinositide and 50–100 μ g of sodium cholate per ml of buffer C (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 100 μ M CaCl₂, 0.005% Tween-20). The monoclonal antibody used for capturing the PtdIns 3-kinase complex was raised against the p85 α subunit of the PtdIns 3-kinase complex. This antibody has been shown to bind to the bcr-related domain of p85 α (P. End, unpublished work).

Western immunoblotting

Western immunoblotting was performed by the method of Kamps and Sefton (1988). Polyclonal antibodies raised against the recombinant proteins p85 α and p85 β (Otsu et al., 1991) were affinity-purified and used in Western immunoblotting at a concentration of 1:500.

Labelling with 5'-*p*-fluorosulphonylbenzoyl[adenine-8-¹⁴C]-adenosine ([¹⁴C]FSBA)

About 5 μ g of purified bovine brain PtdIns 3-kinase was immobilized on phosphopeptide affinity beads and incubated with 2 μ Ci of [¹⁴C]FSBA (NEN; 0.42 mM final concn.) in 100 μ l of incubation buffer consisting of 30 mM Tris/HCl, pH 7.5, 1% BSA, 10 mM MgCl₂ and 0.5% sodium cholate. After incubation for 2 h at room temperature with continuous rotation, the sample was centrifuged and the pellet washed with 3 $\times$ 1 ml of affinity buffer B. Proteins were extracted from the affinity beads with Laemmli sample buffer (45 μ l/tube), separated by SDS/PAGE on a 7.5%-acrylamide gel, dried and exposed for 2 weeks at –70 °C for autoradiography.

Two-dimensional PAGE

Affinity-purified PtdIns 3-kinase complex was dephosphorylated with 0.2 unit of alkaline phosphatase (Boehringer Mannheim,

713 023) in a medium containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 10 mM Tris/HCl, pH 8. Dephosphorylation was maintained for 25 min at room temperature with continuous rotation, and the sample was extracted from the affinity matrix in 20 mM Tris/HCl buffer, pH 6.8, containing 10% SDS and 2.3% dithiothreitol. Two-dimensional PAGE was carried out by the method of O'Farrell et al. (1977), using LKB Ampholines of pH 3–10.

Dephosphorylation of purified PtdIns 3-kinase complex

The effect of dephosphorylation on PtdIns 3-kinase activity of the complex was investigated by using two different phosphatases: pure pig lung phosphotyrosine phosphatase (67E) (Vicendo et al., 1991) and alkaline phosphatase from calf intestine (Boehringer Mannheim, 713 023).

In a first experiment PtdIns 3-kinase complex was immobilized on to phosphopeptide affinity beads and divided into four tubes. Sample 1 was dephosphorylated with 38 units of the specific phosphotyrosine phosphatase from pig lung (1 unit = 1 nmol of *p*-nitrophenol phosphate hydrolysed/min per mg). Sample 2 was first phosphorylated with 59 units of purified EGF-receptor tyrosine kinase [1 unit = 1 pmol of ATP incorporated into poly-(Glu₆Ala₃Tyr)/min], intensively washed for the removal of the receptor and then dephosphorylated as for sample 1. PtdIns 3-kinase activity was measured after each phosphorylation/dephosphorylation treatment and three washes of the bead pellet with the PtdIns 3-kinase assay buffer described above. After these procedures, proteins immobilized on the beads in controls and samples were extracted with Laemmli sample buffer and used for SDS/PAGE analysis.

The time course of dephosphorylation of the affinity-purified PtdIns 3-kinase complex by the phosphotyrosine phosphatase was studied by monitoring in parallel the loss of activity of PtdIns 3-kinase. In this experiment active PtdIns 3-kinase complex was immobilized on phosphopeptide affinity beads, washed with buffer B, resuspended in 70 mM Hepes buffer (pH 7)/0.1 mM dithiothreitol and divided into two Eppendorf tubes. One of the samples was incubated with 19 units of the pig lung phosphotyrosine phosphatase for 15 min at room temperature with rotation, and the other was used as a control being maintained in buffer under the same conditions. After this period tubes were centrifuged for a few seconds, and supernatants were discarded. Pellets were washed with 3 × 1 ml of PtdIns 3-kinase assay buffer, and PtdIns 3-kinase activity in both tubes was measured by the standard assay procedure. Pellets were washed three times in Hepes buffer, and the sample was incubated with 19 units of fresh phosphotyrosine phosphatase for 30 min. The same procedures of washing and measurement of PtdIns 3-kinase activity were used. The third dephosphorylation of PtdIns 3-kinase was kept overnight.

Dephosphorylation by the non-specific protein phosphatase was carried out with alkaline phosphatase from calf intestine (Boehringer Mannheim, 713 023). Affinity-purified PtdIns 3-kinase complex was immobilized on phosphopeptide beads, and incubation with 10 units of alkaline phosphatase was maintained for 10, 20 and 60 min at room temperature. Non-immobilized PtdIns 3-kinase was also dephosphorylated with this alkaline phosphatase, and PtdIns 3-kinase activity was measured after 10 and 20 min of dephosphorylation. These experiments were carried out with independent batches of enzyme incubated for different periods of time.

Binding to the phosphopeptide matrix after dephosphorylation of the PtdIns 3-kinase complex was investigated by using

insoluble alkaline phosphatase from calf intestine (Sigma, P0762). PtdIns 3-kinase was incubated with 10 units of insoluble alkaline phosphatase for 20 min in a medium containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 10 mM Tris, pH 8. The control sample was incubated under the same conditions, but Sepharose beads were added instead of the insoluble alkaline phosphatase. The latter was removed by centrifugation, and PtdIns 3-kinase complex was incubated for 2 h at 4°C with phosphopeptide affinity beads in buffer B. Pellets were washed with assay buffer, and PtdIns 3-kinase activity was measured by standard procedures. Proteins bound to the phosphopeptide affinity beads in the control and the dephosphorylated sample were extracted for SDS/PAGE analysis.

RESULTS

85 and 110 kDa proteins in the PtdIns 3-kinase complex

After Q-Sepharose chromatography two peaks of PtdIns 3-kinase activity were eluted, one at 0.07 M and one at 0.15 M NaCl, named peaks 1 and 2 respectively (Figure 1). Both peaks underwent the same further steps of purification. SDS/PAGE analysis revealed that peak 2 contained many more proteins associated with the PtdIns 3-kinase activity (Figure 1b). Ultimate purification of PtdIns 3-kinase (Table 1) was achieved by using

Figure 1 Q-Sepharose chromatography

The dialysed pool from the phosphocellulose column was applied on to a 50 ml Pharmacia Q-Sepharose column, which was eluted with a 250 ml linear gradient of 0–0.2 M NaCl in buffer A. Two peaks of activity appeared, at 0.07 M and 0.15 M NaCl, respectively named peaks 1 and 2 (a). (b) shows a Coomassie-blue stained SDS/PAGE gel, with positions of molecular-mass markers (kDa) shown to the left.

Table 1 Purification of PtdIns 3-kinase

The procedure followed for purification of bovine brain cytosolic PtdIns 3-kinase is detailed in the Experimental section. The Table is derived from a single purification of peak 1 of PtdIns 3-kinase activity obtained after Q-Sepharose chromatography and is representative of four separate preparations by the same protocol. Protein was determined by the method of Bradford (1976). PtdIns kinase activity was determined with pure sonicated PtdIns as substrate in the medium described in the Experimental section.

	Recovery (%)	Enrichment (fold)	Protein
(NH ₄) ₂ SO ₄ precipitation	30	18	5 g
DEAE-cellulose chromatography	24	29	2.5 g
Phosphocellulose	14.4	414	104 mg
Q-Sepharose	2.0	730	8.2 mg
Mono Q (ATP/MgCl ₂)	1.7	1700	3.0 mg
Affinity chromatography	0.7	10400	0.2 mg

Figure 2 Affinity-purified PtdIns 3-kinase (peak 1)

(a) Silver-stained gel after SDS/PAGE. Lanes: 1, empty lane loaded with SDS sample buffer only; 2, proteins non-bound to the affinity beads; 3, proteins bound to the phosphopeptide affinity beads; 4, sample before affinity chromatography. Positions of molecular-mass markers (kDa) are shown to the left of the Figure. (b) Kinetics of PtdIns 3-kinase bound to the affinity beads (immobilized enzyme) (○) and of PtdIns 3-kinase before affinity purification (●). Each point is the mean of two values (relative S.E.M. < 10%). These kinetics are representative of all the experiments performed (> 3). PtdIns 3-kinase activity of immobilized enzyme was measured in 50 µl samples taken out of the reaction mixture at different times. PtdIns 3-kinase activity before affinity purification was measured with 50 µl of reaction mixture with independent batches of enzyme at different times.

as a final step affinity chromatography on a phosphopeptide which was constructed by using the 17 amino acid residues around tyrosine-751 in the kinase insert domain of the

Figure 3 Binding of PtdIns 3-kinase to substrate vesicles

Overlaid sensorgram showing the binding of purified PtdIns 3-kinase (PI3K) complex to phosphoinositides. The complex (900 RU) was bound to the U10 monoclonal antibody against the bcr-related domain of p85α (P. End, unpublished work), which in turn was bound to RamFc (rat anti-mouse polyclonal antibody). The applied lipid vesicles were prepared as described in the Experimental section.

human PDGF-β receptor (Fry et al., 1992). Phosphorylation of this residue was shown to mediate the association of PtdIns 3-kinase activity with this receptor. All 100% of the PtdIns 3-kinase activity applied to these affinity beads was strongly bound to them, and SDS/PAGE analysis of the bound proteins associated with the activity revealed two bands of 85 and 110 kDa in either peak 1 or 2 (Figure 2a), and partial amino acid sequences of these two bands present in both peaks were consistent with only one form of p85 and one form of p110 (J. Hsuan, personal communication). All further experiments described here were performed with the cleaner peak 1 of PtdIns 3-kinase activity.

The complex formed by both 85 and 110 kDa proteins and the phosphopeptide bound to the matrix was extremely stable and could not be dissociated by solutions containing 2 M NaCl, 2 M urea, 0.5% Triton X-100, 0.2% SDS or 50 mM phosphotyrosine. The complex bound to the solid matrix remained fully active (Figure 2b) and could be used as a bioreactor of immobilized enzyme to produce large amounts of lipid product without dissociation of any of the subunits of the complex. Around 40% of the complex could be eluted from the phosphopeptide matrix with the solution of glycerol/cholate described in the Experimental section. A urea gradient gel (0–8 M urea) of the eluted PtdIns 3-kinase complex showed dissociation of both 85 and 110 kDa proteins at a concentration around 2.5 M urea (results not shown).

Characteristics of the enzyme

It had previously been reported that the three phosphoinositides PtdIns, PtdIns4P and PtdIns(4,5)P₂ can be phosphorylated in the 3-hydroxy position by this kinase activity, showing the highest affinity towards PtdIns(4,5)P₂ and decreasing its affinity for the substrate with the lower polarity of the lipid (Morgan et al., 1990). The lipid products of the reaction with affinity-purified PtdIns 3-kinase complex were analysed by deacylation and h.p.l.c. analysis and comparison with internal standards as described previously (Morgan et al., 1990), confirming that the lipid products of the reaction are PtdIns3P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃, respectively. Binding of PtdIns 3-kinase complex to homogeneous lipid vesicles containing

Figure 4 Western-blot analysis

Polyclonal antibodies raised against the cloned proteins p85 α and p85 β (Otsu et al., 1991) were affinity-purified and used in Western-blot analysis at a concentration of 1:500. Purified baculovirus-expressed p85 α and p85 β were controls in lanes 1 and 2. PtdIns 3-kinase (PI3K) activity of the sample in lane 3 was 5 times that in lane 4. (a) Electroblot probed with anti-p85 α antibody, showing that the p85 protein present in the PtdIns 3-kinase complex from bovine brain is the α -form (> 98%). (b) Electroblot probed with anti-p85 β antibody. Positions of coloured molecular-mass markers (kDa) are indicated to the right of each panel.

Figure 5 Western analysis with a ^{32}P -labelled phosphopeptide probe

Radiolabelled phosphorylated peptide containing the sequence around tyrosine-751 of the human PDGF- β receptor was prepared by using [^{32}P]ATP and following the method described by Fry et al. (1992). This peptide was used as the probe in Western-blot analysis. Affinity-purified bovine brain PtdIns 3-kinase was transferred to a PVDF membrane, which was treated by the renaturing method of Ferrell and Martin (1989). Autoradiography showed the binding of the peptide to the 85 kDa subunit of the complex. Positions of molecular-mass markers (kDa) are shown to the left of the figure.

PtdIns, PtdIns4P or PtdIns(4,5) P_2 , prepared as described above, was monitored with BIAcore. The sensorgrams obtained (Figure 3) revealed that the affinity of the enzyme for the lipid vesicle is

Figure 6 [^{14}C]FSBA labelling of purified PtdIns 3-kinase

Purified bovine brain PtdIns 3-kinase was immobilized on affinity phosphopeptide beads. The incubation medium used for labelling was 30 mM Tris/HCl, pH 7.5, 1% BSA, 10 mM MgCl_2 and 0.5% sodium cholate; 2 μCi of [^{14}C]FSBA (NEN) was dissolved in 100 μl of incubation medium (final concn. 0.42 mM). Autoradiography shows that the ATP analogue intensely labels the 110 kDa subunit. The relative molar labelling of p110:p85 was 7:1. Positions of molecular-mass markers (kDa) are shown to the right of the Figure.

Figure 7 Two-dimensional gel SDS/PAGE: silver-stained gels

(a) Affinity-purified bovine brain PtdIns 3-kinase. (b) The enzyme after dephosphorylation with 0.2 unit of alkaline phosphatase (Boehringer Mannheim 713 023).

higher when PtdIns(4,5) P_2 is present as a constituent of the vesicle, and the binding decreased consecutively with PtdIns4P and PtdIns. The presence of sodium cholate enhanced the binding to the enzyme (sensorgram not shown), which is in agreement with previously reported faster kinetics of the enzyme in the presence of this detergent (Morgan et al., 1990).

Figure 8 Dephosphorylation of bovine brain PtdIns 3-kinase

Purified bovine brain PtdIns 3-kinase was immobilized on affinity phosphopeptide beads and dephosphorylated with pure phosphotyrosyl-protein phosphatase from pig lung (Vicendo et al., 1991). The same batch of immobilized enzyme was dephosphorylated at different times and assayed for PtdIns 3-kinase activity as described in the Experimental section. Symbols: □, control; ◆, dephosphorylated.

α form of the p85 subunit

Both peaks of PtdIns 3-kinase activity after the Mono Q step of purification were electroblotted on to a polyvinylidene difluoride (PVDF) membrane, and Western analysis was carried out as described in the Experimental section. Two different antibodies were used. Since two isoforms of p85, α and β , were revealed by cDNA cloning for bovine brain cDNA libraries (Otsu et al., 1991), specific antibodies against the C-terminal sequence of p85 α and against the recombinant p85 β were raised (Fry et al., 1992). Western analysis of both peaks of activity revealed that the p85 form present in both cases was the α form (Figure 4).

The p85 subunit is the anchor that mediates the binding of p110 to the PDGF receptor phosphopeptide

Affinity-purified PtdIns 3-kinase was transferred to a PVDF membrane and treated by the renaturing method of Ferrell and Martin (1989). The synthetic peptide containing the sequence around tyrosine-751 of the human PDGF- β receptor, which was used to prepare the affinity matrix, was labelled on its tyrosine residue with ³²P and used as the probe in Western analysis. The protein directly bound to the active peptide was the 85 kDa subunit (Figure 5). This result indicates that the 85 kDa subunit would play the role of locating the 110 kDa subunit in the correct position near the activated receptor.

Inhibition of PtdIns 3-kinase with FSBA: ATP-binding site of the p110 subunit

Purified bovine brain PtdIns 3-kinase was immobilized on to affinity phosphopeptide beads. A suspension of these beads in PBS was incubated overnight with different concentrations of the ATP analogue FSBA in PBS. After removing all non-bound FSBA and using different concentrations of PtdIns as substrate, FSBA inhibition was not complete even at concentrations of 10 mM. When 0.5% cholate and 10 mM MgCl₂ were included in the incubation medium with FSBA, total and irreversible inhibition of PtdIns 3-kinase was achieved in the range 400 μ M–10 mM FSBA. This result revealed that the catalytic site of

Figure 9 Dephosphorylation of PtdIns 3-kinase

Purified bovine brain PtdIns 3-kinase was immobilized on affinity phosphopeptide beads. Sample 1 was incubated with 38 units of pure phosphotyrosyl-protein phosphatase (P-Tyr phosphatase) from pig lung (Vicendo et al., 1991) for 30 min at room temperature. Sample 2 was incubated with 50 units of purified EGF receptor (Tyr kinase) in a medium containing 20 μ M ATP, 10 mM MnCl₂ and 100 mM sodium orthovanadate at pH 8 and later dephosphorylated as sample 1. (a) PtdIns 3-kinase activity. (b) Coomassie-Blue-stained SDS/PAGE gel. PtdIns 3-kinase activity was measured in the same batch of immobilized enzyme after each phosphorylation/dephosphorylation treatment as described in the Experimental section. Positions of molecular-mass markers (kDa) are indicated to the right of panel (b).

PtdIns 3-kinase is not directly accessible to the substrate, and a conformational change, which may be promoted by bivalent cations (Mg²⁺) and the structure of the lipid vesicle (presence of the detergent), is necessary to convert PtdIns 3-kinase into its active conformation. Using these optimal conditions for irreversible inhibition 2 μ Ci of [¹⁴C]FSBA was used to label the ATP-binding site, and SDS/PAGE analysis of the proteins showed the labelling of the 110 kDa subunit (Figure 6). Quantification of the electroblotted protein bands by amino acid composition analysis showed a ratio of 1 mol of p110/1 mol of FSBA bound to this subunit, whereas the molar ratio of radiolabelled p110 to radiolabelled p85 was 7:1.

Figure 10 Dephosphorylation of PtdIns 3-kinase

Purified bovine brain PtdIns 3-kinase was dephosphorylated with 10 units of alkaline phosphatase from calf intestine at room temperature. Sample 1 was immobilized on phosphopeptide affinity beads before undergoing dephosphorylation. Sample 2 was not immobilized on the solid matrix. (a) PtdIns 3-kinase activity measured in independent batches of enzyme dephosphorylated with alkaline phosphatase for different periods of time. Lanes: 1, 2 and 3, sample 1 dephosphorylated for different periods of time (10, 20 and 60 min respectively); 4 and 5, control non-dephosphorylated PtdIns 3-kinase (0 and 60 min incubation). (b) Silver-stained SDS/PAGE gel. Positions of molecular-mass markers (kDa) are indicated to the left of panel (b).

Figure 11 Binding of PtdIns 3-kinase to affinity beads

Bovine brain PtdIns 3-kinase was dephosphorylated with 10 units of insoluble alkaline phosphatase from calf intestine (Sigma) and bound to the phosphopeptide affinity beads. (a) Silver-stained SDS/PAGE gel with proteins extracted from affinity beads. Positions of molecular-mass markers are indicated to the left. (b) T.l.c. of lipid products showing PtdIns 3-kinase activity bound to affinity beads in control (C) and dephosphorylated (D) samples.

Dephosphorylation of the PtdIns 3-kinase complex

Two-dimensional PAGE of the proteins bound to the affinity matrix showed up to 10 forms of the p85 subunit and no focusing of the p110 subunit. When the complex underwent intensive dephosphorylation with alkaline phosphatase, the number of 85 kDa forms decreased significantly and p110 focused around pH 6 (Figure 7), showing that phosphorylation is an important covalent modification of both 85 kDa and 110 kDa proteins. Dephosphorylation of the affinity-purified complex with the specific phosphotyrosyl-protein phosphatase purified from pig lung resulted in a 45%-active PtdIns 3-kinase (Figures 8 and 9), and a similar result was obtained when a non-specific alkaline phosphatase from calf intestine was used (Figure 10). On the other hand, treatment of the PtdIns 3-kinase active complex with purified EGF receptor kinase had no effect on the activity of the complex (Figure 9). The loss of activity due to dephosphorylation could be reversed by rephosphorylation of the immobilized complex with purified EGF-receptor kinase (170 kDa). Nevertheless, when the active kinase domain of the EGF receptor, an active fragment of 39 kDa, was used to rephosphorylate the deactivated complex, no PtdIns 3-kinase activity could be recovered. The stability of the p85/p110 complex was extremely high, and no release of any of the subunits was detected after phosphorylation/dephosphorylation treatments, as shown by SDS/PAGE analysis in Figures 9 and 10(b).

Regarding binding capacity of the PtdIns 3-kinase complex to phosphopeptide affinity beads, dephosphorylation of the complex with alkaline phosphatase, although decreasing to 45% PtdIns 3-kinase activity (Figure 11b), did not inhibit its binding to the phosphopeptide matrix as shown in Figure 11(a). The same absorbance at 633 nm in a laser densitometer was detected in the 110 and 85 kDa protein bands of control and dephosphorylated samples after SDS/PAGE analysis.

DISCUSSION

PtdIns 3-kinase activity has been associated with two different proteins of 110 kDa and 85 kDa in preparations of bovine brain (Morgan et al., 1990), rat liver (Carpenter et al., 1990) and bovine thymus (Shibasaki et al., 1991), but in no case was it possible to identify the catalytic subunit as the isolated heterodimer. In this work we identify the 110 kDa subunit of the one containing the ATP-binding site and conferring PtdIns 3-kinase activity to the complex. When this ATP-binding site was blocked by covalent binding to the ATP analogue FSBA, the catalytic activity of the complex was irreversibly lost. By using the purification procedure described here, the partial amino acid sequence of p85 had been obtained and used for screening of bovine brain libraries and cloning of this 85 kDa protein (Otsu et al., 1991). Two similar 85 kDa proteins were cloned, named α and β , but showed no PtdIns 3-kinase catalytic activity when expressed in different eukaryotic systems (Otsu et al., 1991). Of both cloned forms, only the α form is present in the preparation that we purify from bovine brain cytosol. The tissue specificities and receptor-binding selectivities for the two cloned forms of p85 remain to be investigated. The two recombinant p85 proteins were shown to bind a wide variety of activated tyrosine kinase receptors and became highly phosphorylated by these receptors on their tyrosine residues (Gout et al., 1992). In those studies we found also that none of the ultimately purified p85 α and p85 β forms show any PtdIns kinase activity. In this work we find that, when constituting the PtdIns 3-kinase complex, p85 α functions as an anchor with the ability to bind the activated receptor

peptide and thus locates p110 in the correct position near the receptor.

The active PtdIns 3-kinase complex that we purify from bovine brain is highly phosphorylated on both p85 α and p110 subunits, and isoelectric focusing of the proteins in two-dimensional electrophoresis was achieved only after intensive dephosphorylation of the complex with alkaline phosphatase (Figure 7). Dephosphorylation of the purified complex with pure and specific phosphotyrosyl-protein phosphatase from pig lung (Vicendo et al., 1991) produced a 45%-active complex (Figure 8), and the same result was obtained by dephosphorylation with pure alkaline phosphatase from calf intestine (Figure 10). After dephosphorylation, the complex p85-p110 bound to the PDGF-receptor phosphopeptide was still tightly bound together, without release of its subunits. Moreover, dephosphorylation of the complex does not decrease its ability to bind the receptor phosphopeptide. The deactivation of PtdIns 3-kinase after phosphatase treatment indicates that phosphorylation is an important covalent modification of both p85 and p110 proteins and is responsible for maintaining the complex in its active conformation. For other enzymes involved in the metabolism of inositides, such as PLC or Ins(1,4,5) P_3 3-kinase, phosphorylation has been shown to be an important mechanism of regulation. It has been reported that phosphorylation of PLC γ increases PLC activity (Nishibe et al., 1990) and that phosphorylation on tyrosine-783 and -1254 of PLC γ is essential for PDGF-dependent activation of PLC (Kim et al., 1991) and T-cell-antigen receptor stimulation in T-cells (Park et al., 1991). With Ins(1,4,5) P_3 3-kinase, phosphorylation on serine-109 by cyclic-AMP-dependent kinase increases the enzyme's activity, whereas protein kinase C-dependent phosphorylation decreases its V_{max} . (Sim et al., 1990).

The purification procedure shown in the Experimental section was used to obtain a partial amino acid sequence of the 110 kDa subunit and, by using the same cloning strategy as for the p85 protein, p110 has been cloned and expressed in different eukaryotic systems (Hiles et al., 1992). The 110 kDa recombinant protein is active when expressed in insect cells, although no consensus sequence for ATP- or inositol-binding domains was found in its sequence. We are currently carrying out further studies to characterize this new ATP-binding and catalytic site by covalent binding of the active amino acid residues (basic lysine groups are the best candidates for this irreversible inhibition) to the radiolabelled ATP analogue FSBA that we have used in this study for identifying the catalytic subunit. Further studies on the regulation of the PtdIns 3-kinase complex *in vivo* by phosphorylation will provide more information to determine which amino acid residues are involved in its regulation.

We thank Dr. Iciar Martinez for performing the two-dimensional electrophoresis analysis of PtdIns 3-kinase complex and Dr. Anthony D. Smith for the h.p.l.c. analysis of the lipid products of PtdIns 3-kinase activity.

REFERENCES

- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) *Cell* **64**, 281–302
- Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S. and Cantley, L. C. (1990) *J. Biol. Chem.* **265**, 19704–19711
- Fägerstam, L. (1991) A non-label technology for real time biospecific interaction analysis: *Techniques in Protein Chemistry II*, pp. 65–71, Academic Press, New York
- Ferrell, J. E., Jr. and Martin, G. S. (1989) *J. Biol. Chem.* **264**, 20723–20729
- Fry, M., Panayotou, G., Dhand, R., Ruiz-Larrea, F., Gout, I., Nguyen, O., Courtneidge, S. A. and Waterfield, M. D. (1992) *Biochem. J.* **288**, 383–393
- Gout, I., Dhand, R., Panayotou, G., Fry, M. J., Hiles, I., Otsu, M. and Waterfield, M. D. (1992) *Biochem. J.* **288**, 395–405
- Hiles, I. D., Otsu, M., Volinia, S., Fry, F., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hsuan, J. J., Courtneidge, S. A., Parker, P. J. and Waterfield, M. D. (1992) *Cell* **70**, 419–429
- Huang, R., Sorisky, A., Church, W. R., Simons, E. R. and Rittenhouse, S. E. (1991) *J. Biol. Chem.* **266**, 18435–18438
- Jönsson, U. and Malmqvist, M. (1992) *Adv. Biosensors* **2**, 291–336
- Jönsson, U., Fägerstam, L., Ross, H., Rönnberg, J., Sjölander, S., Stenberg, E., Stahlberg, R., Urbaniczky, C., Östlin, H. and Malmqvist, M. (1991) *Biotechniques* **11**, 520–527
- Kamps, M. P. and Sefton, B. M. (1988) *Oncogene* **2**, 305–315
- Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, C. K., Schlessinger, J. and Rhee, S. G. (1991) *Cell* **65**, 435–441
- Löffås, S. and Johnson, B. (1990) *J. Chem. Soc. Chem. Commun.* **21**, 1526–1528
- Macara, I. G., Marinetti, G. V. and Balduzzi, P. C. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2728–2732
- Morgan, S. J., Smith, A. D. and Parker, P. J. (1990) *Eur. J. Biochem.* **191**, 761–767
- Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M., Tonks, N. K., Rhee, S. G. and Carpenter, G. (1990) *Science* **250**, 1253–1256
- O'Farrell, P. Z., Goodman, H. M. and O'Farrell, P. H. (1977) *Cell* **12**, 1133–1141
- Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J. and Waterfield, M. D. (1991) *Cell* **65**, 91–104
- Park, D. J., Rho, H. W. and Rhee, S. G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5453–5456
- Serunian, L. A., Haber, M. T., Fukui, T., Kim, J. W., Rhee, S. G., Lowenstein, J. M. and Cantley, L. C. (1989) *J. Biol. Chem.* **264**, 17809–17815
- Shibasaki, F., Homma, Y. and Takenawa, T. (1991) *J. Biol. Chem.* **266**, 8108–8114
- Sim, S. S., Kim, J. W. and Rhee, S. G. (1990) *J. Biol. Chem.* **265**, 10367–10372
- Stephens, L. R., Hughes, K. T. and Irving, R. F. (1991) *Nature (London)* **351**, 33–39
- Sugimoto, Y., Whitman, M., Cantley, L. C. and Erikson, R. L. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2117–2121
- Talmage, D. A., Freund, R., Young, A. T., Dahl, J., Dawe, C. J. and Benjamin, T. L. (1989) *Cell* **59**, 55–65
- Ullrich, A. and Schlessinger, J. (1990) *Cell* **61**, 203–212
- Vicendo, P., Fauvel, J., Ragab-Thomas, J. M. F. and Chap, H. (1991) *Biochem. J.* **278**, 435–440
- Whitman, M., Kaplan, D. R., Schaffhausen, B., Cantley, L. and Roberts, T. M. (1985) *Nature (London)* **315**, 239–242
- Whitman, M., Downes, C. P., Keeler, M., Keller, T. and Cantley, L. (1987) *Biochem. J.* **247**, 165–174