Purification and characterization of a phosphatidylinositol 3-kinase complex from bovine brain by using phosphopeptide affinity columns

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Specific phosphorylated tyrosine residues in the kinase insert region of the human platelet-derived-growth-factor β -receptor mediate the formation of multienzyme complexes with this receptor. When phosphorylated, tyrosine residue 751 within the kinase insert region mediates binding of PtdIns 3-kinase to this receptor. A 17-amino-acid peptide containing this tyrosine residue was synthesized, phosphorylated by using epidermal-growth-factor receptor and then coupled to an Actigel matrix. The tyrosine-751 phosphopeptide column is used here as a final affinity step in the purification of the PtdIns 3-kinase from bovine brain to apparent homogeneity. The active resin-bound PtdIns 3-kinase is composed of two polypeptides, p110 and p85, which are elutable with SDS-containing buffers and detectable by silver staining of polyacrylamide gels. The 85 kDa protein is shown to be identical with the recently cloned p85 α . Phosphotyrosine is demonstrated to be an essential part of the structure required for binding of both of these proteins and PtdIns 3-kinase activity to this peptide. The active PtdIns 3-kinase complex from bovine brain, but not recombinant p85 subunits, shows specificity for binding to phosphopeptides containing a YXXM consensus sequence. Neither PtdIns 3-kinase activity, nor the complex of p85 and 110 kDa proteins, binds to several other phosphopeptide affinity columns lacking this sequence motif. The selectivity of binding of baculovirus-expressed free p85 α subunit of bovine brain PtdIns 3-kinase, the closely related protein p85 β and purified bovine brain PtdIns 3-kinase to these and other phosphopeptide columns is examined.

INTRODUCTION

A subset of polypeptide growth-factor receptors belong to the family of protein-tyrosine kinases. Activation of these receptors following ligand binding involves autophosphorylation of the receptor as well as phosphorylation of a number of intracellular substrate proteins (reviewed in Ullrich & Schlessinger, 1990). The importance of receptor autophosphorylation had been unclear until recently, when evidence from several laboratories suggested that this event may mediate the formation of complexes between receptor proteins and putative growth-regulatory proteins such as phospholipase C γ (PLC γ) (Meisenhelder *et al.*, 1989), PtdIns 3-kinase (Coughlin *et al.*, 1989), GTPase-activating protein (GAP) (Kaplan *et al.*, 1990), the serine/threonine kinase Raf (Morrison *et al.*, 1989) and members of the *src* family of protein-tyrosine kinases (Kypta *et al.*, 1990) (reviewed in Cantley *et al.*, 1991).

The association of the PtdIns kinase activity with activated receptors is of particular interest, since increased turnover of PtdIns and its phosphorylated derivatives has been implicated in the action of hormones, growth factors and transformation of cells by DNA and RNA viruses (reviewed by Whitman & Cantley, 1988; Carpenter & Cantley, 1990). Several species of PtdIns kinase are known to exist, but none of these enzymes have been characterized by cloning and expression. Fibroblasts contain at least two PtdIns kinase activities, which are distinguishable on the basis of their detergent sensitivity and kinetic properties (Whitman *et al.*, 1987). These two activities were classified as

Type I (inhibited by non-ionic detergents) and Type II (stimulated by non-ionic detergents and inhibited by adenosine). A third distinct species (Type III) has been identified in bovine brain, but remains poorly characterized (Enderman *et al.*, 1987). One species of PtdIns kinase activity in particular has become of major interest in the search for second-messenger systems linked to protein-tyrosine kinases, because this activity was shown to co-immunoprecipitate with activated platelet-derived growth factor (PDGF) receptors (Kaplan *et al.*, 1987; Coughlin *et al.*, 1989) and with the polyoma middle-T antigen/pp60^{c-src} (mT:pp60^{c-src}) complex (Whitman *et al.*, 1985). This activity has been shown to be due to a Type I PtdIns kinase which produces novel inositol lipids phosphorylated at the D-3 position of the inositol ring (Whitman *et al.*, 1988). More recently this enzyme has also been shown to associate with the colony-stimulating-factor-1 receptor (Varticovski *et al.*, 1989), *c-kit* receptor (Lev *et al.*, 1991), the epidermal growth factor (EGF) receptor (Bjorge *et al.*, 1990), the PDGF α -receptor (Yu *et al.*, 1991), the insulin receptor (Ruderman *et al.*, 1990), the hepatocyte growth-factor receptor, Met (Graziani *et al.*, 1991), and with activated non-receptor protein-tyrosine kinases (Fukui & Hanafusa, 1989; Chan *et al.*, 1990; Varticovski *et al.*, 1991).

PtdIns 3-kinase activity has been closely linked to the presence of 81/85 kDa proteins in these immunoprecipitates which can be phosphorylated on tyrosine residues by the associated protein-tyrosine kinase both *in vitro* and *in vivo* (Kaplan *et al.*, 1987; Courtneidge & Heber, 1987; Cohen *et al.*, 1990). Recently a 650-fold purification of PtdIns 3-kinase from bovine brain was

Abbreviations used: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PLC, phospholipase C; GAP, GTPase-activating protein.

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described which, among other proteins present in the purest preparation, contained an 85 kDa protein which was shown to be a substrate *in vitro* for the PDGF and EGF receptors (Morgan *et al.*, 1990). Using sequence information from tryptic peptides derived from this protein we have recently cloned two homologous bovine p85 proteins, denoted p85 α and p85 β (Otsu *et al.*, 1991). Two other groups have independently cloned murine and human p85 α homologues, using different strategies (Escobedo *et al.*, 1991b; Skolnik *et al.*, 1991). Both of these p85 proteins can be demonstrated to bind directly to phosphorylated PDGF receptor *in vitro* (Otsu *et al.*, 1991; Escobedo *et al.*, 1991b). These proteins may function as the receptor-binding subunits of the PtdIns 3-kinase, since neither of them can be shown to encode intrinsic PtdIns 3-kinase activity when expressed in a variety of cell systems (Otsu *et al.*, 1991; Escobedo *et al.*, 1991b). Immunoprecipitation of ¹²⁵I-labelled bovine brain PtdIns 3-kinase with antibodies raised against p85 proteins precipitated an 85 kDa protein together with a second protein of molecular mass 110 kDa (Otsu *et al.*, 1991). PtdIns 3-kinase has recently been purified from rat brain and bovine thymus, and in both cases PtdIns 3-kinase activity was associated with the presence of 110 kDa proteins, but not always 85 kDa proteins, strongly suggesting that the 110 kDa component is the catalytic subunit of the active PtdIns 3-kinase complex (Carpenter *et al.*, 1990; Shibasaki *et al.*, 1991).

PtdIns 3-kinase is one of a growing number of potential signalling proteins which associate with protein-tyrosine kinases activated either by ligand stimulation or as a consequence of cell transformation. A common feature of all these proteins (apart from Raf) is that they contain one or more SH2 domains (*src* homology) (Koch *et al.*, 1991). Both p85 α and p85 β proteins contain two SH2 domains. Experiments from a number of laboratories have suggested that these domains may function by binding to peptide sequences phosphorylated on tyrosine residues and thus mediate the complex formation which follows activation of protein-tyrosine kinases (Anderson *et al.*, 1990; Mayer & Hanafusa, 1990; Moran *et al.*, 1990; Matsuda *et al.*, 1991; Mayer *et al.*, 1991; reviewed by Koch *et al.*, 1991). In support of this, several studies suggest that tyrosine phosphorylation of the PDGF receptor or polyoma mT is essential for its association with proteins such as the PtdIns 3-kinase (Kazlauskas & Cooper, 1989; Talmage *et al.*, 1989), GAP (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990) and PLC γ (Anderson *et al.*, 1990; Margolis *et al.*, 1990). The precise tyrosine residue required for binding of the PtdIns 3-kinase activity (and an 85 kDa phosphoprotein) to the human PDGF receptor has been mapped to tyrosine-751 (Y751), which lies within the kinase insert region of the protein-tyrosine kinase domain (Kazlauskas & Cooper, 1989, 1990; Kazlauskas *et al.*, 1991). The binding sites for other proteins to this receptor (e.g. PLC γ , GAP and *src*-family kinases) have yet to be mapped, but these proteins probably associate via other phosphorylated tyrosine residues.

To define more clearly the polypeptides involved in the active bovine brain PtdIns 3-kinase complex, we have synthesized peptides from the human PDGF β -receptor containing either Y751 or Y857, the two major sites of receptor autophosphorylation. These peptides were phosphorylated *in vitro* by EGF receptor from A431 membranes, purified and then coupled to an Actigel matrix. The resulting phosphopeptide columns were then examined for their ability to act as affinity reagents to complete purification of the previously described partially purified bovine brain PtdIns 3-kinase (Morgan *et al.*, 1990). A peptide derived from the sequence around tyrosine-751 of the PDGF receptor has proved to be very efficient in binding the bovine brain PtdIns 3-kinase, making it possible to take the purification to apparent homogeneity. The ability of other SH2-

containing proteins, including GAP, PLC γ and pp60^{c-*src*}, to bind to these and other phosphorylated peptide columns has also been examined. The relevance of the findings to specificity of binding of different SH2-containing proteins is discussed.

MATERIALS AND METHODS

Cells

A431 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal-calf serum. Maintenance of insect cell culture and infection of *Spodoptera frugiperda* (Sf9) cells were carried out as described in Summers & Smith (1987).

Preparation of A431 membranes

This preparation is modified from that described by Thom *et al.* (1977). Harvesting solution [0.05 M-boric acid (pH 7.2)/0.15 M-NaCl], extraction solution [0.01 M-boric acid (pH 10.2)/0.2 mM-EDTA] and borate solution (0.5 M-boric acid, pH 10.2) were all prepared fresh. A431 cells (2.5×10^8) were washed once with ice-cold harvesting solution and then scraped into fresh harvesting solution. Cells were pelleted by low-speed centrifugation at 200 g for 5 min, and then resuspended by pipetting in 2 pellet vol. of harvesting solution. This was added slowly, with stirring, to 100 pellet vol. of extraction solution. After 10 min, 8 pellet vol. of borate solution was added and stirring continued for a further 5 min. This solution was filtered through nylon gauze (av. mesh size 900 μ m), and spun at 500 g for 10 min at 2 °C to pellet any nuclei/whole cells. Finally, the supernatant was centrifuged at 12000 g for 30 min in a ultracentrifuge SW28 rotor at 4 °C. The membrane pellet was resuspended in a minimum volume of 50 mM-Hepes (pH 7.5), diluted to a final protein concentration of ~2 mg/ml and stored at -70 °C.

Synthesis of peptides

Synthesis of the peptides described in Table 1 was performed on an Applied Biosystems 430A peptide synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and an appropriate amino acid addition program according to Applied Biosystems' recommendations. Peptides were then purified by preparative reverse-phase h.p.l.c. Composition of the peptides was checked by analytical h.p.l.c., amino acid analysis and protein sequencing on a 477A automated pulse-liquid sequencer.

Phosphorylation of peptides

Peptides were freeze-dried to dryness to remove any contaminating chemicals remaining from its synthesis/purification and then dissolved in h.p.l.c.-grade water at a concentration of ~4 mg/ml.

For small-scale phosphorylation, we used: 20 μ g of peptide, 10 μ l of 5 $\times$ kinase buffer [250 mM-Hepes (pH 7.4), 750 mM-NaCl, 0.1% Triton X-100, 10 mM-MnCl₂, 60 mM-MgCl₂, 50%

Table 1. Tyrosine-phosphorylation-site synthetic peptides

Peptide	Sequence
Y740	G E S D G G Y M D M S K
Y751	D M S K D E S V D Y V P M L D M K
Y751.S	C D E S V D Y V P M L
Y857	A R D I M R D S N Y I S K G S T F
Y1313	E F C P D P L Y E V M L K
Y527	R R F T S T E P Q Y Q P G E N L
Y416*	R R L I E D N E Y T A R G

* This peptide was purchased from Sigma Chemical Co. rather than synthesized.

glycerol, 500 mM-sodium orthovanadate], 5 μ l of A431 membrane preparation and ATP/[γ - 32 P]ATP (relative amounts depend on aim of phosphorylation). Water was added to adjust the volume to 50 μ l.

For preparative phosphorylation, 2–3 mg of peptide was dissolved in 1.5 ml of water and added to 450 μ l of 5 $\times$ kinase buffer. The pH was adjusted to 7.0. Then 250 μ l of 0.1 M-ATP and 500 μ l of A431 membranes were added, and the reaction was allowed to proceed for 18 h at room temperature with continual mixing.

Isolation of phosphorylated peptides by reverse-phase h.p.l.c.

A 1 ml portion of buffer A (buffer A: 0.08 % trifluoroacetic acid, 1 % acetonitrile in water; Buffer B: 0.08 % trifluoroacetic acid, 90 % acetonitrile) was added to the kinase reaction and mixed. This solution was then spun for 20 min at 10000 g to pellet the membranes. The supernatant containing the phosphopeptide was then loaded on to a Sep-Pak column (C_{18}) equilibrated with buffer A. The column was washed with 20 ml of buffer A to elute ATP and then the peptide was eluted with 3 $\times$ 1 ml of 3:2 mixture of buffers A and B. The A_{268} of the fractions was monitored, and fractions containing peptide were pooled and then freeze-dried to dryness (note that the phosphorylated Y751 peptide has essentially no absorption at 280 nm). The phosphopeptide was then separated from non-phosphorylated peptide in a 1090 h.p.l.c. system. For preparative separation a C_{18} column (Aquapore OD-300; 250 mm $\times$ 7 mm) equilibrated with 100 % buffer A [214 nm (sensitivity 50 mV)/280 nm (sensitivity 200 mV)] was used with a 2 ml/min flow rate. The peptide was dissolved in 200 μ l of h.p.l.c.-grade water and then loaded via a 500 μ l loop. The column was then washed for 10 min with 100 % buffer A before elution of the peptide and phosphopeptide with a 30 min linear gradient from 100 % buffer A to 60 % buffer A/40 % buffer B, followed by a 5 min linear gradient to 100 % buffer B. Peak fractions were collected manually. The pool fractions were diluted with water, freeze-dried and then stored at -20°C .

Phosphoamino acid analysis of phosphorylated peptides

Peptides phosphorylated in the presence of [γ - 32 P]ATP by using either purified EGF receptor or A431-cell membranes were purified by h.p.l.c. on a C_{18} Sep-Pak column as described above. This material was then hydrolysed at 110°C for 1 h in 1 ml of 6 M-HCl. Then 1 ml of h.p.l.c.-grade water was added and the sample was centrifuged at 10000 g for 10 min to remove debris. The remaining supernatant was frozen and freeze-dried to dryness. The pellet was resuspended in 2 ml of water, frozen and then freeze-dried once more. This material was analysed by two-dimensional thin-layer electrophoresis essentially as described by Cooper *et al.* (1983).

Coupling of peptides to Actigel resin

Peptides were coupled to the matrix essentially as described by the manufacturers. Briefly, 500 μ l (packed volume) of Actigel-ALD Superflow resin (Sterogene, Arcadia, CA, U.S.A.) was washed five times with 100 mM-phosphate buffer (pH 7.8) (coupling buffer). Phosphorylated or non-phosphorylated peptide (1 mg) was dissolved in 200 μ l of coupling buffer and added to the resin. NaCNBH₃ (coupling solution) was added to a final concentration of 100 mM, and this was then mixed at 4°C for 6 h. The resin was washed with 10 column vol. of 500 mM-NaCl and then incubated with 100 mM-Tris/HCl (pH 8.0) for 1 h in the presence of coupling solution to block any sites on the resin that had not reacted. The resin was washed with 500 mM-NaCl and finally with coupling buffer plus 500 μ M-vanadate and 0.02 % NaN₃ and then stored at 4°C . Phosphopeptides bound to the

Actigel matrix were stable for several months under these conditions.

Binding of proteins to the phosphopeptide columns

Proteins were diluted in binding buffer [50 mM-phosphate buffer (pH 7.2), 150 mM-NaCl, 0.02 % Triton X-100, 2 mM-EDTA and 200 μ M-sodium orthovanadate], mixed with the appropriate peptide affinity resin and then allowed to bind for 2 h at 4°C with rotation. The column material was washed repeatedly (> 6 times) with 50 column vol. of the same buffer and then with various elution buffers containing NaCl, urea or detergents as described in the text and legends. Bound proteins were either assayed for PtdIns 3-kinase activity or were removed from the column by boiling in SDS/PAGE sample buffer and then analysed by SDS/PAGE.

PtdIns 3-kinase assay

PtdIns 3-kinase assays were carried out essentially as described by Whitman *et al.* (1987) in 50 μ l containing 50 mM-Tris/HCl (pH 7.5), 50 mM-NaCl, 1 mM-dithiothreitol, 0.5 mM-EDTA, 5 mM-MgCl₂, 100 μ M-ATP (plus 0.5 μ Ci of [γ - 32 P]ATP/assay), 1 mM-PtdIns plus soluble or column-immobilized bovine brain PtdIns 3-kinase. Incubation was for 5 min at room temperature. The reaction was terminated by addition of 100 μ l of 0.1 M-HCl and 200 μ l of chloroform/methanol (1:1, v/v). The mixture was vortex-mixed and then centrifuged to separate the phases. The upper phase was discarded and the lower organic phase washed with 80 μ l of methanol/1 M-HCl (1:1, v/v). After centrifugation, the upper phase was again discarded and the lower phase evaporated to dryness. Reaction products were spotted on thin-layer silica-gel 60 plates [pretreated with 1 % oxalic acid/1 mM-EDTA in water/methanol (3:2, v/v)] and developed in chloroform/methanol/4 M-NH₃ (9:7:4, by vol.).

Preparation of C-terminal-specific antisera

C-Terminal-peptide-specific antisera were prepared against the bovine C-terminal sequences determined by cDNA cloning (Otsu *et al.*, 1991). The peptides TLAYPVYAQQRR for p85 α and TLAHPVRAPGPGPPAAR for p85 β were synthesized by FMOC chemistry and purified by h.p.l.c. The peptides were coupled by using glutaraldehyde to keyhole-limpet haemocyanin and then injected into the lymph nodes of rabbits by methods described in Kypta *et al.* (1988). Positive antisera as determined by enzyme-linked immunoassay were affinity-purified on specific peptide-Actigel affinity columns.

RESULTS

Preparation of Y751-phosphopeptide column

A 17-amino-acid peptide which contains Y751 of the human PDGF- β receptor was chosen for synthesis in an attempt to include all necessary sequence determinants following a survey of the known binding sites for the PtdIns 3-kinase (see Table 1; reviewed by Cantley *et al.*, 1991). In addition to the peptide context of Y751 of the PDGF β -receptor, the sequences around Y315 of polyoma middle T (Talmage *et al.*, 1989) and Y721 of the human colony-stimulating-factor-1 receptor (Shurtleff *et al.*, 1990) were also considered. With the phosphorylation protocol described here, greater than 50 % phosphorylation of the Y751 peptide was achieved by using either purified human EGF receptor or A431 membranes as a source of protein-tyrosine kinase. The phosphorylated Y751 peptide could be clearly identified during reverse-phase h.p.l.c. analysis, where it was eluted approx. 1 min earlier than the non-phosphorylated peptide, since it produced a strong 214 nm absorbance but little or no 280 nm signal (Fig. 1a). Analysis of the absorption properties

Fig. 1. Phosphorylation and purification of Y751 phosphopeptide

(a) H.p.l.c. profile for separation of the phosphorylated from the non-phosphorylated Y751 peptide on a C_{18} reverse-phase column. The trace shows the spectra monitored at 214 nm during the course of the elution. The peaks corresponding to the phosphorylated and non-phosphorylated peptide are indicated by arrows. The smaller peaks observed are derived from the A431 membranes. (b) Spectral analysis of the purified phosphorylated and non-phosphorylated Y751 peptides between 240 and 300 nm as measured by the diode-array detector. The absorption maximum for the peptide is observed to shift to a lower wavelength after tyrosine phosphorylation. (c) Phosphoamino acid analysis of Y751 peptide phosphorylated by either purified EGF receptor (left panel) or A431 cell membranes (right panel). After the phosphorylation reaction, the phosphopeptide was purified by reverse-phase h.p.l.c. as described above. The peptide was subjected to acid hydrolysis and the phosphoamino acids were separated by two-dimensional thin-layer electrophoresis. Internal standards were stained with ninhydrin, and the ^{32}P -labelled phosphoamino acids were detected by autoradiography. The positions of P_i and of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) standards are indicated.

showed that phosphorylation of the Y751 peptide led to a shift in the absorption maximum from 280 to 267 nm (Fig. 1b). For large-scale phosphorylations, A431 membranes were the preferred source of protein-tyrosine kinase activity, since they could be more easily generated. However, as the Y751 peptide contains two serine residues, as well as a single tyrosine residue, it was thought important to demonstrate that the peptide was phosphorylated exclusively at the tyrosine residue. This was established by two separate methodologies: analysis of h.p.l.c.-

Fig. 2. Purification of PtdIns 3-kinase complex on the Y751-phosphopeptide affinity column

Modified purification protocol for bovine brain PtdIns 3-kinase from that previously described by Morgan *et al.* (1990). (a) Peak 1 (P1) and peak 2 (P2) of PtdIns 3-kinase fractions from the second MonoQ step were analysed by SDS/PAGE on a 7.5% gel. Proteins in these two peak fractions were detected by silver staining. The migration positions of molecular-mass markers are indicated. (b) Affinity purification of peak 1 (P1) and peak 2 (P2) PtdIns 3-kinase using the Y751-phosphopeptide column. Silver stain after SDS/PAGE of a 7.5% gel showing PtdIns 3-kinase-associated proteins from MonoQ P1 and P2 which bound to, and were eluted from, the Y751-phosphopeptide column with 0.1% SDS-containing phosphate buffer at 80 °C. Lanes 1, 2 and 3 for both P1 and P2 material indicates the proteins eluted by successive 50 µl elutions.

purified phosphopeptide by phosphoamino acid analysis or by protein microsequencing.

Phosphoamino acid analysis of the Y751 peptide, phosphorylated by either purified EGF receptor or A431 membranes, demonstrated that phosphorylation of the Y751 peptide was occurring exclusively at the tyrosine residue (Fig. 1c). Sequence analysis of the phosphorylated and non-phosphorylated peptides also confirmed that both these peptides were 17 amino acids in length and that their sequences were identical except at cycle 10, where, as expected, no phenylthiohydantoin-tyrosine derivative was observed for the phosphorylated peptide, owing to its modification (results not shown).

Extended purification of bovine brain PtdIns 3-kinase by using a Y751 phosphopeptide affinity column

A 650-fold purification of PtdIns 3-kinase from bovine brain has recently been described (Morgan *et al.*, 1990). The material used here was purified by the same method, except that the gradient for the second MonoQ column was extended to give two distinct peaks containing PtdIns 3-kinase activity (Fig. 2a; S. J. Morgan, unpublished work). Both of these peaks (referred to hereafter as peak 1 and peak 2) contain no PtdIns kinase activity other than PtdIns 3-kinase activity, as determined by h.p.l.c. analysis of deacylated lipid product (results not shown). However, both of these fractions still contain greater than 20 major polypeptides detectable after SDS/PAGE analysis by silver staining (see Fig. 2a). The specific activity of peak-1 PtdIns 3-kinase was calculated as 500 nmol/min per mg of protein, and that of peak-2 PtdIns 3-kinase was 30 nmol/min per mg of protein.

The precise subunit composition of the active PtdIns 3-kinase complex is still a point of some contention, so an attempt was made to address this question by affinity-purifying the PtdIns 3-kinase activity from these two MonoQ pools. The bovine brain PtdIns 3-kinase preparation was diluted 10-fold in binding buffer and allowed to bind batchwise to the Y751-phosphopeptide

Fig. 3. Characterization of the binding of PtdIns 3-kinase activity to Y751-derived peptide columns

A 1 μ g portion of partially purified peak 1 bovine brain PtdIns 3-kinase was applied to 10 μ l of the Y751-derived peptide resins in 100 μ l of binding buffer and allowed to bind, and then the columns were washed extensively with binding buffer as described in the Materials and methods section. The washed column material was then assayed for bound PtdIns 3-kinase activity. Lanes: 1, PtdIns 3-kinase activity bound to non-phosphorylated Y751 column; 2, PtdIns 3-kinase activity bound to phosphorylated Y751 column; 3, PtdIns 3-kinase activity from the supernatant of the column in lane 2, which was able to bind to a fresh 10 μ l of phosphorylated Y751 column; 4, PtdIns 3-kinase activity that remains associated with the Y751 phosphopeptide column from lane 2 after elution of the column with 0.1% SDS at 80 $^{\circ}$ C; 5, PtdIns 3-kinase activity bound to recycled phosphorylated Y751 column as used in lane 2 (eluted as in lane 4 and then extensively washed with binding buffer) after addition of a fresh portion of bovine brain PtdIns 3-kinase in binding buffer; 6, 1 μ g of peak 1 bovine brain PtdIns 3-kinase activity assayed in solution to indicate total activity present. PI3P indicates the migration position of a PtdIns3P standard; Ori indicates the origin of the t.l.c. plate.

affinity resin for 4 h at 4 $^{\circ}$ C. After washing the column extensively with binding buffer, those proteins which remained bound were eluted with SDS-containing buffers and examined by SDS/PAGE. Two major polypeptide species, of approximate molecular masses 85 and 110 kDa (p85 and p110), which bound specifically to the phosphopeptide column, but not to an identical column prepared with unphosphorylated Y751 peptide, were identified in both MonoQ peaks and were observed to be quantitatively depleted from the bovine brain PtdIns 3-kinase preparation (Fig. 2b). Assaying the bound material, we were able to demonstrate that the presence of these two proteins appeared to be sufficient to generate full PtdIns 3-kinase activity (Fig. 3, lane 2). This Y751-phosphopeptide column routinely removed > 90% of the PtdIns 3-kinase activity present in the MonoQ peak-1 or -2 fractions purified from bovine brain (cf. Fig. 3, lanes 2 and 3) after a single incubation. Neither the 85 and 110 kDa proteins nor PtdIns 3-kinase activity bound to a column with an equivalent concentration of non-phosphorylated Y751 peptide (Fig. 3, lane 1) or to a column prepared with phosphotyramine, a phosphotyrosine analogue (results not shown). It should also be noted that binding of the PtdIns 3-kinase complex to the phosphopeptide column did not result in any apparent increase in the total enzyme activity present (Fig. 3, cf. lanes 2 and 6). In fact a slight decrease in activity was often observed, but this was judged to be due to the unstable nature of the highly purified enzyme, which was found to be inhibited by traces of metal ions and reversibly inhibited by oxidation (M. J. Fry, unpublished work). It is estimated that this affinity-purification step results in a 7000–8000-fold purification of PtdIns 3-kinase from bovine brain relative to the material loaded on the DEAE-cellulose (the overall purification achieved from tissue is in fact much greater).

Fig. 4. Identity of p85 species in peaks 1 and 2 of bovine brain PtdIns 3-kinase preparation

Protein samples were separated by SDS/PAGE on 7.5% gels and transferred to nitrocellulose. The blots were then probed with antisera raised against the C-terminal peptide sequences of p85 α or p85 β . (a) Western blot probed with anti-p85 α C-terminal antisera. Lanes: 1, 1 μ g of peak 1 bovine brain PtdIns 3-kinase; 2, 1 μ g of peak 2 bovine brain PtdIns 3-kinase; 3, Cos-1 cell lysate from pMT2 vector transfected cells; 4, Cos-1 cell lysate from pMT2p85 α -transfected cells; 5, Cos-1 cell lysate from pMT2p85 β -transfected cells; 6, Sf9-cell lysate containing p85 α ; 7, Sf9-cell lysate containing p85 β . (b) Western blot probed with anti-p85 β C-terminal antisera. Lanes are as described for panel (a). (c) Competition of peptides with antibodies in Western blots. Samples in lanes 1 and 2 were probed with p85 α -specific antiserum, and samples in lanes 3 and 4 were probed with the p85 β -specific antiserum. Lanes 1 and 2: Sf9-cell lysate containing baculovirus-expressed p85 α . Lanes 3 and 4: Sf9-cell lysate containing baculovirus-expressed p85 β . In the odd-numbered lanes the nitrocellulose was probed with specific antiserum alone. In the even-numbered lanes the antiserum was competed with 100 μ g of p85 α (lane 2) and p85 β (lane 4) specific C-terminal peptides respectively/ml. (d) Anti-p85 α Western blot of bound and soluble PtdIns 3-kinase material after chromatography using the Y751-phosphopeptide column. Samples (each 1 μ g of protein) from peaks 1 (P1) and 2 (P2) of bovine brain PtdIns 3-kinase were immobilized on the Y751-phosphopeptide column. Material which did not bind was collected, and then the resin was washed extensively. Bound proteins were eluted from the column with SDS/PAGE sample buffer. Bound and unbound proteins were separated by SDS/PAGE on a 7.5% gel and then transferred to nitrocellulose. The filter was then probed with anti-p85 α C-terminal antisera and blots were made visible with 125 I-Protein A–Sepharose. Lanes: 1, P1 bound material; 2, P1 material which did not bind to column; 3, P2 bound material; 4, P2 material which did not bind to column.

Elution of p85, p110 and PtdIns 3-kinase activity from the phosphopeptide column

Elution of the active PtdIns 3-kinase complex from the phosphopeptide column proved to be difficult to achieve, owing

Fig. 5. Binding of other SH2-domain-containing proteins to the Y751-phosphopeptide column

BSA and purified PLC γ diluted in lysis buffer or Sf9-cell lysates containing baculovirus-expressed p85 α protein, GAP or pp60^{c-src} were applied to 10 μ l of phosphorylated Y751- (even-numbered lanes) or non-phosphorylated Y751- (odd-numbered lanes) Actigel columns. Incubation with these resins was carried out for 4 h at 4 °C with constant mixing. After extensive washing with binding buffer, bound proteins were eluted from the columns with SDS/PAGE sample buffer at 80 °C for 5 min and then analysed on 7.5%-polyacrylamide gels. Bound proteins were detected by staining with Coomassie Blue. Lanes: 1 and 2, BSA; 3 and 4, GAP; 5 and 6, pp60^{c-src}; 7 and 8, p85 α ; 9 and 10, partially purified bovine brain PLC γ . The migration positions of molecular-mass markers are indicated.

to the high affinity of the interaction. Kazlauskas & Cooper (1990) have previously noted that the binding of cellular p85 proteins to phosphorylated PDGF receptor was stable to treatment with solutions containing ionic detergents, 2 M-NaCl, 1 M-urea or 0.2% SDS. The p85 subunits and PtdIns 3-kinase complex were also found to bind tightly to the Y751-phosphopeptide matrix and were likewise not eluted under any of the above conditions. At 20 °C the 85 and 110 kDa proteins remained bound in the presence of either 2 M-NaCl plus 0.5% Triton X-100, 5 M-NaCl, 6 M-urea, 50 mM-phosphotyrosine or up to 1 mg of free Y751 phosphopeptide/ml. Several alternative elution protocols were investigated without success. An elution medium supplied with the Actigel resin was able to remove both proteins, but led to a complete loss of activity. Interestingly no suitable conditions could be established whereby the 110 kDa, but not the 85 kDa, subunit was released from the column, suggesting that the interaction between the 110 and 85 kDa subunits is also of high affinity. Elution of bound proteins was routinely carried out by heating the resin to 80 °C for 3 min in the presence of 5 mM-phosphate buffer (pH 7.0)/0.1% SDS/0.1 mM-dithiothreitol/10% glycerol. The phosphopeptide column could be simply regenerated after elution by extensive washing in binding buffer (Fig. 3, lanes 4 and 5) and could be successfully used at least ten times before any deterioration in binding was observed.

Analysis of the p85 and 110 kDa proteins bound to the phosphopeptide column

The relationship of the 85 kDa proteins observed to bind to the Y751-phosphopeptide column to the recently cloned p85 α and p85 β proteins was investigated by using polyclonal antisera

generated against the predicted C-terminal 12 and 18 amino acids of p85 α and p85 β respectively. Despite the high degree of overall sequence similarity between p85 α and p85 β , the amino acid sequence over this segment is significantly different and thus p85 α - or p85 β -specific antisera were expected to be produced. Furthermore, the amino acid sequence corresponding to this peptide in p85 α is completely conserved between human, bovine and murine cDNAs, suggesting that antibodies generated against this sequence might be useful for studying the expression of different p85 proteins in species other than bovine (Escobedo *et al.*, 1991b; Otsu *et al.*, 1991; Skolnik *et al.*, 1991). The corresponding region of p85 β in species other than bovine is currently unknown.

The p85 antisera generated against these peptides could specifically immunoprecipitate the appropriate species of expressed recombinant p85 from either COS-1 or Sf9 cells, but were not very efficient at immunoprecipitating PtdIns 3-kinase activity from either cell line or from the partially purified bovine brain PtdIns 3-kinase preparation (M. J. Fry, unpublished work). However, these antisera were found to work well in Western blots. The data presented in Fig. 4 shows that these two antisera specifically recognized expressed p85 proteins present in either COS cells or Sf9 cells. Longer exposures also revealed the endogenous COS p85 protein(s), but no such proteins were detected in Sf9 cells with these antisera. No cross-reactivity was observed even at high concentrations of the recombinant proteins, suggesting that they are specific for p85 α (Fig. 4a) and p85 β (Fig. 4b) respectively. The ability of these antisera to interact with the appropriate p85 species was demonstrated to be completely blocked in the presence of the appropriate peptide used to raise the antisera (Fig. 4c). The p85 species in the two peaks of bovine brain PtdIns 3-kinase activity which bound to the Y751-phosphopeptide column was found to react exclusively with the anti-C-terminal antisera raised against the p85 α -specific sequence (Fig. 4a). After immobilization of the bovine brain PtdIns 3-kinase material on the Y751-phosphopeptide column, all the p85 α -immunoreactive material was bound to the column, with none detectable by either silver staining or Western-blot analysis in the supernatant (Fig. 4d).

For sequence analysis of the PtdIns 3-kinase complex, the 110 and 85 kDa subunits were eluted from the column, after extensive stringent washing, by briefly boiling the resin in 5 mM-phosphate buffer (pH 7.0)/0.1% SDS/0.1 mM-dithiothreitol/10% glycerol. Preparation of both the 85 and 110 kDa proteins for digestion with lysyl-endopeptidase and subsequent sequence analysis are described by Totty *et al.* (1992). Amino acid sequence analysis of a lysyl-endopeptidase C digest of the p85 protein bound to the Y751-phosphopeptide column was performed. This confirmed that the p85 protein present in both peak 1 and peak 2 from the MonoQ column was identical with the previously cloned p85 α (results not shown; Otsu *et al.*, 1991). No peptides corresponding to p85 β were found in either peak. Extensive sequencing of the 110 kDa protein affinity-purified from both MonoQ peak 1 and peak 2 material has led to the isolation of a novel cDNA whose cloning and expression are described elsewhere (Hiles *et al.*, 1992).

Examination of the binding of other SH2-domain-containing proteins to the Y751-phosphopeptide column

In addition to the p85 component of the PtdIns 3-kinase complex, PLC γ , GAP and *src*-family protein-tyrosine kinases have all recently been reported to bind to activated autophosphorylated PDGF β -receptors both *in vivo* and *in vitro* (reviewed by Cantley *et al.*, 1991). We have examined the ability of these proteins to bind to the Y751-phosphopeptide columns. For these experiments, baculovirus expression vectors for GAP

Fig. 6. Specificity of binding of PtdIns 3-kinase complex to Y751-peptide column: comparison with Y857 phosphopeptides

Sf9-cell lysates containing p85 α proteins or 1 μ g of partially purified bovine brain PtdIns 3-kinase (P1 or P2 MonoQ) was allowed to bind to the columns for 4 h at 4 °C as described. The columns were then washed repeatedly with binding buffer, and bound proteins were eluted with SDS-containing buffers and then analysed by SDS/PAGE on 7.5% gels. Bound proteins were detected by silver staining. (a) Proteins bound to Y751-phosphopeptide column. (b) Proteins bound to Y857-phosphopeptide column. The migration position of molecular-mass markers are indicated.

and pp60^{c-src} and PLC γ purified from bovine brain were used. An identical concentration of BSA or baculovirus-expressed EGF receptor was used as non-specific controls.

Both recombinant p85 proteins bound tightly to the phosphorylated, but not the non-phosphorylated, Y751-peptide column. This is shown for p85 α in Fig. 5 (lanes 7 and 8). Neither BSA (Fig. 5, lanes 1 and 2) nor baculovirus-expressed EGF receptor (results not shown) bound to either the phosphorylated or the non-phosphorylated Y751-peptide columns, despite being present at equivalent concentrations to the p85 proteins. Baculovirus-expressed GAP was also found to bind to the Y751-phosphopeptide column (Fig. 5, lane 4). Neither pp60^{c-src} from a Sf9-cell lysate (lanes 5 and 6) nor partially purified PLC γ (Fig. 5, lanes 9 and 10) was found to bind to the Y751 control or phosphopeptide columns. In the case of the pp60^{c-src} this probably reflects a distinct binding-site preference for *src*-family kinases, as this baculovirus-expressed pp60^{c-src} has been shown to be active as a kinase, and is capable of binding to tyrosine-phosphorylated PDGF β -receptors and of forming complexes with polyoma middle-T antigen and p85 (Kypta *et al.*, 1990; Otsu *et al.*, 1991; M. J. Fry, unpublished work). However, although the partially purified PLC γ from bovine brain was highly active and was intact as determined by Western blotting using specific PLC γ antibodies, it was unable to bind *in vitro* to either tyrosine-phosphorylated EGF receptors or PDGF β -receptors (results not shown). The reason for this is unclear, but it may suggest that this PLC γ is in some way covalently modified or that its SH2 domains may already be bound to other proteins present in the preparation.

Specificity of binding of the purified bovine brain PtdIns 3-kinase

In order to evaluate the specificity of the Y751-phosphopeptide column for purifying the PtdIns 3-kinase, other phosphopeptide columns were prepared by using peptides based on the amino acid sequences which surround known protein-tyrosine kinase phosphorylation sites. Tyrosine-857 is the other major

Fig. 7. Binding of recombinant baculovirus-expressed p85 proteins to a panel of phosphopeptide columns

P85 proteins in Sf9-cell lysates were tested for their ability to bind to the various peptide columns. After extensive washing, bound proteins were eluted from the columns, separated by SDS/PAGE on 7.5% gels and then made visible by staining with Coomassie Blue. (a) Bound p85 α . (b) Bound p85 β . Key: CON, 17-amino-acid non-phosphorylated Y751 column; Y751, 17-amino-acid phosphopeptide from the kinase insert region of the PDGF β -receptor; Y751.S, 11-amino-acid version of Y751 phosphopeptide; Y857, 17-amino-acid phosphopeptide derived from the sequence around the second major tyrosine-phosphorylation site in the PDGF β -receptor; pGAT, poly Glu:Ala:Tyr phosphopeptide; Y416 and Y527, 13- and 16-amino-acid phosphopeptides derived respectively from the two major tyrosine-phosphorylation sites of pp60^{c-src}.

autophosphorylation site in the human PDGF β -receptor and has been shown to be required for the binding of GAP, but not for association with the PtdIns 3-kinase (Kazlauskas & Cooper, 1989, 1990; Kazlauskas *et al.*, 1991). For a direct comparison with the Y751 peptide, a 17-amino-acid peptide centred around tyrosine-857 was synthesized (see Table 1). Fig. 6 shows a

Fig. 8. The p85/P110 complex and PtdIns 3-kinase activity show specificity in the range of phosphopeptides to which they will bind

A 1 μ g portion of partially purified bovine brain PtdIns 3-kinase (P1 MonoQ) was allowed to bind to peptide affinity columns (defined in Fig. 7) for 4 h at 4 °C as described. The columns were then washed repeatedly with binding buffer. Bound proteins were either eluted with SDS-containing buffers and then analysed by SDS/PAGE on 7.5% gels or assayed for PtdIns 3-kinase activity bound to the column. (a) Bound proteins were detected by silver staining. The migration of molecular-mass markers is indicated. (b) PtdIns 3-kinase activity bound to various phosphopeptide columns. The 32 P-labelled lipid products were separated by t.l.c. and then detected by autoradiography. PI3P indicates the migration position of a PtdIns3P standard. Ori indicates the origin of the t.l.c. plate.

comparison of the proteins from baculovirus expressing p85 α SF9-cell lysate or from bovine brain PtdIns 3-kinase fractions from MonoQ peak 1 (P1) and peak 2 (P2) binding to either the Y751 (Fig. 6a) or Y857 (Fig. 6b) phosphopeptide columns. Whereas the baculovirus-expressed p85 α is observed to bind to both columns to a similar extent, the 85 and 110 kDa proteins from both peaks of activity are seen only to bind to the Y751-phosphopeptide column. Similarly PtdIns 3-kinase activity is only found associated with the Y751-phosphopeptide column (Fig. 8b).

To determine whether this binding specificity could be ex-

Fig. 9. Binding of PtdIns 3-kinase activity to phosphopeptides containing the YXXM motif

(a) A 1 μ g portion of partially purified peak 1 bovine brain PtdIns 3-kinase was bound to 10 μ l of the indicated peptide columns. After extensive washing, the columns were assayed for bound PtdIns 3-kinase activity. Lanes: 1, PtdIns 3-kinase activity bound to non-phosphorylated Y751 column; 2, PtdIns 3-kinase activity bound to phosphorylated Y751 column; 3, PtdIns 3-kinase activity bound to phosphorylated Y751.S column; 4, PtdIns 3-kinase activity bound to phosphorylated Y857 column; 5, PtdIns 3-kinase activity bound to phosphorylated Y740 column; 6, PtdIns 3-kinase activity bound to phosphorylated Met Y1313 column. PIP indicates the migration position of a PtdIns4P standard. Ori indicates the origin of the t.l.c. plate. (b) Comparison of identified PtdIns 3-kinase binding sites in the peptides tested. The proposed consensus sequence for binding is also shown for comparison (Cantley *et al.*, 1991).

tended, we synthesized several other peptides based on known tyrosine autophosphorylation sites (see Table 1). A shorter 11-amino-acid version of the Y751 peptide, termed Y751.S, was also synthesized in an attempt to refine further the minimal SH2 recognition domain required. Two other peptides containing the YXXM motif were prepared, one based on the sequence around tyrosine-740 of the PDGF- β receptor, a second residue within the PDGF-receptor kinase insert which may play a role in PtdIns 3-kinase binding (Escobedo *et al.*, 1991a), and the second based around tyrosine-1313 of Met, the hepatocyte growth-factor receptor. To introduce a totally random sequence, the synthetic peptide poly(Glu:Ala:Tyr) (6:3:1) was also phosphorylated and coupled to the Actigel matrix. Finally, the peptides surrounding the two major phosphorylation sites from pp60^{c-*src*}, Y416 and Y527, were purchased and synthesized respectively. All peptides were efficiently phosphorylated specifically on tyrosine residues by using the EGF receptor, and then were purified by h.p.l.c. as described above for the Y751 phosphopeptide (results not shown).

Baculovirus-expressed bovine p85 α and p85 β were chosen to test these columns (Otsu *et al.*, 1991; Gout *et al.*, 1992). Binding analysis was carried out under conditions identical with those previously established for the Y751-phosphopeptide column. Somewhat unexpectedly, the baculovirus-expressed p85 subunits bound to all phosphopeptide columns tested (see Figs. 7a and 7b). They did not, however, bind to identical columns containing non-phosphorylated peptides (Figs. 7a and 7b, lane 1; and

results not shown). However, when partially purified bovine brain PtdIns 3-kinase was applied to these columns, it was found to bind exclusively to the phosphopeptide columns containing a YXXM motif (See Figs. 8 and 9a). The Y740, Y751.S and Y1313 phosphopeptide columns containing this YXXM motif all bound bovine brain PtdIns 3-kinase as efficiently as the original Y751-phosphopeptide column as measured by our assay. No binding of PtdIns 3-kinase activity was observed with phosphopeptide columns lacking this motif, i.e. Y857, Y416, Y527 or poly(Glu:Ala:Tyr). This result is in agreement with our previous observation that, whereas both cloned p85 species when expressed in a number of cell systems will bind to either phosphorylated PDGF- β or EGF receptors, the active PtdIns 3-kinase complex will only bind to the PDGF- β receptor under identical conditions (Otsu *et al.*, 1991): That the Y751.S-phosphopeptide column appears to be as efficient at binding the active PtdIns 3-kinase complex as the longer Y751-phosphopeptide column suggests that the consensus sequence recently proposed by Cantley *et al.* (1991) does indeed contain all the sequence data necessary for correct recognition by the PtdIns 3-kinase SH2 domain (Fig. 9b).

DISCUSSION

Phosphoproteins of 81–85 kDa have been proposed to be important components of the enzyme PtdIns 3-kinase and were initially thought to be the catalytic subunit (Courtneidge & Heber, 1987; Kaplan *et al.*, 1987). Partial purification of PtdIns 3-kinase from bovine brain (Morgan *et al.*, 1990) has allowed the cloning of two closely related 85 kDa bovine brain proteins (termed p85 α and p85 β). However, it was shown that these proteins are not sufficient when expressed either alone or together to reconstitute PtdIns 3-kinase activity (Otsu *et al.*, 1991). The two cloned p85 proteins in fact appear to be members of a larger gene family containing at least three members which have been fully or partially cloned (Otsu *et al.*, 1991). Evidence from our own and other laboratories has strongly suggested that the PtdIns 3-kinase holoenzyme consists of at least two subunits and that a component of 110 kDa might be the catalytic subunit (Carpenter *et al.*, 1990; Otsu *et al.*, 1991; Escobedo *et al.*, 1991a,b; Shibasaki *et al.*, 1991). The studies described here began as an attempt at refinement of the PtdIns 3-kinase purification described previously by Morgan *et al.* (1990). Although it was possible to isolate and sequence an 85 kDa protein from this partial purification, the purest fractions contained a major contaminating protein of 110 kDa (Totty *et al.*, 1992), which masked any putative 110 kDa catalytic subunit that might be associated with p85. Since the PtdIns 3-kinase purification could not be easily improved by using conventional chromatographic methods, presumably owing to the strong protein aggregation, affinity-chromatography techniques were evaluated. Several proteins to which the PtdIns 3-kinase binds have been well documented, including the PDGF- β receptor (Kaplan *et al.*, 1987; Coughlin *et al.*, 1989). The interaction is thought to be mediated between the tyrosine-phosphorylated receptor and the SH2 domain(s) of P85 (Kazlauskas & Cooper, 1990; Otsu *et al.*, 1991; Escobedo *et al.*, 1991a; Skolnik *et al.*, 1991). Escobedo *et al.* (1991b) have recently described the use of PDGF- β -receptor affinity chromatography as the means to obtain sufficient p85 α protein to enable protein sequencing and cloning of this protein. However, it was considered that the use of a defined-sequence short-peptide affinity column has several distinct advantages over this approach, since the smaller size of the peptide gives a greater degree of specificity and much higher density of affinity groups per unit volume of the column. Phosphorylated peptide substrates of casein kinase II and glycogen synthase kinase-3

have been successfully used for affinity purification of these protein-serine kinases (Woodgett, 1989). The K_m values of these kinases for their respective phosphopeptides were in the 5–10 μ M range. Since the apparent dissociation constant of p85 and the tyrosine-phosphorylated PDGF receptor has been estimated to be below 1 nM (Escobedo *et al.*, 1991b), it was thought that this interaction might provide the basis for an efficient affinity-chromatography step to enhance significantly our previously described purification of the PtdIns 3-kinase complex from bovine brain (Morgan *et al.*, 1990).

The initial affinity column prepared, which employed a 17-amino-acid sequence around Y751 of the human PDGF β -receptor, proved to have a high affinity for the PtdIns 3-kinase complex, making it possible to remove essentially two proteins from both the peak 1 and peak 2 pool fractions eluted from the last MonoQ column; these were the previously described p85 α protein and a protein of approx. 110 kDa. The 85 kDa species present in both peak 1 and peak 2 is identical with that encoded by the p85 α cDNA recently cloned by our laboratory and others (Otsu *et al.*, 1991; Escobedo *et al.*, 1991b; Skolnik *et al.*, 1991). A p85 β protein could not be detected in our preparations. The function of p85 β remains unclear and may mediate distinct receptor–enzyme interactions from that performed by p85 α . The 110 kDa protein isolated is novel and probably the same as recently suggested by us (Otsu *et al.*, 1991), and by others who have purified PtdIns 3-kinase activity from rat liver (Carpenter *et al.*, 1990), to be the putative catalytic subunit of the PtdIns 3-kinase complex. This suggestion was strengthened by data from Shibasaki *et al.* (1991), who purified two peaks of PtdIns 3-kinase activity from bovine thymus, one of which was composed of two prominent protein species of 85 and 110 kDa, whereas the other consisted of a single major component of 110 kDa. We have used the Y751-phosphopeptide affinity column to purify sufficient amounts of the 110 kDa protein for extensive protein sequencing and have now isolated the cDNA for this protein (Hiles *et al.*, 1992). The reason for the separation of the bovine brain PtdIns 3-kinase into two distinct peaks of activity on the last MonoQ column is unclear. Possible explanations include the presence of distinct 110 kDa subunits, the covalent modification of one of the two proteins of the PtdIns 3-kinase complex in one of the peaks, or the interaction of PtdIns 3-kinase with different co-purifying proteins, potentially via SH2- or SH3-mediated interactions. These possibilities require investigation.

It has been proposed that SH2 domains provide a common regulatory mechanism by which enzymically diverse regulatory proteins can physically associate with activated receptors and thereby couple growth-factor stimulation to intracellular signalling pathways (reviewed by Koch *et al.*, 1991). The data presented here suggest that there is an absolute requirement for tyrosine phosphorylation of these peptide sequences in order for the PtdIns 3-kinase complex and the 85/110 kDa proteins to bind. Moreover, our preliminary results (not shown) obtained using the Pharmacia BIAcore biosensor to measure binding constants for both SH2-domain-containing proteins and isolated SH2 domains to various phosphorylated and non-phosphorylated peptides support the observation that phosphorylation of the peptide is essential for binding.

No increase in PtdIns 3-kinase activity was observed upon binding to any of the phosphopeptide columns described in this paper. This point is worthy of note, because the precise mechanism for the activation of the PtdIns 3-kinase after ligand stimulation of a receptor is currently an area of intense speculation. It is not clear whether the translocation of the PtdIns 3-kinase from the cytosol to a membrane-associated location after its binding to an autophosphorylated receptor, thus bringing it into close proximity to its substrates, is sufficient for activation,

or whether subsequent events are also required. It has also been suggested that binding to an autophosphorylated receptor might induce an activating conformational change in the PtdIns 3-kinase or that tyrosine phosphorylation by the receptor might also activate this enzyme (reviewed by Cantley *et al.*, 1991). The experiment described here dissociates the binding of the PtdIns 3-kinase complex to a tyrosine-phosphorylated receptor sequence from the subsequent phosphorylation of the PtdIns 3-kinase subunits by the receptor and suggests that binding to the activated tyrosine-phosphorylated receptor alone is probably not sufficient to induce a change in PtdIns 3-kinase activity. The events required for PtdIns 3-kinase activation remains an area of intense study.

It is not clear whether different SH2 domains carry sufficient information to specify the particular binding-site-specificity of the whole protein. From the studies described here, the recombinant p85 subunits seem to show very limited specificity in terms of the tyrosine-phosphorylated peptide sequences that they can recognize. A binding-site consensus for the PtdIns 3-kinase of E/D E/D E/D E/D Y M/V P M X X has recently been proposed (Fig. 9b; Cantley *et al.*, 1991). Our purified active enzyme, composed of p85 α complexed with the 110 kDa subunit, appears to exhibit an absolute specificity of binding to phosphopeptides containing a YXXM motif within their sequence where the tyrosine is phosphorylated. All of these peptides conform to this proposed consensus, although our data would indicate that the residues between the absolutely conserved tyrosine and the methionine residues can be more varied than was originally suggested (see Fig. 9b). Whether the conserved acidic residues are important for SH2-domain binding, or simply are required for tyrosine phosphorylation as part of the phosphorylation-site recognition motif for a kinase (Kemp & Pearson, 1990), remains to be established.

A specificity of binding of the PtdIns 3-kinase complex has also been previously observed with respect to receptor binding, with the active PtdIns 3-kinase complex binding to the PDGF receptor but not to the EDG receptor, whereas the expressed 85 kDa subunits were observed to bind indiscriminately to both receptors (Otsu *et al.*, 1991). The human PDGF β -receptor contains two YXXM motifs close together within the kinase insert region. These are the sequences surrounding tyrosine-740 and tyrosine-751, both of which we demonstrate here can bind the bovine brain PtdIns 3-kinase when phosphorylated. Escobedo *et al.* (1991a) have demonstrated that a phosphopeptide containing both of these residues from the murine PDGF β -receptor (where the two equivalent tyrosines to Y740 and Y751 are residues 708 and 719 respectively) can block binding of the PtdIns 3-kinase from NIH 3T3 cells to the murine PDGF β -receptor when either of these residues alone, or both together, are phosphorylated. A role *in vivo* for Y751 in binding of the PtdIns 3-kinase has been demonstrated by mutation of this residue to phenylalanine, which dramatically decreases PtdIns 3-kinase binding to the activated receptor (Kazlauskas & Cooper, 1989, 1990). Whether Y740 is a phosphorylation site *in vivo* remains to be demonstrated. The unphosphorylated peptide, as we observe here, is unable to block binding. Unlike the PDGF β -receptor, the EGF-receptor cytoplasmic domain lacks a kinase insert region, but contains a single YXXM motif located within domain XI of the protein-tyrosine kinase domain (Hanks, 1991). The Y1313 peptide used in this study is from the analogous region of the protein-tyrosine kinase domain of the hepatocyte growth-factor receptor (c-Met) and when phosphorylated is able to bind the bovine brain PtdIns 3-kinase. However, although the Y1313 phosphopeptide is able to bind the PtdIns 3-kinase *in vitro*, it is not known whether these residues are phosphorylated *in vivo* or whether this site would be available to bind SH2-containing

proteins. Studies to determine whether this sequence is important for the observed binding *in vivo* of the PtdIns 3-kinase to the hepatocyte growth factor receptor as demonstrated by Graziani *et al.* (1991) are needed. The differences observed between the phosphopeptide-sequence binding range of p85 and the active PtdIns 3-kinase complex possibly suggest that when the 110 kDa protein is complexed with the p85 α protein it can modulate the binding of the SH2 domain(s) for particular target sequences by altering either the affinity or its sequence specificity. It will be interesting to determine whether isolated bacterially expressed SH2 domains also show a decreased specificity in terms of the tyrosine-phosphorylated sequences to which they will bind.

The structural basis of the interaction between p85 and the 110 kDa protein in the complex remains to be established. Once it was immobilized on an appropriate phosphopeptide column, we were unable to separate the 110 kDa protein from the bound 85 kDa component. Similar findings have been reported by Carpenter *et al.* (1990) for PtdIns 3-kinase purified from rat liver; they were unable to find any native conditions for separating the 85 kDa protein from the 110 kDa protein. As p85 possesses two SH2 domains, it is possible that one of these domains is involved in receptor interactions while the second mediates the interaction between the 110 and 85 kDa subunits. However, since no significant amounts of tyrosine-phosphorylated 110 kDa protein can be detected by Western blotting with anti-phosphotyrosine antibodies, it is unlikely that this occurs due to the interaction of the p85 SH2 domain with a tyrosine-phosphorylated sequence on p110. A recent report has demonstrated the ability of the SH2 domain of the *c-abl* protein to bind to a region of the Bcr-Abl fusion protein, which is only serine- and threonine-phosphorylated (Pendergast *et al.*, 1991), suggesting that the SH2 domains may have a wider binding range than previously suspected and may also regulate the formation of complexes with serine- and threonine-phosphorylated proteins. The possibility exists therefore of an SH2-phosphoserine/phosphothreonine interaction mediating the formation of the p85/p110 active PtdIns 3-kinase complex. Studies into the nature of this interaction will be facilitated by the availability of cloned expressed 110 kDa protein.

In summary, the use of the phosphopeptide affinity techniques described have resulted in highly purified PtdIns 3-kinase, although not in soluble form, and they have greatly increased the use of the purified material for many types of study. The immobilized enzyme is fully active and has been used both for kinetic studies and for the generation of large quantities of 3-phosphorylated phosphoinositides. The techniques described here have allowed the isolation of microgram amounts of the 110 kDa protein associated with p85, resulting in the generation of over 200 amino acids of peptide sequence data from 16 distinct lysyl-endopeptidase proteolytic fragments of p110. cDNA clones for this putative catalytic domain of the PtdIns 3-kinase from bovine cDNA libraries have been isolated for examination of their ability to encode an active catalytic subunit of the PtdIns 3-kinase. In addition, the phosphopeptide columns described in this paper have allowed the further analysis of SH2 domain binding specificity. It will be interesting to determine association and dissociation constants with respect to several phosphopeptides in order to elucidate the different affinities of both whole SH2-domain-containing proteins (including both p85 proteins, PLC γ , *src*-family kinases and GAP), and isolated expressed SH2 domains from these proteins.

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