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Phosphatidylinositol (3,4,5)P_3 Interacts with SH2 Domains and Modulates PI 3-Kinase Association with Tyrosine-Phosphorylated Proteins

Lucia E. Rameh,* Ching-Shih Chen,† and Lewis C. Cantley*

*Department of Cell Biology
Harvard Medical School
and Division of Signal Transduction
Beth Israel Hospital
Boston, Massachusetts 02115

†Division of Medicinal Chemistry and Pharmaceutics
College of Pharmacy
University of Kentucky
Lexington, Kentucky 40536

Summary

Src homology 2 (SH2) domains on the regulatory subunit of phosphoinositide 3-kinase (PI 3-kinase) mediate its binding to specific tyrosine-phosphorylated proteins in stimulated cells. Using a pharmacological and genetic approach, we show that the amount of PI 3-kinase associated with tyrosine-phosphorylated proteins inversely correlates with the amount of PI 3-kinase lipid products present in the cell. An explanation for this observation is provided by our finding that phosphatidylinositol (3,4,5)trisphosphate (PtdIns [3,4,5]P_3) binds directly and selectively to the SH2 domains of the 85 kDa subunit of PI 3-kinase and thereby blocks binding to tyrosine-phosphorylated proteins. The SH2 domain of pp60$^{c\text{-}src}$ also specifically bound PtdIns (3,4,5)P_3, and the binding was competed by a phosphopeptide specific for the Src SH2 domain. These results indicate that production of PtdIns (3,4,5)P_3 at the membrane disrupts the binding of PI 3-kinase to phosphoproteins. This lipid may also recruit other SH2-containing proteins to the membrane to initiate downstream signaling.

Introduction

Src homology 2 (SH2) domains are conserved sequences of approximately 100 amino acids that are able to bind tyrosine-phosphorylated proteins in a phosphorylation-dependent and sequence-specific manner. SH2 domain–containing proteins are often downstream targets of protein-tyrosine kinases (PTKs) (Cantley et al., 1991; Pawson, 1995).

Phosphoinositide 3-kinase (PI 3-kinase) is an SH2-containing enzyme found associated with a variety of receptor and nonreceptor PTKs. The enzyme is a heterodimer of a 110 kDa (p110) catalytic subunit and an 85 kDa (p85) regulatory subunit (Carpenter et al., 1990). It can phosphorylate the D3 position of phosphatidylinositol (PtdIns), PtdIns (4)phosphate (PtdIns [4]P), or PtdIns (4,5)bisphosphate (PtdIns [4,5]P_2) to produce PtdIns (3)P, PtdIns (3,4)P_2, or PtdIns (3,4,5)trisphosphate (PtdIns [3,4,5]P_3), respectively (Whitman et al., 1988; Auger et al., 1989; Carpenter et al., 1990). The p85 subunit is composed of a Bcr homology domain, an Src homology 3 (SH3) domain, two proline-rich regions, and two SH2 domains (Escobedo et al., 1991; Skolnik et al., 1991; Otsu et al., 1991; Kapeller et al., 1994). Activation of this enzyme by extracellular factors involves its recruitment to the membrane, in many cases via SH2–phosphotyrosine (pTyr) interactions (Kapeller and Cantley, 1994). However, the growth factor requirement for PI 3-kinase activation can be circumvented by expressing a constitutively active form of this enzyme, constituted by the p110 subunit bound to a truncated form of p85 that contains only the inter-SH2 region (iSH2) and lacks all the regulatory domains (Hu et al., 1995).

In addition to PI 3-kinase, several other SH2-containing proteins are recruited to the receptor complex in response to stimulation, resulting in a wide range of biological responses. Among the proteins that bind to the platelet-derived growth factor (PDGF) receptor, for instance, are pp60$^{c\text{-}src}$, PI 3-kinase, Nck, GAP, Syp, and phospholipase C-γ (Pawson, 1995). The insulin receptor, on the other hand, has fewer SH2-binding sites than the PDGF receptor and is thought to bind only a few SH2-containing proteins directly, including PI 3-kinase (Ruderman et al., 1990; Backer et al., 1992b). Nonetheless, the insulin receptor is able to activate other SH2-containing proteins through the phosphorylation of adaptor proteins, including the insulin receptor substrates 1 and 2 (IRS-1 and IRS-2). Phosphorylated IRS-1 is able to interact with PI 3-kinase, Grb-2, Syp, and Nck (White and Kahn, 1994).

Several studies suggest that the PI 3-kinase lipid products, PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3, are important regulators of cell proliferation (Cantley et al., 1991). They are undetectable in quiescent cells and appear within seconds after growth factor stimulation (Auger et al., 1989). PtdIns (3)P levels, on the other hand, do not significantly change with cell stimulation. Furthermore, PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3 are elevated in cells transformed by v-*abl*, v-*src*, and polyoma middle T, and decreased levels of these lipids correlate with impaired cell transformation by mutated forms of these oncogenes (Fukui et al., 1991; Varticovski et al., 1991; Cantley et al., 1991; Ling et al., 1992). None of the D3-phosphorylated phosphoinositides is a substrate for phospholipase C, indicating that these lipids are themselves second messengers rather than precursors of inositol phosphate second messengers (Serunian et al., 1989).

Until recently, most of the studies addressing the role of PI 3-kinase on cellular responses were undertaken by transfection of receptor PTKs carrying point mutations that eliminate the binding site for p85 or by microinjection of SH2 domains or neutralizing anti-SH2 antibodies (Backer et al., 1992b; Fantl et al., 1992; Valius and Kazlauskas, 1993; Jhun et al., 1994). These approaches prevent activation of PI 3-kinase, but they provide little information about the direct targets of the lipid products. With the identification of the drugs wortmannin (Yano et al., 1993; Okada

et al., 1994) and Ly294002 (Vlahos et al., 1994) as potent PI 3-kinase inhibitors, it is possible to complement these experiments by directly and acutely inhibiting the activity of PI 3-kinase in intact cells.

While investigating the effect of these inhibitors, we made the surprising observation that inhibition of insulin-induced PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3 formation correlates with increased association of PI 3-kinase protein with tyrosine-phosphorylated proteins. Investigation of this phenomenon led to the observation that PtdIns (3,4,5)P_3 directly binds to the SH2 domains of PI 3-kinase in competition with phosphoproteins. We also show that other SH2 domains can bind PtdIns (3,4,5)P_3, raising the possibility that some physiological responses of PI 3-kinase are mediated by SH2-containing proteins.

Results

PI 3-Kinase Inhibition by Wortmannin and Ly294002 Stabilizes the Association of PI 3-Kinase with Tyrosine-Phosphorylated Proteins

To test whether PI 3-kinase activation is important for insulin-induced association of PI 3-kinase with pTyr-containing proteins, we incubated CHO cells transfected with human insulin receptor (HIR cells) with the PI 3-kinase inhibitors Ly294002 or wortmannin for 15 min prior to insulin stimulation. In agreement with previous studies (Ruderman et al., 1990), insulin caused an increase in the amount of the 85 kDa subunit of PI 3-kinase that can be precipitated with anti-pTyr antibody in the absence of PI 3-kinase inhibitors (Figure 1a). Surprisingly, we observed a further increase in the amount of p85 present in anti-pTyr immunoprecipitates from insulin-stimulated cells that had been treated with either Ly294002 (50%–80% increase) or wortmannin (150%–250% increase). The greater effect of wortmannin compared with Ly294002 is consistent with wortmannin being more effective than Ly294002 in blocking the insulin-dependent stimulation of PtdIns (3,4,5)P_3 production at the concentrations used (see below). While wortmannin inhibition of PI 3-kinase is irreversible, Ly294002 binding to PI 3-kinase is reversible and can be washed away during the immunoprecipitation procedures. Therefore, the amount of PI 3-kinase activity present in the anti-pTyr immunoprecipitates from Ly294002-treated cells reflects the amount of p85 present (Figures 1a and 1c, lane 3). On the other hand, the anti-pTyr immunoprecipitates from the wortmannin-treated cells show very little PI 3-kinase activity, despite the fact that they contain more p85 protein (Figures 1a and 1c, lane 4).

The effects of Ly294002 and wortmannin on the association of PI 3-kinase with tyrosine-phosphorylated proteins were also investigated by immunoprecipitating with an anti-p85 antibody. This antibody has been shown to immunoprecipitate tyrosine-phosphorylated IRS-1 (185 kDa) and, to a lesser extent, tyrosine-phosphorylated insulin receptor (95 kDa) in response to insulin (Backer et al., 1992a; Kelly and Ruderman, 1993). Treatment of HIR cells with wortmannin or Ly294002 enhanced the amount of PI 3-kinase–associated p185 (Figure 1d) and p95 (data not shown) detectable by anti-pTyr antibodies. Ly294002 and wortmannin did not significantly affect the total amount of p85 precipitated or the amount of tyrosine phosphorylation in total cell lysates and anti-pTyr immunoprecipitates (Figures 1e, 1f, and 1b). In agreement with previous results, very little pTyr was detected at the position of p85 (data not shown). These results all indicate that inhibition of PI 3-kinase increases its association with tyrosine-phosphorylated proteins.

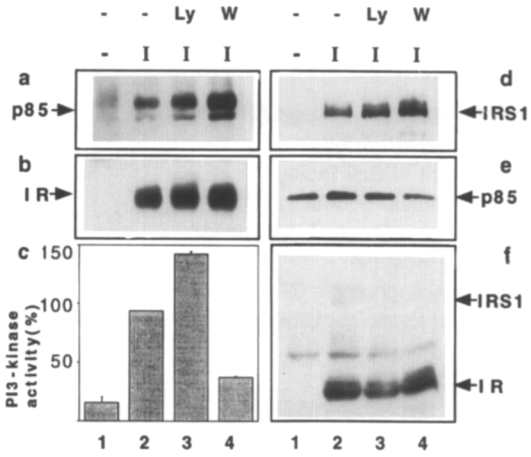

Figure 1. p85 Association with Tyrosine-Phosphorylated Proteins Is Stabilized by Ly294002 and Wortmannin Treatment In Vivo

Serum-starved HIR cells were pretreated with DMSO (minus), 10 μM Ly29402 (Ly), or 100 nM wortmannin (W) and stimulated with 100 nM insulin (I) for 1 min. Cell lysates were prepared as described and precipitated with anti-pTyr (a-c) or anti-p85 antibodies (d and e). Proteins present in the immune complexes (a, b, d, and e) or in the cell lysates (f) were separated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted sequentially with anti-p85 antibody (a and e) and then anti-pTyr antibody (b, d, and f). The anti-pTyr precipitates were also assayed for PI 3-kinase activity (c). The insulin receptor (IR), IRS-1, and p85 bands are indicated. The data presented are representative of more than three experiments. Error bars in (c) represent the standard error from three experiments.

To understand better the effect of these PI 3-kinase inhibitors, we performed a time course of insulin stimulation in cells pretreated with dimethyl sulfoxide (DMSO), Ly294002, or wortmannin. Increased p85 association with tyrosine-phosphorylated proteins in cells pretreated with Ly294002 (data not shown) and wortmannin (Figure 2a) was observed as early as 1 min after insulin stimulation. A correlation between PI 3-kinase inhibition and increased p85 association with tyrosine-phosphorylated proteins was observed when cells were treated with different doses of wortmannin (Figure 2b). The effect of wortmannin was observed at concentrations as low as 1 nM. Lipid analysis revealed that insulin-stimulated in vivo production of PtdIns (3,4)P_2 (Figure 2c) and PtdIns (3,4,5)P_3 (Figure 2d) was almost totally abolished by wortmannin treatment and only partially blocked by Ly294002 at the concentrations used in the experiments presented in Figures 1 and 2a. Thus, decreasing the amount of PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3 correlates with an increase in the association of p85 with tyrosine-phosphorylated proteins.

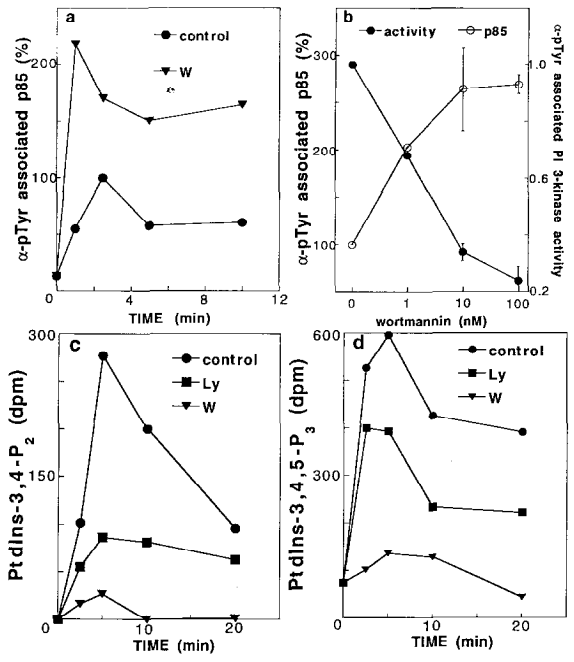

Figure 2. Increased p85 Association with Tyrosine-Phosphorylated Proteins Correlates with PI 3-Kinase Inhibition In Vivo

(a) Serum-starved HIR cells were pretreated with DMSO carrier (circles) or 100 nM wortmannin (triangles) for 15 min, stimulated with 100 nM insulin at time zero, and lysed in NP-40 buffer at the indicated times. Lysates were precipitated with anti-pTyr antibody, blotted for p85, and quantified as described. The standard errors (of three experiments) for the 1 min insulin treatment were ± 13% (control) and ± 10% (wortmannin). The data presented are representative of three experiments.

(b) Serum-starved HIR cells were treated with different concentrations of wortmannin prior to insulin stimulation (100 nM). After 5 min, cells were lysed in NP-40 buffer, and the lysates were precipitated with anti-pTyr antibody. The precipitates were assayed for PI 3-kinase activity (closed circles) and blotted for p85 protein (open circles) as in Figure 1. PI 3-kinase-specific activity was calculated by dividing the relative activity by the relative amount of p85 protein. Error bars indicate the range from two experiments.

(c and d) Serum-starved HIR cells were labeled with ^{32}P inorganic phosphate for 4 hr, treated with DMSO (circles), 10 μM Ly294002 (squares), or 100 nM wortmannin (triangles), and then stimulated with insulin (time zero). Cells were lysed at the indicated time, and lipids were extracted, deacylated, and analyzed by high pressure liquid chromatography (HPLC) as described. The peaks corresponding to PtdIns (3,4)P_2 (c) and PtdIns (3,4,5)P_3 (d) were quantified and normalized to the total amount of ^{32}P label in PtdIns (4)P plus PtdIns (4,5)P_2. The data presented are representative of three experiments.

Overexpression of a Constitutively Active Form of PI 3-Kinase Reduces the Amount of PI 3-Kinase Associated with Tyrosine-Phosphorylated Proteins

To confirm the results obtained with PI 3-kinase inhibitors, we transiently transfected HIR cells with a Myc-tagged version of p85 or the iSH2 region of p85 that binds and activates p110 (Klippel et al., 1993). Immunoprecipitation with anti-Myc antibody has shown that the iSH2 is able to associate with endogenous p110 and cause constitutive activation of the PI 3-kinase activity (B. Duckworth, A. Toker, and L. C. C., unpublished data). The PtdIns (3,4,5)P_3 levels in the iSH2-transfected cells were comparable with those at the peak of PDGF stimulation of the parental cells (data not shown). Transient expression of these cDNAs in HIR cells resulted in protein levels that were 5- to 10-fold higher than endogenous p85, as analyzed by blotting total cell lysates from these cells with anti-p85 antibody (Figure 3a).

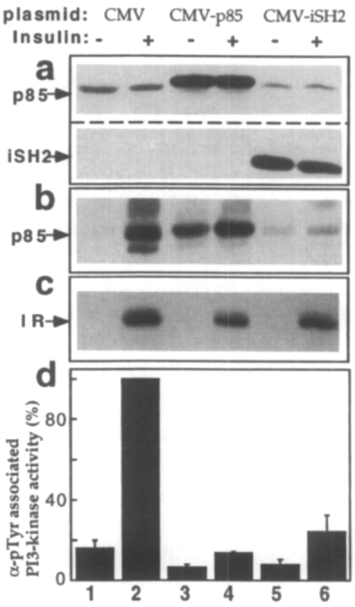

Figure 3. The Amount of p85 Associated with Tyrosine-Phosphorylated Protein Complex Is Decreased by Overexpression of the iSH2 Region of p85

HIR cells were transiently transfected with control plasmid (CMV) or plasmids expressing the full-length p85 (CMV-p85) or iSH2 (CMV-iSH2) under the CMV promoter. Cells were or were not stimulated with 100 nM insulin for 5 min and were lysed in NP-40 buffer. Total cell lysates (a and c) and anti-pTyr precipitates (b) were separated by 7.5% SDS-PAGE and blotted using anti-p85 (a and b) or anti-pTyr (c) antibodies. Total protein concentration of the lysates from CMV-p85- and CMV-iSH2-transfected cells were, respectively, 70% and 80% of the CMV-transfected cells. The p85, iSH2, and insulin receptor (IR) bands are indicated on the left. The data shown are representative of two experiments. (d) shows the percentage of PI 3-kinase activity present in anti-pTyr precipitates from two separate experiments, normalized by the activity present in insulin-stimulated cells transfected with control vector. Error bars indicate the range of the two experiments.

Expression of the iSH2 domain dramatically reduced the amount of p85 that associates with tyrosine-phosphorylated proteins in response to insulin stimulation (compare lanes 2 and 6 in Figure 3b). Cells overexpressing full-length p85, on the other hand, had increased levels of basal and insulin-stimulated anti-pTyr-associated p85 (Figure 3b), even though the levels of tyrosine-phosphorylated insulin receptor (Figure 3c) and IRS-1 (data not shown) were similar to those in cells transfected with control vector. Overexpression of iSH2 also decreased the amount of insulin-stimulated PI 3-kinase activity in anti-pTyr immunoprecipitates (Figure 3d). In agreement with previous observations that excess p85 competes with endogenous p85/p110 for binding to tyrosine-phosphorylated proteins, overexpression of p85 reduced the PI 3-kinase activity in antipTyr immunoprecipitates from insulin-stimulated cells (Figure 3d).

Therefore, expression of a constitutively active form of PI 3-kinase decreases the amount of pTyr–p85 complexes, while overexpression of p85 (dominant negative) increases the amount of pTyr–p85 complexes.

Together, these results suggest that PI 3-kinase lipid products interfere with the interaction between tyrosine-phosphorylated proteins and the p85 subunit of PI 3-kinase. Since SH2 domains mediate this interaction, we explored the possibility that phosphoinositides directly associate with the SH2 domains of p85 to cause release from phosphoproteins.

PI 3-Kinase Lipid Products Bind to the SH2 Domains of p85

To test whether PI 3-kinase lipid products associate directly with p85, we incubated a glutathione S-transferase (GST)–p85 fusion protein immobilized on glutathione–Sepharose beads with phosphatidylserine lipid vesicles containing [^{32}P]PtdIns (3)P, [^{32}P]PtdIns (3,4)P_2, and [^{32}P]PtdIns (3,4,5)P_3 in the presence of 0.02% NP-40. Recombinant proteins from different domains of p85 as well as the whole protein were tested. The percentage of PtdIns (3,4,5)P_3 that remained associated with the various GST beads after a fast wash with 0.5% NP-40 is plotted in Figure 4a. SH2-containing GST beads retained a substantial portion of the original [^{32}P]PtdIns (3,4,5)P_3, about 6% for p85 and 13% for its carboxy-terminal SH2 domain. In contrast, the SH3 domain and GST alone failed to bind a significant amount of lipid under these conditions. Phenyl phosphate (20 mM) competed for [^{32}P]PtdIns (3,4,5)P_3 binding to the amino- and carboxy-terminal SH2 domains, but did not affect the low level of binding to GST or GST–SH3 and only partially blocked binding to full-length p85 (discussed further below).

To investigate whether the ability to bind D3-phosphorylated lipids is a feature shared by other SH2 domains, we performed the lipid binding assay using GST fusion proteins containing SH2 domains from different proteins (Figure 4a). Src SH2 was able to bind 10% of the PtdIns (3,4,5)P_3 from the original lipid mixture, and this interaction was almost totally blocked by phenyl phosphate. Abl SH2 and Lck SH2 were capable of binding to lipids better than the control GST and GST–SH3 domain. As with other SH2 domains, phenyl phosphate blocked this lipid binding.

A comparison between the phenyl phosphate–inhibitable [^{32}P]PtdIns (3,4,5)P_3 binding and the phenyl phosphate–inhibitable [^{32}P]PtdIns (3)P binding (data not shown) to the carboxy-terminal SH2 domain (using a phosphorimager to quantitate the ^{32}P) revealed a 3- to 5-fold selection for PtdIns (3,4,5)P_3 (after normalizing for the abundance of the ^{32}P-labeled lipids in the starting mixture). The binding was not affected by the presence or absence of physiological concentrations of divalent cations (1 mM Mg^{2+} and 1 μM Ca^{2+}; data not shown).

The relative abilities of the various SH2 domains to bind [^{32}P]phosphoinositides was in agreement with their relative abilities to bind tyrosine-phosphorylated proteins from insulin-stimulated cells (Figure 4b), except for Abl and Lck SH2 domains, which bound lipids more weakly than proteins. These results indicate that phosphoinositide products of PI 3-kinase directly bind to SH2 domains.

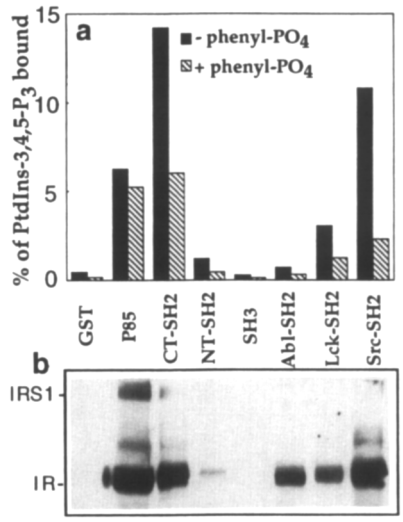

Figure 4. Phosphoinositide Products of PI3-Kinase Bind to SH2 Domains

(a) Binding of PtdIns (3,4,5)P_3 to different domains of p85 or to SH2 domains of different proteins. GST fusion protein beads were incubated with phosphatidylserine vesicles (118 μM) containing ^{32}P-labeled PtdIns (3,4,5)P_3 (0.1 μM), PtdIns (3,4)P_2 (0.03 μM), and PtdIns (3)P (0.03 μM) in the presence or absence of 20 mM phenyl phosphate (see Experimental Procedures). The beads were washed with 0.5% NP-40, and the lipids that remained associated were extracted with chloroform:methanol and separated on a thin-layer chromatography plate. The amount of radioactivity present in the PtdIns (3,4,5)P_3 spot was determined and plotted as a percentage of the total PtdIns (3,4,5)P_3 present in the original lipid mixture. The data shown are representative of more than three experiments. CT-SH2, carboxy-terminal SH2 domain; NT-SH2, amino-terminal SH2 domain.

(b) Binding of tyrosine-phosphorylated proteins to various SH2 domains. GST fusion protein beads were incubated with lysates from insulin-stimulated HIR cells at 4°C for 1 hr. Beads were washed as described, and the proteins were denatured, separated by 7.5% SDS-PAGE, and blotted with anti-pTyr antibodies. The insulin receptor (IR) and IRS-1 bands are indicated.

SH2 Domains Preferentially Bind PtdIns (3,4,5)P_3

The ability of the D3-phosphorylated phosphoinositides to bind to the Src SH2 and p85 carboxy-terminal SH2 domains was not affected when the assay was performed using phosphatidylserine vesicles containing different lipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, or cholesterol, in addition to PtdIns (3,4,5)P_3 in a 10:1 molar ratio. These results indicate that SH2 domain binding to lipids is restricted to the phosphoinositide family. To determine whether SH2 domains are able to associate preferentially with a specific phosphoinositide, we incubated vesicles containing a mixture of all the [^{32}P]phosphoinositide products of PI 3-kinase, PtdIns 4-kinase, and PtdIns (4)P 5-kinase with different GST–SH2 domains in 0.02% NP-40 for 1.5 hr and then washed them with 0.5% NP-40. The relative amount of each lipid in the pellet was divided by the amount in the starting

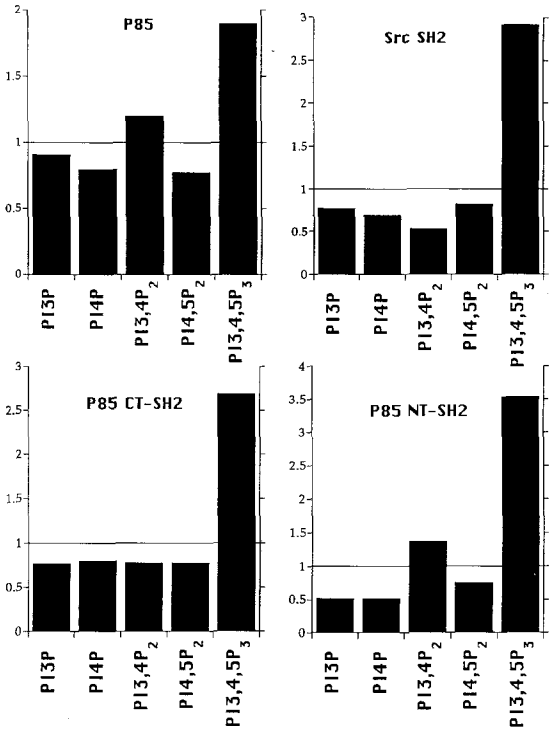

Figure 5. Selectivity of SH2 Domains for PtdIns (3,4,5)P_3

A mixture of sonicated [^{32}P]phosphoinositides was incubated with various GST-SH2 beads, as described in Experimental Procedures. The lipids that remained associated with SH2 domains after washing the beads were extracted, deacylated, and analyzed by HPLC. The peaks corresponding to each lipid were quantified, and the data were normalized as described in Experimental Procedures. The normalization was such that if all lipids bound equally well, then each would have a selectivity of 1. Shown is a representative result from three similar experiments.

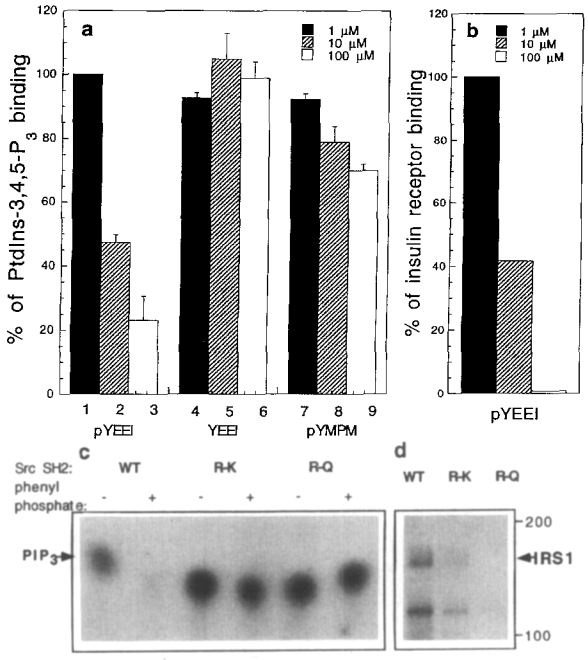

Figure 6. PtdIns (3,4,5)P_3 Interaction with Src SH2 Competes with pTyr Binding, but Does Not Require the Arginine of the FLVR Sequence

(a)PtdIns (3,4,5)P_3 binding to GST-Src SH2 beads in the presence of 1 μM (closed bars), 10 μM (hatched bars), or 100 μM (open bars) of the peptides pYEEI (lanes 1–3), YEEI (lanes 4–6), or pYMPM (lanes 7–9) was performed and analyzed as described for Figure 4a (see Experimental Procedures for complete peptide sequences). The results were plotted as a percentage of the PtdIns (3,4,5)P_3 bound to the beads in the presence of 1 μM pYEEI peptide (which is not significantly different from the content in the absence of peptide). Error bars indicate standard error from three experiments (lanes 1,2,3, and 8) or the range from two experiments (lanes 4, 5, 6, 7, and 9).

(b) Insulin receptor binding to the Src SH2 domain in the presence of 1, 10, or 100 μM of the peptide pYEEI. GST-Src SH2 beads were incubated with lysates from insulin-stimulated HIR cells, as described, in the presence of different concentrations of peptide, and the relative amount of phosphorylated insulin receptor bound was determined as described in Figure 4b.

(c) PtdIns (3,4,5)P_3 binding to wild-type (WT) Src SH2 domain or mutants Arg-178→Lys (R-K) and Arg-178→Gln (R-Q) in the presence or absence of 20 mM phenyl phosphate was analyzed as described for Figure 4a. A thin-layer chromatographic showing the PtdIns (3,4,5)P_3 (PIP_3) spot is presented (which is representative of three experiments).

(d) Anti-pTyr blot showing binding of wild-type Src SH2 domain and mutants to tyrosine-phosphorylated proteins from insulin-stimulated cells. Results were obtained as described for Figure 4b. The migration positions of IRS-1 and molecular mass markers are indicated on the right.

mixture to provide an indication of the selectivity of each SH2 domain for individual phosphoinositides (Figure 5). All three SH2 domains preferentially bound PtdIns (3,4,5)P_3. Full-length p85 was less selective for PtdIns (3,4,5)P_3, probably because of the additional site in the inter-SH2 of p85 that binds PtdIns (4)P and PtdIns (4,5)P_2 (End et al., 1993). Of all the SH2 domains investigated, the Abl SH2 domain had the lowest affinity (Figure 4) and the least selectivity (1.8-fold selection for PtdIns [3,4,5]P_3 over other phosphoinositides; data not shown). No detectable PtdIns (3,4,5)P_3 was found associated with either GST alone or the GST–p85 SH3 in these experiments (data not shown).

PtdIns (3,4,5)P_3 Association with the Src SH2 Domain Is Competed by an SH2-Specific Phosphotyrosine Peptide, but Is Not Affected by Mutation of an Arginine Residue in the pTyr-Binding Pocket

To characterize further the interaction between SH2 domains and PtdIns (3,4,5)P_3, we tested whether pTyr-containing peptides that specifically bind to the pTyr pocket of the Src SH2 domain can compete with the lipid. As seen in Figures 6a and 6b, phosphopeptides containing the sequence pYEEI competed at 10 μM with PtdIns (3,4,5)P_3, as well as with pTyr-containing proteins, for Src SH2 binding. A phosphopeptide with lower affinity for the Src SH2 domain (pYMPM; Songyang et al., 1993) competed at higher concentrations, while an unphosphorylated peptide containing the sequence YEEI did not significantly affect the lipid–SH2 interaction at concentrations up to 100 μM. Consistent with previous observations that a pYMPM-containing peptide binds specifically to the SH2 domains of p85 (Fantl et al., 1992; Songyang et al., 1993), this peptide competed 50% of the phenyl phosphate–sensitive lipid binding to the p85 amino-terminal SH2 at 10 μM,

while at this concentration no competition was observed using the pYEEI peptide (data not shown). Thus, phosphopeptide competition for lipid binding occurs at the well-characterized phosphopeptide-binding sites.

Binding of pTyr-containing peptides to SH2 domains is known to involve the arginine residue present in the FLVR sequence that is conserved among these domains. To determine whether lipid binding to SH2 domains also involves this arginine residue, GST–Src SH2 domain mutants were tested for their ability to bind the ^{32}P-labeled PI 3-kinase lipid products. Substitution of the Arg-178 with lysine or glutamine did not decrease the ability of this SH2 domain to bind PtdIns (3,4,5)P_3 (Figure 6c). However, consistent with the requirement of Arg-178 for pTyr binding, the ability of phenyl phosphate to compete for the lipid interaction was almost completely abolished by the Arg-178→Lys mutation and was totally eliminated by the Arg-178→Gln mutation. As expected, the Arg-178→Lys mutant (in which the positive charge of the residue is maintained) was impaired in its capacity to bind tyrosine-phosphorylated proteins, and the Arg-178→Gln mutant (in which the positive charge is eliminated) was completely defective in pTyr-binding function (Figure 6d). These results indicate that, while pTyr binding and PtdIns (3,4,5)P_3 binding are competitive, they do not utilize all the same positively charged residues of the pTyr-binding pocket. These results also confirm that the phenyl phosphate competition for lipid binding occurs via the pTyr pocket.

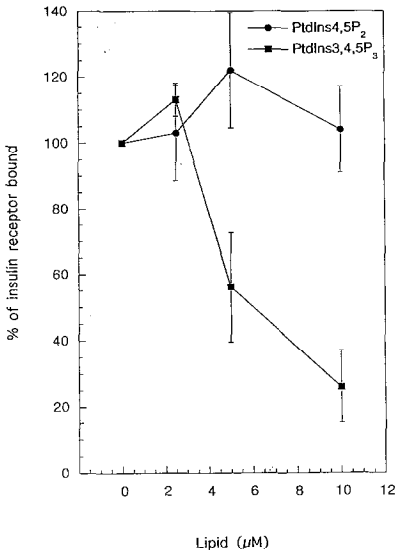

Figure 7. PtdIns (3,4,5)P_3 Blocks p85 Carboxy-Terminal SH2 Binding to Phosphorylated Insulin Receptor

GST beads containing the carboxy-terminal SH2 domain of p85 were incubated with different concentrations of sonicated PtdIns (4,5)P_2 (circles) or chemically synthesized DiC_{16} PtdIns (3,4,5)P_3 (Liscovitch et al., 1994) (squares), as described. Lysates from insulin-stimulated HIR cells were added so that the final concentration of NP-40 was 0.5%. After 10 min, the beads were washed and the proteins associated were analyzed by anti-pTyr blotting. The scanned image of the insulin receptor band was quantified as described. The results are expressed as a percentage of the total amount of insulin receptor associated with SH2 beads in the absence of lipids. Error bars represent the range from duplicate samples.

PtdIns (3,4,5)P_3 Blocks In Vitro Binding of p85 Carboxy-Terminal SH2 to Phosphorylated Insulin Receptor

We also investigated whether PtdIns (3,4,5)P_3 and PtdIns (4,5)P_2 could interfere with the ability of SH2 domains to bind pTyr-containing proteins from a cell lysate. Figure 7 shows that synthetic dipalmitoyl PtdIns (3,4,5)P_3 (presented as NP-40-mixed micelles) blocks the binding of GST–p85 carboxy-terminal SH2 to the insulin receptor with a 50% maximal effect at approximately 5 μM (PtdIns [3,4,5]P_3 also inhibited p85 carboxy-terminal SH2 binding to IRS-1 by 50% at 5 μM; data not shown). In agreement with the results in Figure 5, PtdIns (4,5)P_2 micelles were less effective at blocking binding. Similar results were obtained when PtdIns (3,4,5)P_3 was presented in phosphatidylserine vesicles (data not shown). We also investigated the ability of inositol polyphosphates to block the Src SH2 domain binding to insulin receptor and found that inositol (1,3,4,5)P_4, the head group of PtdIns (3,4,5)P_3, had a much lower affinity than PtdIns (3,4,5)P_3: inositol (1,3,4,5)P_4 inhibited 29% of the insulin receptor binding at 1 mM concentration, while PtdIns (3,4,5)P_3 inhibited 90% at 80 μM. Nevertheless, it did bind better than inositol (1,4,5)P_3, the head group of PtdIns (4,5)P_2, which inhibited only 14% of the insulin receptor binding at 1 mM concentration (data not shown). These results indicate that the diacylglycerol component of the lipid provides additional affinity.

Discussion

The data presented in this paper demonstrate a novel protein–lipid interaction between SH2 domains and a phosphoinositide product of PI 3-kinase, PtdIns (3,4,5)P_3. The p85 amino-terminal and carboxy-terminal SH2 domains and the Src SH2 domain had specificity for binding PtdIns (3,4,5)P_3 over other phosphoinositides. The Lck and Abl SH2 domains also had significant, although weaker, binding to PtdIns (3,4,5)P_3. The ability of phenyl phosphate and tyrosine-phosphorylated peptides to block the PtdIns (3,4,5)P_3–SH2 interaction indicates that lipid binding and phosphopeptide binding are mutually exclusive. The arginine at position 178 of the Src SH2 domain is required for pTyr binding, but not for PtdIns (3,4,5)P_3 binding, indicating that lipid and phosphoprotein interactions with SH2 domains are not coordinated by the same residues inside the pocket.

Evidence that PI 3-kinase lipid products compete with pTyr-containing proteins for binding to the p85 subunit of PI 3-kinase in vivo is provided by experiments with PI 3-kinase inhibitors and by overexpression of dominant positive PI 3-kinase. Blocking the production of PtdIns (3,4,5)P_3 with wortmannin or Ly294002 enhances pTyr–p85 protein interactions, while expression of constitutively active PI 3-kinase reduces pTyr–p85 protein interactions.

These results suggest some interesting models for the involvement of PI 3-kinase in signal transduction. It is likely

that local concentrations of PtdIns (3,4,5)P_3 will be enough to bind to the SH2 domain of p85 since the lipid is synthesized locally by the catalytic subunit of this protein. This could be an effective mechanism of dissociating PI 3-kinase from tyrosine-phosphorylated proteins once the lipid product has reached an optimal level, providing a negative feedback control mechanism. However, it is also possible that the PtdIns (3,4,5)P_3 recruits a subgroup of SH2-containing proteins to specific compartments of the cell. For example, binding of Grb-2 to PtdIns (3,4,5)P_3 could be an alternative mechanism to binding of Grb-2 to receptors for recruiting the Grb-2–SOS complex to the plasma membrane. PtdIns (3,4,5)P_3 binding to the SH2 domain of $pp60^{c\text{-}src}$ could also be important for the localization or activation of this enzyme (or for both). Experiments to test these ideas are in progress.

To date, PI 3-kinase has been implicated in a plethora of cellular responses, including mitogenesis (Whitman et al., 1985; Kaplan et al., 1987; Coughlin et al., 1989; Cantley et al., 1991), chemotaxis (Kundra et al., 1994; Wennstrom et al., 1994), membrane ruffling (Wennstrom et al., 1994), activation of p70S6 kinase (Chung et al., 1994), insulin-dependent Glut4 recruitment (Cheatham et al., 1994; Clarke et al., 1994), glycogen synthesis (Cross et al., 1994; Welsh et al., 1994), activation of integrins in platelets (Kovacsovics et al., 1995), histamine release (Yano et al., 1993), receptor down-regulation (Joly et al., 1994), and blocking apoptosis (Yao and Cooper, 1995). In addition, the yeast homolog of PI 3-kinase, Vps34p (which produces PtdIns [3]P but not PtdIns [3,4]P_2 or PtdIns [3,4,5]P_3), is required for trafficking of proteins from the Golgi to the vacuole (Herman and Emr, 1990; Hiles et al., 1992). It seems unlikely that a single target for the D3 phosphoinositides mediates these diverse responses. Thus, it is an attractive idea that various SH2 domains bind to specific D3 phosphoinositides to mediate distinct responses.

The fact that PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3 are normally undetectable in quiescent cells and appear within seconds to minutes of stimulation with various growth factors or hormones is consistent with these molecules acting as second messengers (Auger et al., 1989). In addition to the evidence presented here that SH2 domains bind to PtdIns (3,4,5)P_3, we have previously shown that both PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3 activate protein kinase C types ε, η, and δ (Toker et al., 1994). Activation of native protein kinase C-ζ (Nakanishi et al., 1993), but not recombinant protein kinase C-ζ (Toker et al., 1994), has also been reported. Interestingly, PtdIns (3,4)P_2 is more effective than PtdIns (3,4,5)P_3 in activating the Ca^{2+}-independent protein kinase C isoforms (Toker et al., 1994), while PtdIns (3,4,5)P_3 is more effective than PtdIns (3,4)P_2 in binding to the Src and p85 SH2 domains (Figure 5). Recently, PtdIns (3)P was implicated in the activation of the Akt Ser/Thr protein kinase via a Plekstrin homology domain (Franke et al., 1995). These results raise the possibility that the different lipid products of PI 3-kinase preferentially activate different targets in the cell and begin to provide a framework for understanding the diverse cellular responses in which PI 3-kinase has been implicated.

Experimental Procedures

Cell Culture

CHO cells transfected with wild-type HIR (White et al., 1988) were cultured in RPMI medium containing 10% FCS and 0.4 mg/ml G418 until they reached 80% confluence. Cells were serum starved in RPMI containing 0.5% bovine serum albumin (BSA) for 48 hr. For the PI 3-kinase inhibitor experiments, cells were treated with DMSO carrier, 10 μM Ly294002 (Lilly) in DMSO, or 100 nM wortmannin (Sigma) in DMSO for 15 min prior to insulin stimulation.

Transient Transfection

HIR cells were cultured in 100 mm dishes until they reached 70% confluence. We used 8 μg of plasmid DNA (pRC [Invitrogen] containing the cytomegalovirus [CMV] promoter, CMV-p85, or CMV-iSH2) and 48 μl of lipofectamine reagent (GIBCO BRL) to transfect each plate in the absence of serum. After 18 hr, the medium was replaced by RPMI-0.5% BSA. Approximately 45 hr after the beginning of the transfection, the cells were or were not stimulated with 100 nM insulin and lysed as described below. The CMV-iSH2 plasmid is a construct containing the Myc tag sequence fused to the carboxy-terminal sequence of the iSH2 region (amino acids 428-678) of the human p85 cDNA, cloned into a vector containing the CMV promoter. The CMV-p85 plasmid was constructed by fusion of the Myc tag sequence to the amino-terminal sequence of the bovine p85 cDNA and cloning into a vector containing the CMV promoter.

Immunoprecipitation and Immunoblot

Cells were lysed in 1 ml of NP-40 lysis buffer (137 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP-40) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM PMSF) and phosphatase inhibitor (1 mM sodium vanadate). Cells were scraped after rocking for 15 min at 4°C, and the lysates were cleared by centrifugation at 15,000 rpm for 10 min. Variations in the total protein concentration of different cell lysates (measured using the Bio-Rad protein assay reagent) were less than 7%.

Antibody and protein A-Sepharose were added to lysates, and the mixture was incubated at 4°C for 3 hr. Immunoprecipitates were washed three times with PBS-1% NP-40, twice with 0.5 M LiCl, 0.1 M Tris-HCl (pH 7.5), and twice with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA and were assayed for PI 3-kinase activity, using PtdIns (4,5)P_2 as a substrate as previously described (Serunian et al., 1991) or were denatured in Laemmli's buffer for 10 min at 100°C.

Proteins present in the immunoprecipitates were separated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. After blocking with TBS (20 mM Tris-HCl [pH 8.0], 137 mM NaCl) containing 5% lowfat milk, the membranes were incubated with primary antibody for 1 hr in TBS-0.05% Tween 20 for 45 min with secondary antibody conjugated with HRP and developed using the chemiluminescence detection system (Dupont). The anti-p85 antibody was obtained from UBI, and the anti-pTyr antibody (4G10) was a gift from Dr. T. Roberts. The scanned images of autoradiograms were quantified using the NIH image program. For the kinetics experiments (Figure 2a), the amount of p85 was calculated based on a p85 standard curve obtained from immunoblots of p85 containing serial dilutions of the protein.

In Vivo Labeling of Lipids and HPLC Analyses

HIR cells were labeled with 0.5 mCi/ml of inorganic [^{32}P]phosphate for 4 hr. After being treated with inhibitors as described above, cells were stimulated with insulin for 2.5, 5, 10, or 20 min at 37°C. One 100 mm plate was used for each timepoint. Cells were harvested with 800 μl of methanol, 1 M HCl (1:1). Lipids were extracted with 400 μl of chloroform, deacylated, and analyzed by high pressure liquid chromatography (HPLC) on a SAX column, as described previously (Serunian et al., 1991). The peaks corresponding to the deacylated products of PtdIns (3,4)P_2 and PtdIns (3,4,5)P_3 were identified with the use of ^{3}H standards, and the radioactivity present in each peak was quantified with the use of an on-line radiomatic detector (Packard) and normalized to the total amount of ^{32}P label in PtdIns (4)P plus PtdIns (4,5)P_2. For the experiment shown in Figures 2c and 2d, the peak amounts of [^{32}P]PtdIns (3,4)P_2 and [^{32}P]PtdIns (3,4,5)P_3 in the insulin-

stimulated cells were approximately 0.1% and 0.5%, respectively, of the total [^{32}P]phosphoinositides in the cell.

GST Fusion Proteins

pGEX vectors containing the cDNA sequences encoding the SH2 domains, the p85 protein, p85 carboxy-terminal SH2, p85 amino-terminal SH2 (Yoakim et al., 1992), or p85 SH3 (Kapeller et al., 1994) were expressed in Escherichia coli as GST fusion proteins by isopropyl-β-D-thiogalactopyranoside induction. Bacterial lysates (prepared as described in Sambrook et al. [1989]) were incubated with glutathione-Sepharose beads for 2 hr at 4°C and washed several times with 30 mM HEPES (pH 7.0), 100 mM NaCl, 1 mM EDTA (HNE) containing 0.5% NP-40, followed by HNE.

Protein Binding Assay

HIR cells were stimulated with insulin for 10 min and lysed in 1% NP-40-HNE buffer. Lysates were centrifuged for 10 min at 14,000 rpm, and supernatants were incubated with different GST-Sepharose beads at 4°C for 1 hr. Beads were washed as described for the immunoprecipitation procedures, resuspended in an equal volume of 2 × Laemmli's buffer, and incubated for 10 min at 100°C. Proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and blotted using anti-pTyr antibody (4G10). For the competition experiments, the GST beads were incubated with lipids (sonicated in HNE), inositol (1,4,5)P_3 (Amersham), or inositol (1,3,4,5)P_4 (Sigma) for 1 hr at room temperature prior to the addition of the cell lysates.

Lipid Binding Assay

[^{32}P]PtdIns (3)P, [^{32}P]PtdIns (3,4)P_2, and [^{32}P]PtdIns (3,4,5)P_3 were prepared by incubating the precursor of these lipids with purified PI 3-kinase in the presence of excess phosphatidylserine as previously described (Carpenter et al., 1990). After 2 hr at room temperature, the reaction was stopped with 10 mM EDTA, and lipids were isolated by chloroform:methanol extraction as described previously (Serunian et al., 1991). For the lipid selection experiments, a 200 μM solution of presonicated crude brain phosphoinositides was incubated with purified PI 3-kinase, PtdIns 4-kinase, and PtdIns (4)P 5-kinase in the presence of 15 mM HEPES (pH 7.0), 200 μM [^{32}P]ATP (1.1 μCi/mmol), and 5 mM $MgCl_2$. The lipids were extracted with chloroform:methanol and dried. Dry lipids were resuspended in 10 mM HEPES (pH 7.0), 1 mM EDTA, sonicated, and added to 40 μl of Sepharose beads containing approximately 12 μg of the indicated GST fusion protein in the absence or presence of 20 mM phenyl phosphate in a solution containing 0.02% NP-40-HNE buffer. A titration experiment has shown that at 0.02% NP-40 lipid binding is optimal. Incubation was carried out for 1.5 hr at room temperature, after which the beads were quickly washed twice with 1 ml of 0.5% NP-40-HNE (2-3 min total). Lipids that remained associated with the beads were extracted with chloroform:methanol (1:1) and resolved by thin-layer chromatography (TLC) using 1-propanol:2 M acetic acid (65:35 v/v). A sample of the original lipid mixture was also run on the TLC. The peptide competition experiments were done in the presence of the peptides RENEpYMPMAPQIH (pYMPM), EPQpYEEIPIYL (pYEEI), or AEEEEIYEEIEAKKKK (YEEI), as described above (pY indicates pTyr). The results were quantified using a phosphorimager (Bio-Rad).

For the lipid selectivity experiments (Figure 5), the bound lipids were deacylated and analyzed by HPLC as described previously (Serunian et al., 1991). The radioactivity in each lipid was then divided by the radioactivity in the same lipid in the starting mixture to determine the fraction of each lipid bound to a given SH2 domain. To compare the relative selectivities of the various SH2 domains for PtdIns (3,4,5)P_3, the data were normalized such that if all the lipids bound equally well, each would have a selectivity of 1. Total radioactivity bound in the experiments presented is as follows: p85, 12,473 dpm; p85 carboxy-terminal SH2, 9,603 dpm; p85 amino-terminal SH2, 5,607 dpm; Abl SH2, 2,724 dpm; Src SH2, 7,437 dpm; GST, 1,219 dpm; p85 SH3, 1,440 dpm; total (before SH2 selection), 11,4390 dpm. The radioactivity from the GST and p85 SH3 experiments did not appear above background in any of the phosphoinositide elution positions. The proportions of [^{32}P]phosphoinositides in the starting mixture were as follows: PtdIns (3)P, 39%; PtdIns (4)P, 14.6%; PtdIns (3,4)P_2, 9.5%; PtdIns (4,5)P_2, 25.5%; PtdIns (3,4,5)P_3, 11.3%.

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