Structural and Biochemical Evaluation of the Interaction of the Phosphatidylinositol 3-Kinase p85α Src Homology 2 Domains with Phosphoinositides and Inositol Polyphosphates*

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Src homology 2 (SH2) domains exist in many intracellular proteins and have well characterized roles in signal transduction. SH2 domains bind to phosphotyrosine (Tyr(P))-containing proteins. Although tyrosine phosphorylation is essential for protein-SH2 domain interactions, the binding specificity also derives from sequences C-terminal to the Tyr(P) residue. The high affinity and specificity of this interaction is critical for precluding aberrant cross-talk between signaling pathways. The p85α subunit of phosphoinositide 3-kinase (PI 3-kinase) contains two SH2 domains, and it has been proposed that in competition with Tyr(P) binding they may also mediate membrane attachment via interactions with phosphoinositide products of PI 3-kinase. We used nuclear magnetic resonance spectroscopy and biosensor experiments to investigate interactions between the p85α SH2 domains and phosphoinositides or inositol polyphosphates. We reported previously a similar approach when demonstrating that some pleckstrin homology domains show binding specificity for distinct phosphoinositides (Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C., Waterfield, M. D., and Panayotou, G. (1996) *EMBO J.* 15, 6241–6250). However, neither SH2 domain exhibited binding specificity for phosphoinositides in phospholipid bilayers. We show that the p85α SH2 domain Tyr(P) binding pockets indiscriminately accommodate phosphoinositides and inositol polyphosphates. Binding of the SH2 domains to Tyr(P) peptides was only poorly competed for by phosphoinositides or inositol polyphosphates. We conclude that these ligands do not bind p85α SH2 domains with high affinity or specificity. Moreover, we observed that although wortmannin blocks PI 3-kinase activity *in vivo*, it does not affect the ability of tyrosine-phosphorylated proteins to bind to p85α. Consequently phosphoinositide products of PI 3-kinase are unlikely to regulate signaling through p85α SH2 domains.

Src homology 2 (SH2)[1] domains are conserved, noncatalytic sequences of about 100 amino acids that adopt a common three-dimensional fold. These domains are commonly found in signal transduction proteins that regulate a variety of cellular processes, such as phospholipid metabolism, protein phosphorylation, and dephosphorylation, protein trafficking, and gene expression (1). SH2 domains mediate high affinity binding to phosphotyrosine (Tyr(P)) residues in proteins such as activated membrane receptors and cytosolic adaptor proteins. Three to five amino acids C-terminal to the target Tyr(P) residue bind to a groove on the SH2 domain surface and confer the specificity of interaction that is necessary to avoid aberrant signaling (2, 3). The role of SH2 domains in Tyr(P)-dependent protein recruitment is critical for the assembly of active complexes of signaling proteins (4, 5).

The p85α/p110α Class I$_A$ phosphoinositide 3-OH kinase (PI 3-kinase) contains two SH2 domains in its regulatory p85α subunit (6). Upon cell stimulation, the SH2 domains bind to tyrosine-phosphorylated, membrane-bound growth factor receptors. As a result, p85α/p110α is recruited to the vicinity of its phosphoinositide substrates (7). The p110α PI 3-kinase activity then produces 3′-phosphorylated phosphoinositides. In this manner, p85α/p110α mediates a dramatic increase in the basal concentration of phosphatidylinositol 3,4,5-trisphosphate (PtdIns (3,4,5)P$_3$) and phosphatidylinositol 3,4-bisphosphate in the plasma membrane shortly after cell stimulation (8, 9).

It is now clear that p85α/p110α phosphorylates phosphoinositides to produce second messengers, which control the membrane recruitment and activation of numerous signaling proteins, notably including regulators of apoptosis (10–12). Many of the target proteins of these second messengers contain pleckstrin homology (PH) domains and have been shown to bind specifically to PtdIns (3,4,5)P$_3$ and/or phosphatidylinositol 3,4-bisphosphate *in vitro* and/or *in vivo* (13–19). Indeed, nu-

[1] The abbreviations used are: SH2, Src homology 2; di-C$_6$-PIP$_3$, *rac*-dihexanoylphosphatidyl-D/L-*myo*-inositol 3,4,5-trisphosphate; HSQC, heteronuclear single quantum coherence; GST, glutathione *S*-transferase; Ins, D-*myo*-inositol; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI, phosphoinositide; Ptd, phosphatidyl; Tyr(P), phosphotyrosine; PBS, phosphate-buffered saline.

merous interactions between distinct phosphoinositides and PH domains have now been demonstrated and appear to be essential for the function of various cytoskeletal or signal transduction proteins (reviewed in Ref. 20).

However, a few reports have suggested that PH domains are not unique targets of second messenger phosphoinositides produced by PI 3-kinase. It has been proposed that PtdIns $(3,4,5)P_3$ can also bind to SH2 domains. These proposals followed the observation of an inverse correlation between the amount of p85α/p110α associated with tyrosine-phosphorylated proteins and the level of PI 3-kinase lipid products present in the cell (21). Consequently, a model was proposed in which the PtdIns $(3,4,5)P_3$ produced by PI 3-kinase activation could compete for Tyr(P)-bound p85α SH2 domains and directly result in the relocalization of p85α/p110α at the plasma membrane. Similarly, the production of PtdIns $(3,4,5)P_3$ may regulate additional proteins such as the tyrosine kinase Src and phospholipase C γ-1 (21, 22). It has been shown that *in vitro* the p85α C-terminal SH2 (C-SH2) domain can bind to PtdIns $(3,4,5)P_3$ (21). However, it was not demonstrated that recombinant p85α C-SH2 domain can act as a faithful model of p85α activity. Indeed, we noted with intrigue that the reported interaction of PtdIns $(3,4,5)P_3$ with the p85α C-SH2 domain could be significantly inhibited by phenyl phosphate, but that such inhibition was not observed in the case of the reported interaction between PtdIns $(3,4,5)P_3$ and full-length p85α (21). The work presented herein arose from our attempts to clarify these apparently conflicting observations and to resolve certain issues central to these models describing distinct phosphoinositide-SH2 domain interactions.

Prerequisites for the models above are that the SH2 domains that interact with phosphoinositides must (*a*) demonstrate a significant binding affinity for these ligands and (*b*) discriminate between the numerous phosphoinositides present in the plasma membrane. Because we had access to appropriate reagents and assay techniques, we set out to determine whether the p85α SH2 domains indeed display clear binding specificity and affinity for distinct phosphoinositides. We report the first high resolution structural studies of model phosphoinositide-SH2 domain interactions, which we performed by nuclear magnetic resonance (NMR) spectroscopy. We also employed two sensitive biosensor assays; one to measure interactions between proteins and phospholipid bilayers containing phosphoinositides and another to measure directly the competition between Tyr(P)-containing ligands and phosphoinositides for binding to SH2 domains. In addition, we report *in vivo* studies in which we sought a correlation between the association of p85α with activated growth factor receptors or tyrosine-phosphorylated proteins and the intracellular level of PI 3-kinase products.

EXPERIMENTAL PROCEDURES
Protein Expression and Purification

The p85α C-SH2 domain (amino acids Glu-614–Arg-724) was expressed and purified as described previously (23). A pGEX-2T plasmid encoding glutathione *S*-transferase (GST) (Amersham Pharmacia Biotech) fused to the p85α N-terminal SH2 (N-SH2) domain (amino acids Pro-314—Tyr-431) was kindly provided by Dr. R. Stein (Ludwig Institute for Cancer Research, London), and the protein was prepared and purified essentially as described previously (24, 25). Transformed *Escherichia coli* BL21 (DE3) cells were grown at 37 °C to a culture density $A_{600} \sim 0.8$. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a concentration of 0.2 mM. Cells were harvested after 4 h, resuspended in phosphate-buffered saline (PBS), and lysed by a French press. For NMR spectroscopy and biosensor Tyr(P) competition assays, the GST moiety was removed by thrombin cleavage. In contrast, intact fusion protein was used in the liposome binding assays. Further protein purification was accomplished by gel filtration in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.02% NaN$_3$. ^{15}N-

isotopically enriched samples for NMR spectroscopy were prepared as above except that cells were grown in a minimal M9 medium using ^{15}NH$_4$Cl (Isotec Inc.) as the sole nitrogen source. NMR samples were prepared in 50 mM deuterated Tris-HCl (Cambridge Isotope Laboratories), pH 7.5, 50 mM NaCl, 2 mM dithiothreitol (for C-SH2 only), 10% (v/v) D$_2$O.

Ligands Tested for Binding to p85α SH2 Domains

The water-soluble ligands tested included D-*myo*-inositol 1,4,5-trisphosphate (D-Ins $(1,4,5)P_3$), L-Ins $(1,4,5)P_3$, L-α-glycerophospho-D-*myo*-inositol 4,5-bisphosphate, and phenyl phosphate obtained from Sigma; D-Ins $(1,3,4,5)P_4$ and L-Ins $(1,3,4,5)P_4$, synthesized and purified by ion-exchange chromatography as published (26, 27); *rac*-dihexanoylphosphatidyl-D/L-*myo*-inositol 3,4,5-trisphosphate (di-C$_6$-PIP$_3$), synthesized using published techniques (28); and the nine-residue phosphopeptide SVDY(P)VPMLD (Y(P) is phosphotyrosine) (Genosys Ltd.). The phospholipids tested included PtdIns, PtdIns (4)P, and PtdIns $(4, 5)P_2$ (obtained from Lipid Products, Redhill, Surrey, UK) and PtdIns $(3,4,5)P_3$, which was prepared as described previously (29) and kindly provided by Professor R. Gigg. The additional liposome components described were purchased from Sigma.

NMR Spectroscopy Experiments

For NMR spectroscopy, SH2 domain samples were prepared at 0.5 mM concentration in 600 μl. Interactions were monitored via spectra recorded during titration of the SH2 domain with 1.5-μl aliquots of test ligand (prepared at 20 mM in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl). NMR experiments were performed at 15 °C on a Varian UNITY-plus spectrometer operating at a ^{1}H frequency of 600 MHz. Two-dimensional gradient enhanced sensitivity ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) experiments were performed using a pulse sequence kindly provided by Professor L. E. Kay (30). Sign discrimination in t$_1$ was achieved using the States-time-proportional phase incrementation method. The HSQC spectra were acquired with 16 scans, 64 increments in t$_1$, and sweep widths of 10000 Hz (^{1}H) and 2400 Hz (^{15}N). Three-dimensional ^{15}N-^{1}H HSQC total correlation spectroscopy and nuclear Overhauser effect spectroscopy experiments were recorded to verify the published resonance assignments for the N-SH2 domain (31, 32).

NMR data were processed using NMRpipe software (33). Phase-shifted, sine-squared shaped weighting functions and zero-filling were applied before Fourier transformation. NMR spectra were analyzed using XEASY (34) and AZARA software (AZARA v.II, W. Boucher, Department of Biochemistry, University of Cambridge, UK).

Biosensor Experiments

Preparation of Liposomes for Biosensor Studies—Large unilamellar liposomes with a phospholipid composition approximating the inner leaflet of the plasma membrane were prepared as described previously (35). By weight, the liposomes contained 30% phosphatidylcholine, 15% sphingomyelin, 20% cholesterol, 15% phosphatidylethanolamine, 10% phosphatidylserine, and 10% of the phosphoinositide to be tested. Liposomes were used in 10 mM HEPES, pH 7.4, 80 mM KCl, 15 mM NaCl, 0.7 mM NaH$_2$PO$_4$, 1 mM EGTA, 0.466 mM CaCl$_2$, 2.1 mM MgCl$_2$.

Liposome Binding Studies Using the Biosensor—The basic operating procedures of the surface plasmon resonance BIAcore biosensor (BIACORE AB, Uppsala) have been published (36). The ability of immobilized GST fusion SH2 domains to bind to phosphoinositides in liposomes was examined using the method described previously (35).

Phosphoinositide Phosphotyrosine Peptide Competition Studies Using the Biosensor—A precoated streptavidin biosensor chip (SA-5, BIACORE AB) was used to immobilize the N-terminal-biotinylated, Tyr(P) peptide *N*-biotinyl-DMSKDESVDY(P)VPMLDMK (Y(P) is phosphotyrosine). The Tyr(P) peptide was loaded in the buffer used throughout the assay: 20 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, and 4 mM dithiothreitol. Solutions of 0.5 μM N- or C-SH2 domain were injected over the surface at a flow rate of 5 μl/min at 25 °C, and the maximum response was recorded. Competition experiments were performed by incubating the SH2 domains with a competitor ligand before injection. Efficacious competition resulted in a diminished response. Between injections, protein remaining bound to the biosensor was removed by a 5-μl pulse of 0.05% SDS solution.

Data analysis of the competition measurements was performed with the BIAcore-2000 software package (BIACORE AB). In calculations of the half-maximal inhibitory constants (IC$_{50}$), the control response from injection of SH2 domain over the biosensor surface lacking the Tyr(P) peptide was subtracted from the experimental response to yield the corrected response, *R*. Data was plotted as corrected response units
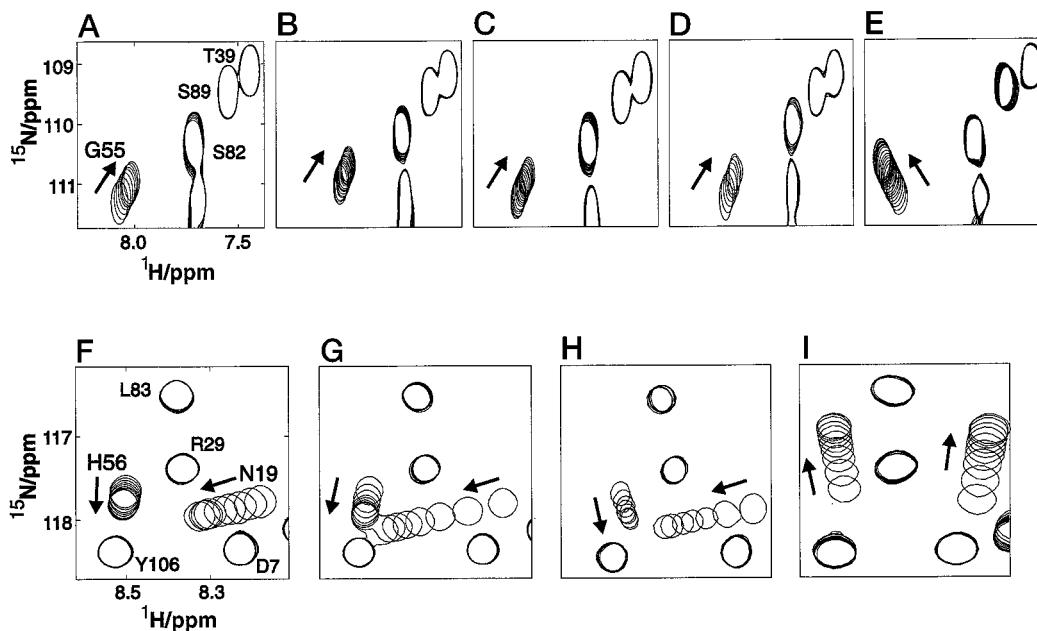
FIG. 1. **NMR investigation of PI 3-kinase p85α subunit SH2 domains with inositol polyphosphate and phosphoinositide ligands.** Superimposed contour plots of highlighted regions of two-dimensional ^{1}H-^{15}N HSQC NMR spectra obtained from titrations of 0.5 mM ^{15}N-labeled samples of the p85α N-SH2 domain (*A–E*) and C-SH2 domain (*F–I*) with increasing ligand concentration (0–1.0 mM). The ligands used for each experiment are D-InsP$_3$ (*A* and *F*), D-InsP$_4$ (*B* and *G*), L-InsP$_4$ (*C*), di-C$_6$-PIP$_3$ (*D* and *H*), and phenyl phosphate (*E* and *I*). Cross-peaks represent correlations between the ^{15}N and ^{1}H resonances of polypeptide backbone amide groups. A single contour level is plotted per step in the titration. Only a subset of cross-peaks change position during the titrations. For each set of titrations, the same spectral region is shown, revealing that di-C$_6$-PIP$_3$ and the inositol polyphosphates induce similar selective chemical shift perturbations in all titrations.

versus concentration of competitor and were fitted to the following equation using a nonlinear least-squares analysis: $R = R_{max}/[1 + (C/IC_{50})^P]$, where R_{max} is the response for SH2 binding in the absence of competitor, C is the concentration of competitor, and P is the Hill coefficient.

In Vivo Assays

Cell Culture—Mouse NIH3T3 fibroblasts were grown at 37 °C in a humidified atmosphere containing 10% CO$_2$ in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Life Technologies, Inc.) and penicillin/streptomycin (Life Technologies, Inc.). The cells were grown to confluence in 150-mm dishes and serum-starved in Dulbecco's modified Eagle's medium containing 0.5% (v/v) heat-inactivated fetal calf serum for 16 h.

Immunoprecipitations—Cells grown on 150-mm dishes were incubated for 15 min at 37 °C with wortmannin (100 nM in Me$_2$SO) or an equivalent volume of Me$_2$SO and subsequently stimulated with recombinant PDGF-β (100 nM) for 10 min at 37 °C. The dishes were then placed on ice, washed once in ice-cold PBS buffer (Life Technologies, Inc.), and lysed for 20 min on ice in 1 ml of lysis buffer (20 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 1%(w/v) Triton X-100, 2 mM EDTA, 10 mM NaF, 10 mM Na$_2$HPO$_4$, 10% (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 7 mM diisopropylphosphofluoridate, 1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 20 μM leupeptin, 18 μM pepstatin, 21 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM Na$_3$VO$_4$, 10 mM β-glycerophosphate, 1 mM tetrasodium pyrophosphate, 1 mM sodium molybdate). The cells were then scraped from the dishes and centrifuged for 20 min at 15,000 × g and 4 °C. The supernatant was collected and incubated with the relevant antibody with constant agitation at 4 °C for 2 h. Protein G-Sepharose CL-4B (Amersham Pharmacia Biotech) at 10 μl of bead slurry/sample was then added, and the incubation continued for 1 h at 4 °C on a wheel. The immunoprecipitates were washed three times in lysis buffer and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting or assayed for PI 3-kinase activity.

PI 3-Kinase Assays—PI 3-kinase activity was assayed on immunoprecipitates resuspended in 25 μl of 2× kinase buffer (40 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM dithiothreitol). PtdIns stored in CHCl$_3$ solution was dried, sonicated for 15 min in 50 mM Tris-HCl, pH 7.4, and added to a concentration of 0.2 mg/ml. The reactions (50-μl final volume) were started by the addition of 40 mM ATP, 10 μCi of [γ-^{32}P]ATP

(3000 Ci/mmol, Amersham Pharmacia Biotech), and 3.5 mM MgCl$_2$. Kinase reactions were stopped by the addition of 100 μl of 1 M HCl. For phospholipid extraction, 200 μl of 1:1 (v/v) CHCl$_3$/CH$_3$OH was added. The organic phase was collected and re-extracted with 40 μl of 1:1 (v/v) 1 N HCl/CH$_3$OH. The samples were then dried, resuspended in 30 μl of CHCl$_3$/ CH$_3$OH 1:1 (v/v), and spotted onto prechanneled silica gel 60 TLC plates (Whatman) that had been pretreated in 1% (w/v) oxalic acid, 1 mM EDTA, H$_2$O/CH$_3$OH (60:40 (v/v) and baked for 15 min at 110 °C. The plates were developed in propanol, 2 M acetic acid 65:35 (v/v), and the radioactive spots were quantified using a PhosphorImager (Molecular Dynamics).

Western Blotting—After SDS- polyacrylamide gel electrophoresis, polyacrylamide gels (7.5%) were transferred onto polyvinylidene difluoride membranes (Gelman Sciences) using a semi-dry blotter (Amersham Pharmacia Biotech). The membranes were then blocked for 1 h in PBS buffer containing 3% (w/v) nonfat dry milk, 0.1% (w/v) polyethylene glycol 20000. The relevant primary antibodies were diluted in PBS buffer and 0.05% (w/v) Tween 20 (PBS/Tween) and incubated with the membranes for 2 h. After extensive washing in PBS/Tween, the blots were incubated for 1 h with goat anti-mouse or anti-rabbit antibodies coupled to horseradish peroxidase (Dako) at 1:2000 dilution. The membranes were then washed in PBS/Tween, and the bands were detected using ECL (Amersham Pharmacia Biotech).

RESULTS

The Identification by NMR Spectroscopy of a Binding Site for Phosphoinositides and Inositol Polyphosphates on the p85α SH2 Domains—NMR spectroscopy was used to investigate the structural details of the interactions between the p85α SH2 domains and a range of candidate phosphoinositide and inositol polyphosphate ligands. ^{15}N-^{1}H HSQC NMR spectra were recorded during titrations of ^{15}N-labeled SH2 domain with unlabeled test ligands. It is well established that observations of chemical shift perturbations upon titration with a ligand can be a sensitive probe of the ligand binding site of a protein (37, 38).

During the titration, changes in ^{15}N and ^{1}H chemical shift values for each residue were monitored by measuring changes in the cross-peak positions of assigned resonances. For both p85α SH2 domains, the introduction of any of the phosphoinositide or inositol polyphosphate ligands tested resulted in
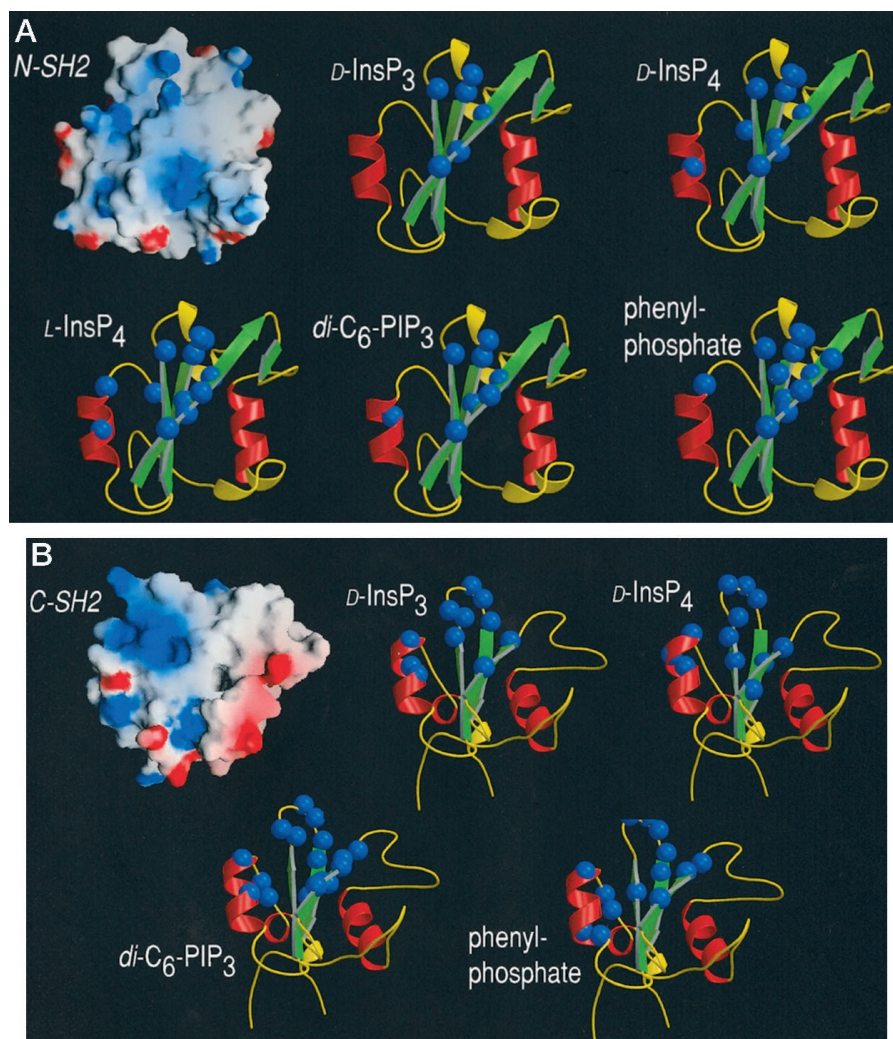
FIG. 2. **The ligand binding sites of p85α SH2 domain revealed in NMR titrations.** Aligned structures of the p85α N-SH2 domain (*A*) and C-SH2 domain (*B*), made using MOLSCRIPT (47), Raster3D (48), and GRASP (49), are shown. Secondary structure elements are represented in schematic form. The *blue balls* represent N-H groups, which displayed large chemical shift changes (modular vector sum ^{1}H and ^{15}N shift changes >40 Hz) upon the addition of the ligand. In all cases, the ligand binds between α-helix A (23, 39) and the opposing face of the β-sheet. In the upper left of each *panel*, the SH2 domain surface is colored by electrostatic potential to highlight the positively charged ligand binding site (*blue*); the *red* color corresponds to negatively charged surface. This conserved binding pocket is partly formed by a number of Arg and Lys residues, including the invariant Arg at the βB5 position (23).

significant chemical shift perturbations for a limited set of cross-peaks (Fig. 1). For both the p85α N- and C-SH2 domains, it was observed that the overall pattern of chemical shift changes induced by the addition of the ligands are similar in both direction and magnitude. The small differences in the perturbation direction seen in the case of phenyl phosphate (Fig. 1, *panels E* and *I*) result from additional ring-current shift effects induced by its aromatic group. Most notably, there seem to be very few differences when comparing the effects of D-Ins $(1,4,5)P_3$, D-Ins $(1,3,4,5)P_4$, L-Ins $(1,3,4,5)P_4$, or di-C$_6$-PIP$_3$. The results with D-Ins $(1,4,5)P_3$ and L-α-glycerophospho-D-*myo*-inositol 4,5-bisphosphate were also qualitatively similar to the data obtained with the other inositol polyphosphates (data not shown).

By mapping of the chemical shift perturbation data onto the three-dimensional protein structures, the ligand binding sites of the p85α N- and C-SH2 domains were determined. In all cases, it was seen that the SH2 domain residues affected by the ligands are similar and are localized to a discrete region of the protein structure (Fig. 2). The results obtained for di-C$_6$-PIP$_3$, the inositol polyphosphates, and phenyl phosphate show that all these ligands bind to the region corresponding to the Tyr(P) binding pocket seen in the high resolution structures of p85α SH2 domains (39, 40). From quantitative analysis of the chemical shift variation in the NMR studies, the equilibrium dissociation constants (K_D) of the interactions of the SH2 domains with di-C$_6$-PIP$_3$ and inositol polyphosphates were found to be between 0.5 and 1 mM. However, these averaged values contain ranging contributions from different residues involved in the

binding and therefore are best considered as approximation estimates. The absence of chemical shift perturbations for the majority of SH2 domain resonances suggests that the ligands tested neither induced long range conformational changes nor resulted in local or global unfolding of the protein structure.

Phosphoinositides and Inositol Polyphosphates Compete Poorly for the Binding of the p85α SH2 Domains to Tyrosine-phosphorylated Proteins—Because the results from NMR spectroscopy showed that phosphoinositides and inositol polyphosphates can all bind to the Tyr(P) binding pockets of both p85α SH2 domains, an assay was performed to assess whether these interactions were sufficiently strong to displace Tyr(P)-containing ligands. A biosensor-based competition assay was used to measure the binding of p85α SH2 domains to an immobilized Tyr(P) peptide with the sequence DMSKDESVDY(P)VPM-LDMK (Y(P) is phosphotyrosine). This phosphopeptide corresponds to the autophosphorylation site at Tyr^{751} of the PDGF-β receptor and is known to bind to both p85α SH2 domains with high affinity ($K_D \sim 200$ nM) (3). The biosensor results revealed that when present in relatively high concentrations, di-C$_6$-PIP$_3$ or any of the inositol polyphosphate ligands tested can compete for the interaction between the p85α SH2 domains and the immobilized Tyr(P) peptide ligand. However, it was readily apparent that free Tyr(P) peptide was a much more effective competitor than any of the other ligands tested, by a factor ~1000 (Fig. 3).

By curve-fitting the data to obtain IC$_{50}$ values for the interactions, it was observed that for each SH2 domain the phosphoinositide and inositol polyphosphates tested were similarly
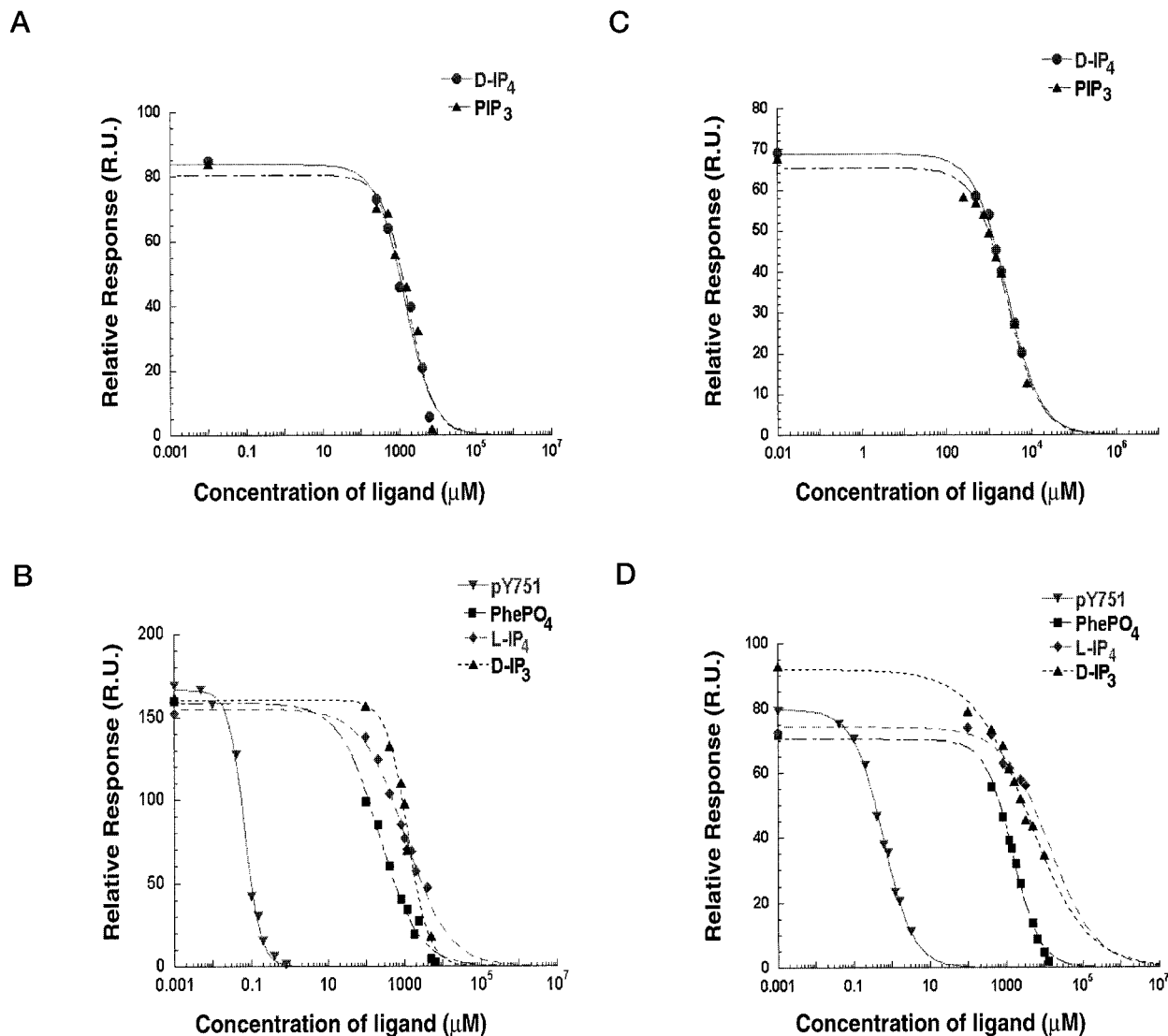
FIG. 3. **Biosensor competition assay.** Biosensor results showing the ability of ligand compounds to compete for the binding of p85α C-SH2 (*panels A* and *B*) and N-SH2 (*panels C* and *D*) domains to a tyrosine-phosphorylated ligand corresponding to the Tyr-751 (*pY751*) site of the cytoplasmic domain of the PDGF receptor. IC$_{50}$ values were determined by data fitting as described under "Experimental Procedures" and are listed in Table I. *Phe*, phenyl

competitive. Thus, no preference for ligand binding for either SH2 domain was observed. For example, for the C-SH2 domain, approximately the same IC$_{50}$ values were obtained for di-C$_6$-PIP$_3$, D-Ins (1,3,4,5)P$_4$, L-Ins (1,3,4,5)P$_4$, and D-Ins (1,4,5)P$_3$ (Fig. 3, *panels A* and *B* and Table I). Although the N-SH2 domain exhibited a pattern of interactions similar to that of the C-SH2 domain, the N-SH2 domain generally interacted even more weakly with all the compounds tested (Fig. 3, *panels C* and *D*). The IC$_{50}$ values obtained for the phenyl phosphate interactions were slightly smaller than for the phosphoinositide or inositol polyphosphate ligands but considerably larger than for the free Tyr(P) peptide (Fig. 3 and Table I). The latter result reflects that residues C-terminal to the Tyr(P) are also essential for the known physiological high affinity interaction.

The p85α C-SH2 Domain Does Not Display Distinct Binding Specificity for Phosphoinositides in Phospholipid Bilayers— The results presented above did not suggest that the p85α SH2 domains undergo specific interactions with water-soluble phosphoinositide or inositol polyphosphate ligands. However, to eliminate the possibility that the previous assays were not representative of interactions with phosphoinositides available *in vivo*, a second biosensor assay was performed. Using this alternative assay, it was shown previously that the Btk PH domain binds to phospholipid bilayers containing PtdIns (3,4,5)P$_3$ but not to those containing other negatively charged phosphoinositides (35). Subsequently, this result has been supported by numerous reports of a high affinity interaction between the Btk PH domain and PtdIns (3,4,5)P$_3$ or D-Ins (1,3,4,5)P$_4$ (15, 41, 42).

Thus, a second biosensor assay was performed to establish whether the p85α C-SH2 domain could bind to specific phosphoinositides when presented in large unilamellar liposomes (solely the C-SH2 domain was tested because the previous assay yielded IC$_{50}$ values that were smaller for the C-SH2 domain than for the N-SH2 domain). In brief, GST fusion SH2 domain was immobilized on an anti-GST antibody-coated surface, as described previously (35). Solutions of liposomes with differing phosphoinositide compositions were then injected over the surface, and the responses were observed. The experimental conditions used were exactly the same as those for the previously reported study of phosphoinositide interactions with the Btk PH domain (35). However, in contrast with the results obtained for the Btk PH domain, the p85α C-SH2 domain did not bind significantly to any of the phosphoinositide-containing

liposomes tested. When the liposome injection dosages were increased to more than 50 times the quantity sufficient to give clear binding signals when assaying PH domains (35), it was possible to observe a low level of nonspecific binding between the SH2 domain and the entire array of liposomes tested. However, the C-SH2 domain did not display a preference for binding to liposomes containing PtdIns $(3,4,5)P_3$ (Fig. 4). Furthermore, it was observed that under these rather extreme conditions, even a control GST protein exhibited a basal level of nonspecific binding to all the liposomes tested (data not shown).

<div align="center">TABLE I</div>

Inhibition of binding of phosphoinositide 3-kinase p85α subunit SH2 domains to an immobilized phosphotyrosine peptide in a biosensor assay

N-Biotinyl-DMSKDESVDY(P)VPMLDMK (corresponding to the Tyr-751 autophosphorylation site of the cytoplasmic domain of the platelet-derived growth factor receptor) was fixed to a precoated streptavidin biosensor chip. Solutions of 0.5 μM p85α N- or C-SH2 domain were injected over the surface at a flow rate of 5 μl/min at 25 °C, and the maximum response was recorded. Competition experiments were performed by incubating the SH2 domains with a competitor ligand before injection of the protein solution.

Protein	Ligand tested[a]	IC$_{50}$
		mM
C-SH2	Tyr(P)^{751} peptide	0.07×10^{-3}
C-SH2	di-C$_6$-PIP$_3$	1.4
C-SH2	D-InsP$_4$	1.1
C-SH2	L-InsP$_4$	1.1
C-SH2	D-InsP$_3$	1.2
C-SH2	PhPO$_4$	0.2
N-SH2	Tyr(P)^{751} peptide	0.59×10^{-3}
N-SH2	di-C$_6$-PIP$_3$	2.7
N-SH2	D-InsP$_4$	2.7
N-SH2	L-InsP$_4$	13.6
N-SH2	D-InsP$_3$	3.8
N-SH2	PhPO$_4$	1.4

[a] Tyr(P)^{751} peptide, SVDY(P)VPMLD; D-InsP$_4$, D-myo-inositol 1,3,4,5-tetrakisphosphate; L-InsP$_4$, L-*myo*-inositol 1,3,4,5-tetrakisphosphate; D-InsP$_3$, D-myo-inositol 1,4,5-trisphosphate; PhPO$_4$, phenyl phosphate.

However, in contrast with the results obtained for the Btk PH domain, it was observed that the p85α C-SH2 domain did not display a preference for binding to liposomes containing PtdIns $(3,4,5)P_3$ (Fig. 4). Indeed, the C-SH2 domain shows only a low level of binding to an array of liposomes with different phosphoinositide compositions, with no clear preference emerging for any of the phosphoinositides tested.

The Inhibition of PI 3-Kinase Activity Does Not Increase the Levels of Phosphotyrosine-bound p85α in Vivo—After the *in vitro* assays reported above, an *in vivo* experiment based on that reported previously (21) was performed to search for a correlation between the intracellular levels of Tyr(P)-bound p85α and PI 3-kinase products. This involved measuring the levels of p85α bound to the PDGF receptor and/or tyrosine-phosphorylated proteins after cell stimulation in the presence or absence of wortmannin. Because wortmannin inhibits PI 3-kinase activity (43), these experiments are taken to be in the presence or absence of 3′-phosphorylated phosphoinositides.

The stimulation of NIH3T3 fibroblasts by PDGF induced both the association of p85α with the PDGF receptor (Fig. 5*A*) and the appearance of p85α in anti-phosphotyrosine immunoprecipitates (Fig. 5*B*), as judged by the anti-p85α immunoblotting of anti-PDGF receptor and anti-phosphotyrosine immunoprecipitates, respectively. These experiments were then repeated, the only difference being the pretreatment of the fibroblasts with 100 nM wortmannin. The wortmannin treatment did not significantly affect the amount of p85α present in either anti-PDGF receptor immunoprecipitates (Fig. 5*A*) or in anti-phosphotyrosine immunoprecipitates (Fig. 5*B*). For control purposes, the efficacy of wortmannin with respect to PI 3-kinase inhibition was confirmed by the total inhibition of PI 3-kinase activity present in anti-phosphotyrosine immunoprecipitates after PDGF stimulation (Fig. 5*C*).

<div align="center">DISCUSSION</div>

We sought to verify whether the products of PI 3-kinase activity, 3′-phosphorylated phosphoinositides, can interact with the SH2 domains derived from the p85α regulatory subunit of PI 3-kinase itself. Such interactions have been proposed

FIG. 4. **Biosensor assay of p85α SH2 domain interactions with phosphoinositide-containing liposomes.** Biosensor measurements for interactions between liposomes and the p85α C-SH2 domain. The specific phospholipid tested in the liposome composition is indicated next to each sensorgram. *PC*, phosphatidylcholine
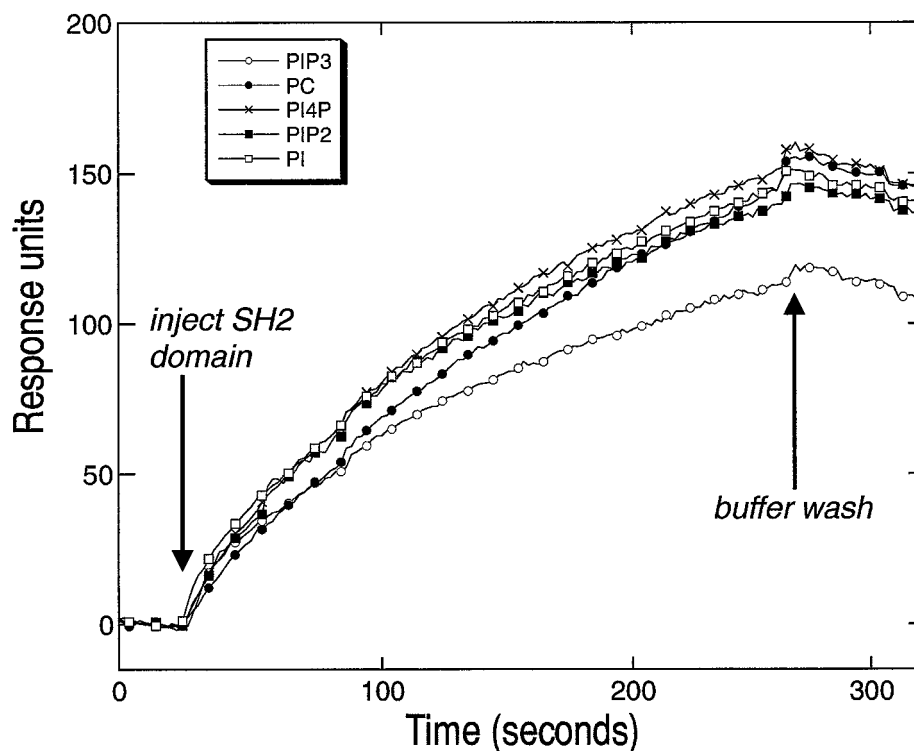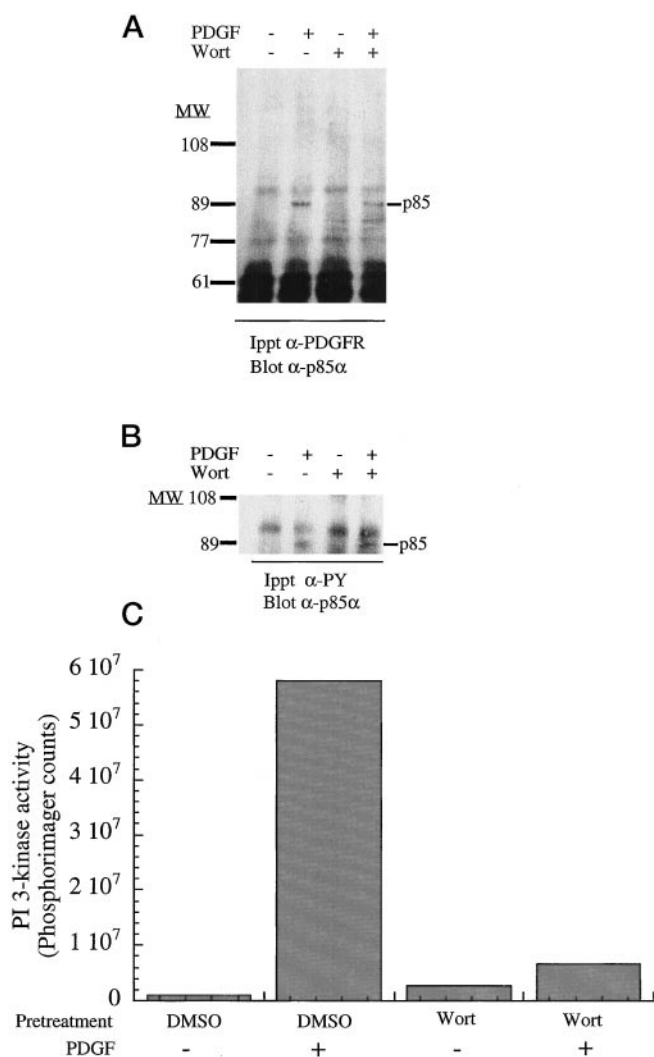
FIG. 5. **Lack of correlation of p85-phosphotyrosine interactions with wortmannin treatment.** Wortmannin, a PI 3-kinase catalytic domain inhibitor, does not promote the association of p85α with the PDGF receptor or, more generally, with tyrosine-phosphorylated proteins. Serum-starved NIH3T3 fibroblasts were treated with wortmannin (*Wort*, 100 nM) or Me₂SO (vehicle) for 15 min and stimulated with PDGF (100 nM) for 10 min, and cell lysates were immunoprecipitated with anti-PDGF receptor (*Ippt α-PDGFR*, *A*) or anti-phosphotyrosine (*Ippt α-PY*, *B* and *C*) antibodies and protein G-Sepharose. *A* and *B*, anti-phosphotyrosine immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and anti-p85α immunoblotting. *C*, the immunoprecipitates were assayed for PI 3-kinase activity. Radioactive lipids were analyzed by TLC. The results are representative of three independent experiments.

as competitors of the association of PI 3-kinase with tyrosine-phosphorylated proteins and as regulators of other SH2 domain-containing proteins, *e.g.* Src and phospholipase C γ-1 (21, 22). Although the SH2 domain-mediated phosphoinositide-dependent regulation of p85α/p110α, Src, or phospholipase C activity has interesting implications, it is a model yet to be clearly established.

Therefore, our primary experimental aim was to discover whether the p85α N- and C-SH2 domains can bind to distinct phosphoinositides with high affinity, specificity, and stereoselectivity. Furthermore, we investigated whether the well defined interactions between the p85α SH2 domains and Tyr(P)-containing ligands could be competed by phosphoinositides or inositol polyphosphates. We tested both phosphoinositides and inositol polyphosphates as potential ligands of SH2 domains. Inositol polyphosphates were in part used as conveniently wa-

ter-soluble analogues of the head groups of phosphoinositides but may also represent physiological ligands. In our opinion this usage of inositol polyphosphates is valid because protein-phosphoinositide interactions appear to be predominantly governed by the charge status, phosphorylation positions, and stereochemistry of the inositol ring (42, 44, 45). For example, it has been demonstrated that D-Ins $(1,4,5)P_3$ can functionally replace PtdIns $(4, 5)P_2$ in the activation of the dynamin GTPase (35) and that the Btk PH domain binds specifically to both D-Ins $(1,3,4,5)P_4$ and PtdIns $(3,4,5)P_3$ (35, 41) *in vitro* and to PtdIns $(3,4,5)P_3$ *in vivo* (15).

In search of specific phosphoinositide binding preferences of the p85α SH2 domains, we chose to compare their interactions with D-Ins $(1,4,5)P_3$ and D-Ins $(1,3,4)P_4$ or PtdIns $(4,5)P_2$ and PtdIns $(3,4,5)P_3$. This choice was based on the knowledge that PtdIns $(4,5)P_2$ is abundant in the plasma membrane of resting cells, whereas PtdIns $(3,4,5)P_3$ is only present in appreciable quantities after cell stimulation (8, 9). We also compared the binding of the p85α SH2 domains to the physiological D- and nonphysiological L-enantiomers of the inositol polyphosphates, because stereoselectivity should be exhibited in the case of true, biological interactions.

We observed that numerous different phosphoinositides and inositol polyphosphates can bind to the p85α SH2 domains, albeit weakly. Using NMR spectroscopy, we found that di-C₆-PIP₃ and all the inositol polyphosphates tested bound to the SH2 domains in the Tyr(P) binding pockets that accommodate protein ligands. However, the SH2 domains failed to display clear preferences for distinct phosphoinositides or inositol polyphosphates presented in solution. Similarly, the C-SH2 domain did not demonstrate a distinct binding specificity for phosphoinositides presented in phospholipid bilayers. Surface representations of the SH2 domain structures that display their calculated electrostatic potentials show that the Tyr(P) binding pockets of the N- and particularly of the C-SH2 domains are highly positively charged. Thus, the lack of binding specificity or stereoselectivity shown by the SH2 domains for the test ligands may reflect the likelihood that their interaction is largely based on electrostatic interactions that have little dependence on distinct structural features. Such interactions are thus very different from those of high specificity observed between SH2 domains and physiological Tyr(P)-containing ligands.

In addition, in a competition assay we observed that despite an overlap of binding sites, phosphoinositides and inositol polyphosphates only poorly displaced SH2 domains from a Tyr(P) peptide ligand. Furthermore, among the ligands tested, there was no significant variation in the efficacy of competition. From this assay, it also emerged that the N-SH2 domain bound to di-C₆-PIP₃ and inositol polyphosphates similarly to, but even more weakly than, the C-SH2 domain. This observation may perhaps be explained by two factors. First, the Tyr(P) binding pocket produces a greater density of positive charge on the surface of the C-SH2 domain compared with the surface of the N-SH2 domain (see Fig. 2), thus favoring interactions of the former with negatively charged ligands. Second, it has been observed that the unoccupied Tyr(P) binding pocket of the C-SH2 domain is relatively exposed, whereas that of the N-SH2 domain is not fully formed in the absence of a peptide ligand (39) and may therefore be less accessible to phosphoinositides.

However, the similar patterns of ligand binding observed for both p85α SH2 domains suggest that all the ligands contact the SH2 domains in the same, rather nonspecific manner. We conclude that although *in vitro* both p85α SH2 domains may interact weakly with PtdIns $(3,4,5)P_3$ and inositol polyphosphates, the lack of specificity of these interactions and their

inability to compete effectively with Tyr(P) peptide ligands suggest that they do not represent physiologically significant interactions. Rather, it seems that *in vitro* the SH2 domain Tyr(P) binding pocket has a tendency to bind somewhat indiscriminately to negatively charged ligands. This promiscuity is further witnessed in a crystal form of the p85α C-SH2 domain in which the Tyr(P) binding pocket accommodates an aspartate side chain.[2] Although the mode of interaction is highly reminiscent of Tyr(P) binding (coordination of the aspartate carboxylate group with both R36 and R18), the aspartate side chain is clearly not a consensus ligand.

Finally, we demonstrated that although *in vivo*, wortmannin blocks the activity of PI 3-kinase, it does not affect the ability of activated PDGF receptors (or other tyrosine-phosphorylated proteins) to bind the p85α regulatory subunit. These results are in agreement with characterizations of wortmannin activity (46) but contrast with previous reports of an inverse correlation between the level of 3′-phosphorylated phosphoinositides in the cell and the association of PI 3-kinase with tyrosine-phosphorylated proteins (insulin receptor and insulin receptor substrate) (21). Therefore we suggest the levels of PI 3-kinase products in the plasma membrane are unlikely to regulate signal transduction events through interactions with SH2 domains. Rather, we consider that the immediate targets of PI 3-kinase activity are represented by those proteins that display high affinity, distinct binding specificity, and stereoselectivity for 3′-phosphorylated phosphoinositides, such as the PH domain-containing proteins Akt, Btk, PDK-1, and phospholipase C γ-1.

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Structural and Biochemical Evaluation of the Interaction of the Phosphatidylinositol 3-Kinase p85 α Src Homology 2 Domains with Phosphoinositides and Inositol Polyphosphates

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