Specific Binding of the C-terminal Src Homology 2 Domain of the p85α Subunit of Phosphoinositide 3-Kinase to Phosphatidylinositol 3,4,5-Trisphosphate

LOCALIZATION AND ENGINEERING OF THE PHOSPHOINOSITIDE-BINDING MOTIF

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Phosphoinositide second messengers, generated from the action of phosphoinositide 3-kinase (PI3K), mediate an array of signaling pathways through the membrane recruitment and activation of downstream effector proteins. Although pleckstrin domains of many target proteins have been shown to bind phosphatidylinositol 3,4,5-trisphosphate (PIP3) and/or phosphatidylinositol 3,4-bisphosphate (PI3,4P2) with high affinity, published data concerning the phosphoinositide binding specificity of Src homology 2 (SH2) domains remain conflicting. Using three independent assays, we demonstrated that the C-terminal (CT-)SH2 domain, on the PI3K p85α subunit displayed discriminative affinity for PIP3. However, the binding affinity diminished precipitously when the acyl chain of PIP3 was shortened. In addition, evidence suggests that the charge density on the phosphoinositol ring represents a key factor in determining the phosphoinositide binding specificity of the CT-SH2 domain. In light of the largely shared structural features between PIP3 and PI3,4P2, we hypothesized that the PIP3-binding site on the CT-SH2 domain encompassed a sequence that recognized PI3,4P2. Based on a consensus PI3,4P2-binding sequence (KXXXXXXKK; K denotes Arg, Lys, and His), we proposed the sequence 19RNLAKILRLK28 as the PIP3-binding site. This binding motif was verified by using a synthetic peptide and site-directed mutagenesis. More importantly, neutral substitution of flanking Arg18 and Arg28 resulted in a switch of ligand specificity of the CT-SH2 domain to PI3,4P2 and PI3,4P2, respectively. Together with computer modeling, these mutagenesis data suggest a pseudosymmetrical relationship in the recognition of the phosphoinositoid head group at the binding motif.

Substantial evidence indicates that the lipid products of phosphoinositide 3-kinase (PI3K1), phosphatidylinositol 3,4,5-trisphosphate (PIP3) and phosphatidylinositol 3,4-bisphosphate (PI3,4P2), facilitate transmembrane signaling, in part, by serving as membrane-localization elements to recruit target proteins to specific sites (1, 2). Among various targets reported for these lipid second messengers, a series of signaling proteins containing Src homology 2 (SH2) or pleckstrin homology (PH) domains are of particular interest in light of their role in signal transduction (SH2 domains (3, 4); PH domains (5–12)). Conceivably, understanding the mode of this unique phosphoinositide recognition will shed light on the mechanism by which PI3K regulates downstream signaling pathways. Although recent investigations demonstrate that distinct PH domains have evolved selectivity for different phosphoinositides to provide discriminatory regulation (5), data pertaining to the interactions between SH2 domains and PIP3 are conflicting (3, 13). It has been proposed that PIP3 binds selectively to the p85 C-terminal (CT-)SH2 domain of PI3K, thereby blocking the binding of pTyr to tyrosine-phosphorylated proteins (3). This model, however, was refuted by a recent NMR study that showed lack of specific binding of PIP3 to p85 SH2 domains (13). Nevertheless, this question remains outstanding, because different structural analogues of PIP3 were used as binding ligands in these investigations, i.e. optically active diC16-PIP3 (3) versus a diastereomeric mixture of diC6-PIP3 (13).

Thus, this study is aimed at clarifying these conflicting observations, of which the biochemical impetus is multifold. First, binding specificity of protein modular domains for distinct phosphoinositides is central to the mechanism by which the specificity of PI3K signaling is maintained. Second, SH2 domains play a crucial role in phosphotyrosine (pTyr)-dependent protein recruitment for the assembly of initial signaling complexes (14, 15). The displacement of pTyr by PIP3 in the SH2 domain binding may lead to a redistribution of PI3K in the

1 The abbreviations used are: PI3K, phosphoinositide 3-kinase; PIP3, 1-α-phosphatidyl-D-myoinositol 3,4,5-trisphosphate, dipalmitoyl (all phosphoinositides used in this study were all dipalmitoyl derivatives in optically active form unless otherwise mentioned); PI3,4P2, 1-α-phosphatidylinositol 3,4-bisphosphate (other phosphoinositides are abbreviated similarly); PI, 1-α-phosphatidylinositol 3-phosphate; Ins1,3,4,5P4, 1-α,3,4,5-tetraakisphosphate; SH2 domains, Src homology 2 domains; NT-SH2, N-terminal SH2; CT-SH2, C-terminal SH2; PH domain, pleckstrin homology domain; GST, glutathione S-transferase; pTyr, phosphotyrosine; PLC, phospholipase C; diC8, dihexanoyl; diC16, dipalmitoyl.

2 The atomic coordinates and structure factors (code 1QAD) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
phosphorylation of the CT-SH2 domain displayed clear binding specificity and affinity for PI(3,5)P_2, we hypothesized that the PI(3,5)P_2-recognition motif in the CT-SH2 domain encompassed a sequence that bound PI(3,5)P_2. Based on a consensus sequence for PI(3,5)P_2 binding (KXXXXXXXK) reported by Yin and co-workers (16), we proposed an internal peptide fragment, RKK(18)RKK(28)AGCAACGCAAACAAAGCCG-3', as the putative PI(3,5)P_2-binding site. This binding motif was verified by two independent approaches. First, a synthetic peptide containing this sequence exhibited distinctive binding specificity and affinity for PI(3,5)P_2. In addition, it was able to inhibit PI(3,5)P_2 binding to the full-length protein in a dose-dependent manner and acquired an ordered conformation upon PI(3,5)P_2 binding. Second, substitution of any of the five basic residues with Ala within the PI(3,5)P_2-binding site substantially diminished the binding affinity of the CT-SH2 domain to PI(3,5)P_2. More importantly, mutations at the flanking Arg residues (Arg(18) and Arg(28)) resulted in a switch in ligand specificity of the CT-SH2 domain to PI(3,4,5)P_3 and PI(3,4)P_2, respectively. In conjunction with these mutagenesis data, computer modeling suggests a pseudosymmetrical relationship in the interaction between the phosphoinositol head group and the binding motif. Together, these results confirm the previous observation that the p85a CT-SH2 domain binds to PI(3,5)P_2 with distinct specificity.

EXPERIMENTAL PROCEDURES

Materials—All phosphoinositides used in this study were in the optical active form with enantiomeric excess greater than 99%. 1-O-(1,2-Di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol 3,4,5-trisphosphate (PI(3,4,5)P_3), 1-O-(1,2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol 3,4,6-trisphosphate (PI(3,4,6)P_3), 1-O-(1,2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol 3,5,6-trisphosphate (PI(3,5,6)P_3), 1-O-(1,2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol 4,5,6-trisphosphate (PI(4,5,6)P_3), 1-O-(1,2-di-O-octanoyl-sn-glycero-3-phosphoryl)-D-myoinositol 3,4,5-trisphosphate (dIc_2(PI(3,4,5)P_3)), 1-O-(1,2-di-O-butanoyl-sn-glycero-3-phosphoryl)-D-myoinositol 3,4,5-trisphosphate (dIc_4(PI(3,4,5)P_3)), 1-O-(1,2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol 3,4-bisphosphate (PI(3,4)P_2), 1-O-(1,2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol 4,5-bisphosphate (PI(4,5)P_2), 1-O-(1,2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol 3-monophosphate (PI(3)P), 1-O-(1,2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol 4-monophosphate (PI(4)P), and 1-O-(1,2-di-O-palmitoyl-sn-glycero-3-phosphoryl)-D-myoinositol (PI) were prepared as previously reported (17, 18). 1-[(3H)]dIc_2(PI(3,4,5)P_3) was prepared according to a modified procedure for the nonradioactive counterpart. 2 All synthetic phosphoinositides were characterized by ^1H and ^13C NMR and fast atom bombardment mass spectrometry. No appreciable impurity was noted within the detection limits. The peptide used in this study was synthesized by an Applied Biosystem peptide synthesizer in the Macromolecular Structure Analysis Facility and was characterized by fast atom bombardment mass spectrometry in the Mass Spectrometry Facility at University of Kentucky.

Preparation of the N- and C-terminal SH2 Domains of the p85 Subunit of PI3K—The pGEX vector containing the cDNA sequence encoding the p85a NT-SH2 domain or the p85a CT-SH2 domain was expressed in Escherichia coli as a GST fusion protein by isopropyl-1-thio-β-D-galactopyranoside induction (3). The bacterial lysates were incubated with glutathione-coupled Sepharose 4B beads (Sigma Chemical Co.) for 2 h at 4 °C and washed three times with 30 ml Hepes, pH 7.0, containing 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40, followed by the fusion protein without Nonidet P-40 twice. The beads were washed and centrifuged, and the bead-washed probe was used for the subsequent fluorescence titration and gel filtration analyses. For the preparation of free SH2 domains, the immobilized protein was subjected to Factor Xa hydrolysis at 4 °C overnight, followed by centrifugation at 2000 × g for 5 min. The supernatant that contained the free SH2 domain was recovered, and concentrated by ultrafiltration. The homogeneity of isolated SH2 domains was indicated by a single band on 15% SDS-polyacrylamide gel electrophoresis with molecular masses of 14 and 20 kDa for NT-SH2 and CT-SH2 domains, respectively.

Site-directed Mutagenesis—Individual point mutation were introduced into the plasmid pGEXCT-SH2 encoding the wild type protein by using the QuikChange site-directed mutagenesis kit from Stratagene and each of the following mutagenesis primers: R18A, 5'-GGGGGAGCCACCAAGCCAAAAACGGC-3'; K20A, 5'-GCAACACAGGAACGCGCCGGCAGAAC-3'; R28A, 5'-GGCGGAACCTATTGGCGAGAAAAGAG-3'; R28A, 5'-CTATTGCGAGGGGCGAGATGGC-3', and R29A, 5'-GGCGAGGAAGAGGATGCATTTC-3'. The desired point mutation and sequence flanking the mutagenic primer- annealing site was confirmed by DNA sequencing analysis.

Lipid Binding Assay—A mixture of [32P]PI(3)P, [32P]PI(3,4)P_2, and [32P]PI(4,5)P_2, was prepared by incubating the type 1 brain extract (Sigma) with purified p85 in the presence of excess phosphatidylinosine as previously described (19). The mixture (100 μl) was incubated at 37 °C for 1 h and stopped by adding 5 μl of 1 mM EDTA and 25 μl of 5 mM HCl, followed by 160 μl of CHCl_3/CH_3OH (1:1, v/v). After mixing by vortex for 30 s, the phases were separated by centrifugation at 6000 × g for 5 min. The organic layer was dried by a stream of nitrogen. The dried lipids were resuspended in 200 μl of 10 mM Hepes, pH 7.0, containing 1 mM EDTA, and sonicated in a water bath-type sonicator for 5 min. The lipid micelles (10 μl) were added to 40 μl of Sepharose beads containing ~12 μg of the GST fusion protein in 30 mM Hepes, pH 7.0, containing 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40. The incubation was carried out for 1 h at room temperature, and the beads were quickly washed twice with 1 ml of the same buffer. Lipids that were associated with the Sepharose beads were extracted with CHCl_3/CH_3OH (1:1, v/v), dried, and spotted onto 1% oxalic acid-treated TLC plates. The plates were developed with n-propanol:2 M acetic acid (65:35) for overnight. Autoradiograms were loaded onto autoradiography film and compared with standards. The autoradiograms were scanned by a Fotodyne image system and quantified using the IMAGE program from the National Institutes of Health (version 1.59).

Micelle Preparation—Homogeneous phosphoinositide micelles were prepared according to a published procedure (20, 21). In brief, pure phosphoinositides were suspended in buffered solution and sonicated in a bath-type sonicator for 5 min at room temperature.

Gel Filtration Analysis—The analysis was based on the principle that binding of a protein (or peptide) to PI(3,4,5)P_3-containing micelles will change its elution profile on gel filtration chromatography. Protein binding to micellar phosphoinositides was indicated by the co-elution of the protein at the void volume with the lipid vesicles, while free protein was retained. The SH2 full-length protein (or the peptide fragment) was incubated with lipid vesicles consisting of varying amounts of pure PI(3,4,5)P_3 in a final volume of 100 μl, for 30 min at room temperature. The mixture was chromatographed on a Sephacryl S-200 column (1 × 10 cm) equilibrated with 10 mM Tris/HCl, pH 7.5, containing 75 mM KCl. For the full-length protein, 1 mM dithiothreitol was included in the elution buffer. The column was eluted with the same buffer at 0.5 ml/min, and fractions of 0.45 ml were collected. Protein concentrations were analyzed by the Bradford assay. The lipid vesicle-bound peptide/protein was eluted in the void volume and was well separated from the free peptide/protein. The amount of micelle-bound peptide/protein was calculated as the difference between the total amount applied to the column and the amount of the free peptide/protein. The apparent dissociation constant (K_d) was estimated according to Equation 1 as follows.

\[ K_d = \frac{[	ext{peptide}]_0}{[	ext{phospholipid}]_0} \cdot \frac{[\text{peptide-phospholipid complex}]}{[\text{peptide-lipid complex}]} \] (Eq. 1)

Fluorescence Spectroscopy—Interactions between free SH2 domains and PI(3,5)P_2 were assessed by monitoring the tryptophan fluorescence with an excitation wavelength at 292 nm. The buffer used for the fluores-
The immobilized SH2 domains were exposed to a mixture containing [32P]PIP3, [32P]PI(3,4)P2, and [32P]PI(3)P as described. The solution contained 40 μM of the CT-SH2 peptide fragment in the presence of six molar equivalents of PIP3. The following settings were used: wavelength range, 200–250 nm; bandwidth, 1 nm; step resolution, 0.5 nm; scan speed, 10 millidegrees/min. Each spectrum represented an average of ten scans with baseline subtraction.

RESULTS

The p85 CT-SH2 Domain Displays a High Degree of Specificity in Phosphoinositide Binding—To demonstrate the phosphoinositide binding specificity of p85α SH2 domains, we used three independent analyses, i.e. radioligand binding, fluorescence titration, and gel filtration. In the radioligand experiment, phosphoinositide vesicles containing equal amounts of [32P]PIP3, [32P]PI(3,4)P2, and [32P]PI(3)P were exposed to the respective GST fusion proteins immobilized onto glutathione-coated beads. It has been shown that GST did not interfere with PIP3 binding (3). After incubation, the immobilized protein was separated from the incubation mixture by centrifugation. SH2 domain-bound phosphoinositides were extracted and analyzed by TLC then by autoradiography (Fig. 1).

This competitive radioligand binding experiment shows that both NT- and CT-SH2 domains exhibited preferential binding to PIP3. However, the PIP3-binding affinity of the NT-SH2 domain was substantially lower than that of the CT-SH2 counterpart, by nearly two orders of magnitude. To confirm this observation, we further used fluorescence titration and gel filtration to examine the differential recognition of phosphoinositides by these SH2 domains. Both methods, especially gel filtration, have been applied to the analysis of phosphoinositide binding in a number of proteins including profilin (20, 21), gelsolin (22), PLCγ PH domain (23), dynamin PH domain (24), the Listeria monocytogenes ActA protein (22), and actophorin (26).

To eliminate potential interference by the GST moiety in these analyses, we isolated the free SH2 domain by subjecting the respective immobilized fusion protein to Factor Xa hydrolysis, followed by centrifugation. Fig. 2A shows the quenching effect of PIP3 on the tryptophan (Trp) fluorescence of the CT-SH2 domain, which, upon excitation at 292 nm, displayed an emission maximum at 330 nm. Micellar PIP3 attenuated the Trp fluorescence in a dose-dependent and saturable manner, whereas PIP3 alone did not show appreciable emission (data not shown). Accordingly, the $K_d$ values were determined by fluorescence titration as described under “Experimental Procedures.” Represented are means ± S.D. of three independent experiments.

Other phosphoinositides examined showed significantly lower binding affinity, with $K_d$ values ranging from 4- to 20-fold higher than that of PIP3. These results were consistent with the above competitive binding data. In contrast, when the NT-SH2 domain was analyzed, its Trp fluorescence was very little perturbed by PIP3 (not shown). The fluorescence quenching could not be saturated even with 60 molar excess of PIP3, confirming its low binding affinity with the NT-SH2 domain.

With regard to the gel filtration analysis (not shown), apparent $K_d$ values for the CT-SH2 domain binding were estimated to be 23 ± 3 μM for PIP3 ($n = 3$), 110 ± 25 μM for PI(4,5)P2 ($n = 3$), and 300 μM for PI(3,4,5)P3. Again, with the NT-SH2 domain, no significant protein adsorption was noted with lipid vesicles consisting of PIP3 or any other phosphoinositide examined.

Binding Affinity of the CT-SH2 Domain to PIP3 Is Affected by the Size of Acyl Chains—To examine whether the diacylglycerol moiety played a role in the interaction of PIP3 with the CT-SH2 domain, we analyzed the binding of the CT-SH2 domain to the diCn and diCn derivatives of PIP3 and Ins(1,3,4,5)P4, the head group of PIP3, by fluorescence titration. An inverse relationship was noted between the acyl chain length and the $K_d$ value, i.e. diCn-PIP3, 23 ± 3 μM ($n = 3$); diC4-PIP3, 93 ± 5 μM ($n = 3$); diC7-PIP3, > 500 μM. With regard to Ins(1,3,4,5)P4, no significant binding to the CT-SH2 domain was noted. These data indicate the involvement of the acyl chain of PIP3 in the interaction with the CT-SH2 domain.
Elucidation of the PIP₃-binding Motif on the p85 CT-SH2 Domain.—Previously, we reported that PIP₃ could be recognized by a synthetic peptide in a selective manner (27), suggesting that PIP₃ binding might be achieved by a contiguous peptide fragment. Because PIP₃ contains all the structural features of PI(4,5)P₂ with an additional 3-phosphoryl function on the inositol ring, we hypothesized that the recognition motif for PIP₃ encompassed the PI(4,5)P₂-binding sequence with additional basic residue(s) flanking either the N or C terminus for interacting with the 3-phosphate. Previously, Yin and co-workers (16) delineated a consensus sequence for PI(4,5)P₂ binding, i.e., KXXXKKK (K denotes Lys, Arg, and His), by analyzing a series of PI(4,5)P₂-binding proteins (16). By examining the amino acid sequence of the CT-SH2 domain (Fig. 3), we proposed the internal sequence RNKAENLLRGRKR, corresponding to amino acids 18–29 on the helix αA, as the putative PIP₃-binding motif. This binding motif was verified by two independent approaches, i.e., a synthetic peptide and site-directed mutagenesis.

**Direct Interactions of PIP₃ with a Peptide Fragment Derived from the CT-SH2 Domain**—To examine whether the proposed sequence could interact with PIP₃, we synthesized the peptide corresponding to amino acids 11–29 (WNVGSSNRRKAENLLRGRKR) of the CT-SH2 domain. Interactions of this synthetic CT-SH2 peptide with various phosphoinositides were assessed by gel filtration. It is noteworthy that the CT-SH2 peptide displayed a high degree of selectivity in phosphoinositide recognition. It bound PIP₃ with $K_d$ of $30 \pm 7 \mu M (n = 3)$, which was in line with that of the full-length CT-SH2 protein. More importantly, the binding affinity of other phosphoinositides examined was one to two orders of magnitude lower than that of PIP₃. The $K_d$ values were: PI(4,5)P₂ (350 ± 30 μM, n = 3), PI(3,4)P₂ (>500 μM), PI(3)P (>1 mM), and PI(4)P (>1 mM). Phospholipids lacking the phosphoinositol head group, such as PI, phosphatidylserine, and phosphatidylcholine, showed no appreciable binding to the peptide. This differential recognition strongly argues against the possibility that the binding arose from nonspecific charge-charge interactions. This specific PIP₃-peptide interaction was further supported by two lines of evidence.

First, this peptide fragment inhibited the binding of PIP₃ to the full-length CT-SH2 domain in a dose-dependent manner. To facilitate the radioligand binding assay, the CT-SH2 domain was biotinylated with p-diazobenzoyl biocytin, which allowed the protein to be immobilized onto streptavidin-coated beads. As shown, the CT-SH2 peptide fragment blocked the binding of $[^{3}H]$diC₈-PIP₃ to the immobilized CT-SH2 domain in a competitive manner. The IC₅₀ was estimated to be $62 \mu M$ (Fig. 4A). The concentration range for the complete inhibition lied within one log unit, indicating that the binding of PIP₃ to the peptide was specific.

Second, the CT-SH2 peptide acquired a partially ordered structure by moving from an unbound form to a PIP₃-bound state. Fig. 4B shows the circular dichroism spectra of the peptide alone (trace a) and in the presence of 6 μM equivalents of diC₈-PIP₃ (trace b) in the far UV range between 200 and 250 nm. The spectra underwent marked changes when PIP₃ was added, in which absorption around 222 nm was indicative of α-helical structures.

**Site-directed Mutagenesis Alters the Ligand Specificity of the CT-SH2 Domain**—Comparing the putative PIP₃-binding motif (RNKAENLLRGRKR) with the consensus sequence for PIP₂ binding (KXXXXXXXKKK) we hypothesized that Arg₁₈ was involved in direct interactions with the 3-phosphoryl group of the inositol ring. Accordingly, neutral substitution of Arg₁₈ would result in a sequence conforming to the consensus sequence for PI(4,5)P₂ binding. This modification might change the ligand specificity of the resulting CT-SH2 domain from PIP₃ to PI(4,5)P₂. To address this issue, we performed site-directed mutagenesis to replace each of the five basic residues within the region (Arg₁₈, Lys₂₀, Arg₂₆, Lys₂₈, and Arg₃₀) with alanine. By designing mutagenic primers to induce single base pair changes in the cDNA sequence for the p85 CT-SH2 domain, we generated these five mutant clones to which we assigned the names R₁₈A, R₂₀A, R₂₆A, K₂₈A, and R₃₀A according to the wild type amino acid altered, its position in the wild type sequence, and the amino acid substituted in its place. All of these mutants were successfully expressed in E. coli as GST fusion proteins and purified by affinity chromatography on glutathione beads. The fusion proteins were treated with Factor Xa to isolate the mutated CT-SH2 domains. Milligram quantities of these mutant proteins were obtained. Each of these proteins showed a single protein band on SDS-polyacrylamide gel electrophoresis with molecular mass identical to that of the wild type protein (data not shown).

The effect of these mutations on the interactions with phosphoinositides is summarized in Fig. 5.

In line with our hypothesis, R₁₈A exhibited enhanced binding affinity with PI(4,5)P₂ accompanied by a 16-fold decrease in PIP₃ binding affinity vis à vis the wild type, resulting in a switch in ligand preference from PIP₃ to PI(4,5)P₂. In contrast,
the impact of this amino acid replacement on PI(3,4)P₂ binding was limited, i.e. ~20% increase in $K_d$. Neutral substitution of any of the other four basic residues with alanine also attenuated PIP₃ binding, although to a lesser extent as compared with R18A. It is noteworthy that mutations at Arg²⁶, Lys²⁸, and Arg²⁹ had a positive effect on PI(3,4)P₂ binding (50% to 86% reduction in $K_d$ values). Moreover, switch in ligand specificity from PIP₃ to PI(3,4)P₂ was noted in R29A, underscoring the importance of Arg²⁹ in interacting with the 5-phosphoryl function on the inositol ring. To the best of our knowledge, this is the first report on the change in phosphoinositide binding specificity by site-directed mutagenesis.

**The CT-SH2 Domain Displays Relaxed Regiospecificity in Interacting with the Three Phosphoryl Functions on the Head Group of PIP₃**—To shed light on the mode of phosphoinositide recognition, we synthesized three permuted isomers of PIP₃ (PI(3,4,6)P₃, PI(3,5,6)P₃, and PI(4,5,6)P₃), and examined their binding affinity with the CT-SH2 domain vis-à-vis PIP₃ by fluorescence titration (Fig. 6).

It is interesting to note that the CT-SH2 domain exhibited a high degree of tolerance with regard to the regioisomeric arrangement of the three phosphate functions. As shown, except PI(4,5,6)P₃, they bound to the CT-SH2 domain with comparable affinity despite variation in the position of phosphoryl groups. This observation suggests that the charge density on the phosphoinositol ring represents a key factor in the binding, i.e. the effective binding is facilitated by three adjacent phosphoryl functions, regardless of their relative positions on the ring.

**DISCUSSION**

Although the PI(4,5)P₂-binding motif on various proteins has been extensively studied (e.g. α-actinin (28), actophorin (26), cofilin (29), gelsolin (16, 30), PLCβ2 (31), profilin (32), villin (30)), information concerning the mode of PIP₃ recognition has focused mainly on a subset of PH domains (33–36). This study is aimed at resolving a controversial issue of whether p85α SH2 domains could interact with PIP₃ in a selective manner. The present data demonstrate that the CT-SH2 domain, but not the NT-SH2 domain, displays specific binding to PIP₃. Our data indicate that the acyl chain of PIP₃ plays a crucial role in CT-SH2 domain binding. Decrease in the chain length precipitously attenuates the binding affinity with PIP₃. The $K_d$ values increase from 23 μM to over 500 μM when the acyl group is shortened from C₁₆ to C₄. This observation provides a plausible explanation for the lack of specific interaction between diC₆-PIP₃ and the CT-SH2 domain in the NMR analysis (13). In addition to the short acyl chain, only 25% of the diastereomeric mixture of diC₆-PIP₃ represents the physiologically relevant isomer, of which the impact on the binding affinity with the SH2 domain remains unclear.

In light of our hypothesis that the PIP₃-binding motif contains a PI(4,5)P₂-binding sequence (KXXXXXXKXX), we propose the internal peptide fragment 18RNKAENLLRGKR²⁹ as the PIP₃-binding site. This motif resides in the helix αA that is readily accessible to the milieu. Furthermore, it is located near the pTyr-binding motif FLVR. The close proximity between these two motifs is in line with the observation that PIP₃ binding and tyrosine-phosphorylated peptide binding to the CT-SH2 domain were mutually exclusive (3).

This PIP₃-binding site was confirmed by two independent approaches that employed a synthetic peptide and site-directed mutagenesis, respectively. The synthetic peptide displays discriminative binding affinity with PIP₃ and competes with the full-length protein for PIP₃ binding in a dose-dependent manner. Moreover, the $K_d$ value of the synthetic peptide with PIP₃...
is consistent with that of the full-length CT-SH2 domain (30 versus 23 μM), indicating that this contiguous peptide fragment contributes to the PIP₃ binding.

The mutagenesis study indicates that neutral substitution of any of the five basic residues within the binding sequence resulted in a considerable decrease in the binding affinity with PIP₃. It is especially noteworthy that modification of the flanking Arg₁⁸ and Arg₂⁹ leads to changes in the ligand specificity to P(4,5)P₂ and P(3,4)P₂, respectively. This observation underlines the importance of these two residues in interacting with the 3- and 5-phosphoryl functions on the inositol ring. Together, these data suggest a working hypothesis that depicts the mode of interaction between the binding site and the phosphoinositide head group of PIP₃ (Fig. 7A).

Fig. 7. A, a working model depicting the pseudosymmetrical relationship in the recognition of the phosphoinositide head group of PIP₃ at the binding motif. B, surface rendering of the p85α CT-SH2 domain. Coordinates were obtained from the Protein Data Bank data base (code 1QAD). The electrostatic potential was mapped to the solvent accessible surface of CT-SH2 using program GRASP. The unit of the surface potential was kT. Docking of PIP₃ was performed using INSIGHTII. The three basic residues Lys²⁰, Arg²⁶, and Lys²⁸ form a single basic patch on the surface of the CT-SH2 domain, representing part of the PIP₃-binding site.

As shown, the electrostatic interactions between the basic residues and phosphate functions exhibit a high degree of symmetry surrounding the central Arg²⁶. Disruption of this pseudosymmetrical relationship not only decreases the PIP₃ binding affinity, but also alters the phosphoinositide specificity. To gain insight into this molecular interaction, we mapped the electrostatic potential onto the solvent accessible surface of the CT-SH2 domain (Fig. 7B). As shown, Lys²⁰, Arg²⁶, and Lys²⁸ cluster to create a single basic patch on the surface of the CT-SH2 domain with direct interaction with the phosphoryl functions at positions 3, 4, and 5, respectively, on the phosphoinositide ring of PIP₃. The docking experiment indicates a perfect fit of three-point attachment between these two components. Consequently, mutation at any of these three basic residues diminished the binding affinity. Furthermore, this computer modeling suggests that Arg₁⁸ and Arg₂⁹ play a crucial role in governing the ligand specificity by regulating the helical structure. These two Arg residues, located at the N- and C-terminal ends of αA, are distant from the basic triad, and the guanidino moieties are oriented away from the phosphoinositol head group. Because alanine has a strong helix propensity, it is assumed that substitution of either Arg residue with alanine might affect the helicity of αA. As a consequence, the mutation R18A disrupts the interaction of the nearby Lys²⁰ with the 3-phosphate while maintaining the structural integrity of Arg²⁶ and Lys²⁸. This conformational modification results in a change in the ligand specificity to P(4,5)P₂. Likewise, the R29A mutation would impose a conformational effect limited to
Lys28, resulting in a switch of the binding preference to PIP3.

Our binding data indicate that the regioisomeric arrangement of the phosphoinositide functions does not have a significant impact on the binding to the CT-SH2 domain. Apparently, the charge density on the phosphoinositol ring represents a key factor in determining the phosphoinositide binding specificity of the CT-SH2 domain. This ligand specificity is achieved through the participation of all three phosphates on the inositol ring, regardless of their relative positions on the ring.

It is noteworthy that the mode of PIP3 recognition by the CT-SH2 domain is distinctively different from that proposed for the Grp1 PH domain (36). The signature motif for phosphoinositide binding on the PH domain consists of three separate sequences in the phosphoinositide binding specificity and affinity between these two protein modular domains. Moreover, sequence analysis indicates that the SH2 domains of a number of signaling proteins (37) contain internal sequences homologous to the FLVR sequence.

The bold residues denote basic amino acids, and the underlined region represents the putative PIP3-binding motif.

Among these proteins, the N-terminal (NT) SH2 domain of PLCγ1 has been shown to bind PIP3 (3, 4). Thus, this sequence may be of predictive value for the putative PIP3-binding site, which remains to be investigated.

In summary, this study demonstrates the specific binding of the p85α CT-SH2 domain to PIP3 and identifies the phosphoinositide-binding site. Data from site-directed mutagenesis suggest that interactions between PIP3 and its recognition site display a high degree of symmetry. Disruption of this pseudo-symmetrical relationship results in a change in ligand preference to either PIP4,5P2 or PIP3,4P2. This switch in binding specificity suggests a mechanism whereby phosphoinositide-binding proteins have evolved distinct binding selectivity, which constitutes the focus of the present study.

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