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Structural Basis for Recognition of the T Cell Adaptor Protein SLP-76 by the SH3 Domain of Phospholipase C γ 1

Lu Deng[†], C. Alejandro Velikovsky[†], Chittoor P. Swaminathan, Sangwoo Cho, Roy A. Mariuzza^{*}, and R. Huber

Center for Advanced Research in Biotechnology, W. M. Keck Laboratory for Structural Biology, University of Maryland Biotechnology Institute, Rockville, MD 20850 USA

Abstract

The enzyme phospholipase C γ 1 (PLC γ 1) is essential for T cell signaling and activation. Following T cell receptor ligation, PLC γ 1 interacts through its SH2 and SH3 domains with the adaptors LAT and SLP-76, respectively, to form a multiprotein signaling complex that leads to activation of PLC γ 1 by Syk tyrosine kinases. To identify the binding site for PLC γ 1 in SLP-76, we used isothermal titration calorimetry to measure affinities for the interaction of PLC γ 1-SH3 with a set of overlapping peptides spanning the central proline-rich region of SLP-76. PLC γ 1-SH3 bound with high specificity to the SLP-76 motif ¹⁸⁶PPVPPQRP¹⁹³, which represents the minimal binding site. To understand the basis for selective recognition, we determined the crystal structures of PLC γ 1-SH3 in free form, and bound to a 10-mer peptide containing this site, to resolutions of 1.60 Å and 1.81 Å, respectively. The structures reveal that several key contacting residues of the SH3 shift toward the SLP-76 peptide upon complex formation, optimizing the fit and strengthening hydrophobic interactions. Selectivity results mainly from strict shape complementarity between protein and peptide, rather than sequence-specific hydrogen bonding. In addition, Pro193 of SLP-76 assists in positioning Arg192 into the compass pocket of PLC γ 1-SH3, which coordinates the compass residue through an unusual aspartate. The PLC γ 1-SH3/SLP-76 structure provides insights into ligand binding by SH3 domains related to PLC γ 1-SH3, as well as into recognition by PLC γ 1 of signaling partners other than SLP-76.

Keywords

crystal structure; SH3; calorimetry; T cell signaling; phospholipase C γ 1

The enzyme phospholipase C γ 1 (PLC γ 1) is an essential signal transducing element in T cell activation and development.^{1,2} This multidomain protein comprises two Src homology 2 (SH2), one Src homology 3 (SH3), one pleckstrin homology (PH), and two catalytic domains (lipase and C2).³ PLC γ 1 interacts with tyrosine-phosphorylated sites on target proteins through its SH2 domains, and with proline-rich sequences through its SH3, resulting in formation of specific complexes that mediate T cell signaling.^{1,2,4} In addition, PLC γ 1 plays a pivotal role in cellular growth and proliferation through interactions with various growth factor receptors, such as epidermal growth factor receptor,^{3,5–7} and has been shown to function as a guanine nucleotide exchange factor for the nuclear GTPase PIKE.⁸

Following engagement of the T cell receptor (TCR) by MHC/peptide ligands, the trans-membrane adaptor protein, linker for activation of T cells (LAT), becomes phosphorylated at

multiple tyrosine residues by Src or Syk family protein kinases.^{1,2} Phosphorylation of LAT creates binding sites for the SH2 domains of other signaling molecules, notably PLC γ 1 and the soluble adaptor Gads. In addition, PLC γ 1 and Gads interact through their SH3 domains with the adaptor SLP-76.^{4,9–11} The resulting multiprotein signaling complex, comprising LAT, Gads, PLC γ 1, and SLP-76, leads to phosphorylation of PLC γ 1 by Syk tyrosine kinases.^{1,2} Once activated in this manner, PLC γ 1 translocates to the plasma membrane and catalyzes production of the second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, which, respectively, trigger calcium flux and contribute to protein kinase C and Ras activation.^{3,5}

SLP-76 is composed of three distinct domains: (1) an N-terminal domain bearing tyrosine residues that become phosphorylated following TCR engagement, enabling SLP-76 to bind the SH2-containing proteins Vav and Nck; (2) a C-terminal SH2 domain which binds phosphorylated ADAP; and (3) a central proline-rich region that contains binding sites for the SH3 domains of Gads and PLC γ 1.^{1,2} The binding site for Gads-SH3 has been precisely mapped to residues 233–241 of the SLP-76 proline-rich domain.¹² In addition, three-dimensional structures of Gads-SH3 in complex with peptides representing this site have been determined.^{13,14} By contrast, the binding site for PLC γ 1-SH3 has only been localized to a 67 amino acid residue stretch between the N-terminal domain of SLP-76 and the Gads binding site.¹⁰ This segment, designated the P-I region, is required for TCR-induced activation of PLC γ 1 and NFAT.^{10,15} Likewise, although the NMR structure of human PLC γ 1-SH3 in the unliganded form has been reported,¹⁶ no structural information is available on the interaction of PLC γ 1-SH3 with SLP-76. Indeed, no three-dimensional structure has been determined for PLC γ 1-SH3 in complex with a target sequence from any of the multiple signaling partners that this SH3 is known to recognize, which include MIST,¹⁷ SOS1,¹⁸ CAIR-1/BAG-3,¹⁹ and dynamin.²⁰

Here, we used isothermal titration calorimetry (ITC) to delineate the binding site for PLC γ 1-SH3 in the proline-rich region of SLP-76. We then determined the crystal structure of rat PLC γ 1-SH3, both in the free form and bound to a peptide representing this site.

Identification of the SLP-76 sequence motif recognized by PLC γ 1-SH3

Our first goal was to identify the binding site for PLC γ 1-SH3 in the central proline-rich domain of SLP-76 (residues 157–420). Immunoprecipitation and glutathione-S-transferase (GST) pull-down experiments previously localized this site to a 67 residue segment (the P-I region) encompassing amino acid residues 157–223.¹⁰ However, this region includes 20 proline residues and seven XPXXP core motifs, several of which overlap (Table 1).

PLC γ 1-SH3 has been classified as a class II SH3 domain, signifying that it preferentially binds peptides containing XPXXPXR sequences, where X is any amino acid.^{21,22} Phage-displayed peptide libraries have further shown that the PLC γ 1-SH3 consensus motif is PPVPPRPXXTL.²³ The same study identified other polyproline sequences, not conforming to the class II motif, that also bind PLC γ 1-SH3. However, as phage display methods do not readily permit quantitative comparisons of affinity, we used ITC to determine dissociation constants (K_d) for the interaction of PLC γ 1-SH3 with 11 overlapping peptides (P1 through P11) of 12–18 residues each that were designed to span the entire P-I region of SLP-76 (Table 1).

The highest affinities were measured for peptides P5 and P6, which bound PLC γ 1-SH3 with K_d values of 11.9 μ M and 11.8 μ M, respectively (Table 1, Figure 1). These are typical affinities for known SH3–peptide interactions.^{21,22} Both peptides contain the core sequence XPXXPXR present in the library consensus motif XPPVPPRPXXTL.²³ Moreover, PLC γ 1-SH3 is highly specific for this site in SLP-76, since it binds weakly, or not at all, to other peptides bearing the XPXXP motif (P1–P3, P7–P11). The only exception is P4 (K_d 33.1 μ M); however, this peptide overlaps with P5 and P6 at nine positions (¹⁸⁴QQPPVPPQR¹⁹²).

To define the shortest SLP-76 sequence retaining full affinity for PLC γ 1-SH3, as determined using the 15-mers P5 and P6, we tested truncated peptides P12 (12-mer) and P13 (8-mer) (Table 1). Both bound this SH3 with K_d values (12.5 μ M for P12 and 12.9 μ M for P13) essentially identical with those for P5 and P6. Thus, the minimal binding site for PLC γ 1-SH3 in SLP-76 is ¹⁸⁶PPVPPQRP¹⁹³.

Structures of PLC γ 1 in free form and bound to an SLP-76 motif peptide

The crystal structures of unbound PLC γ 1-SH3, and of PLC γ 1-SH3 in complex with the SLP-76-derived 10-mer ¹⁸⁵QPPVPPQRP¹⁹⁴ (SLP-76 185–194), were determined by the molecular replacement method and refined to resolutions of 1.60 Å and 1.81 Å, respectively (Table 2). The overall fold of rat PLC γ 1-SH3 can be described as a β -barrel typical of all known SH3 domains,^{21,22} formed by two antiparallel, three-stranded β -sheets (Figure 2(a)). The root-mean-square deviation (r.m.s.d.) of main chain atoms, excluding the five N-terminal residues, is 1.97 Å for unliganded rat and human (PDB code of 1HSQ)¹⁶ PLC γ 1-SH3. The structure of the PLC γ 1-SH3 domain bound to the SLP-76 peptide ligand retains the same compact scaffold as its free form (Figure 3(a)). The r.m.s.d. of main chain atoms of free and peptide-bound PLC γ 1-SH3 is 0.55 Å, indicating that the structure of the SH3 domain does not substantially change upon binding its cognate ligand. The most obvious difference is a local rearrangement of the n-Src loop (nomenclature according to Musacchio²²) adjacent to the β c strand (residues 823–828; main chain atom r.m.s.d. of 0.77 Å). Binding of the SLP-76 peptide causes a small displacement of Gly827 and Trp828, which in turn initiates a conformational rearrangement of residues 823–826, with the C α atom of Asp825 shifted by 1.82 Å relative to its position in the unbound SH3 (Figure 3(b)).

The electron density map of the PLC γ 1-SH3/SLP-76 complex allowed unambiguous interpretation of the entire SLP-76 peptide chain. The sequence ¹⁸⁶PPVPP¹⁹⁰ (XPXXP) forms the core of this SH3 ligand, with the peptide docked onto the relatively flat surface of the SH3 domain in a class II binding mode (Figure 2(a)).^{21,22} The positively charged arginine at position 192, highly conserved in class II ligands, establishes the orientation of the SLP-76 peptide through formation of a salt-bridge with Asp808 of PLC γ 1-SH3. Because Arg192 determines peptide orientation, it is termed the “compass residue”.²² The polyproline type II (PPII) helix starts from the second residue (Pro186) and ends at the last residue (Met194) with three residues per turn. There are three shallow pockets, or slots, evenly distributed along the surface of PLC γ 1-SH3 to accommodate the peptide, two XP sites for binding the XP dipeptide moieties, and the neighboring compass slot, formed by residues from the RT and n-Src loops, the β c strand and the $_310$ helix, for binding Arg192.^{21,22} The PLC γ 1-SH3/SLP-76 complex buries a total solvent-accessible surface of 871 Å², of which 394 Å² is contributed by PLC γ 1-SH3 and 477 Å² by SLP-76. The surface of PLC γ 1-SH3 buried in the interface is mostly hydrophobic, except for the compass slot, which is negatively charged (Figure 2(b)).

As with other known PPII helices, a continuous hydrophobic strip is formed around the surface of the bound SLP-76 peptide, while the backbone carbonyl groups present three hydrogen-bonding groups towards PLC γ 1-SH3 (Figure 2(a)). The first peptide residue, Gln185, is held in an extended conformation. The first two PII helix residues, Pro186 and Pro187 are inlaid into the first XP slot formed by the side-chains of Phe800, Asn844 and Tyr845. The second XP slot holds SLP-76 residues Pro189 and Pro190 from the second turn of the PPII helix. Asn844 and Tyr845 form a wall separating the two XP slots, while Gln805 and Trp828 partition the second XP slot from the compass slot (Figure 2(b)). The third residues of the first two PPII helix turns, Val188 and Gln191, are positioned directly above the two walls and are oriented with their side-chains facing the solvent. Three short hydrogen bonds are present between the main chain carbonyl oxygen atoms of Pro187, Val188, and Pro190, and the hydroxyl oxygen atom of Tyr845, the side-chain amido nitrogen atom of Asn844, and the indole nitrogen atom

of Trp828, respectively (Figure 2(a)). The side-chain of Arg192, the first residue of the third turn of the PPII helix, is tightly intercalated into the negatively charged compass pocket *via* salt-bridge interactions with Asp808 from the RT loop of PLC γ 1-SH3. The side-chain conformation of Arg192 is further stabilized by a hydrogen bond to the side-chain of Glu809. A water molecule was found to assist binding by bridging the main chain nitrogen atom of Arg192 of SLP-76 and the side-chain N η^2 and N η^1 atoms of Arg806 of PLC γ 1-SH3 (Figure 2(a)). The other two residues of the third PPII helix turn (Pro193 and Met194) point away from the SH3, towards the N terminus of the peptide ligand from a symmetry-related molecule. Thus, the pyrrolidine ring of Pro193 is parallel with that of Pro187 from a symmetry-related peptide; in addition, the carbonyl oxygen atom of Pro193 makes hydrogen-bond contact with the main chain nitrogen atom of Val188 from the same symmetry-related peptide (not shown).

Binding specificity of PLC γ 1-SH3

Despite sequence differences of up to 75%, all SH3 domains of known three-dimensional structure adopt a similar β -barrel fold, with their proline-rich ligands forming classical PPII helices. However, subtle geometrical differences among these domains impart a significant degree of selectivity towards specific proline-rich targets.^{21,22} As shown in Figure 3(c), residues of PLC γ 1-SH3 involved in ligand binding were identified by calculating differences in solvent accessibility between the free and complexed molecules. No major changes in binding site topology are evident upon engagement of SLP-76, with most side-chains retaining the same conformation as in the unbound SH3. Thus, the r.m.s.d. of residues (all atoms) involved in ligand binding is only 0.83 Å, indicating that relatively small adjustments suffice to accommodate the SLP-76 peptide. It is, however, notable that the side-chains of several contacting residues, in particular those of Phe800, Trp828 and Trp840, shift towards the SLP-76 peptide, optimizing the fit of the binding groove to the ligand and strengthening hydrophobic interactions (Figure 3(c)).

The PLC γ 1-SH3/SLP-76 structure suggests that the selectivity of the interaction is primarily determined by strict geometric, or shape, complementarity between protein and peptide, rather than by sequence-specific hydrogen bonding. Thus, the hydrogen bonds formed by Asn844, Tyr845 and Trp828 are directed to main chain atoms of the peptide, and are therefore independent of peptide sequence. Although it has been proposed that the specificity of SH3–ligand interactions may arise from recognition of non-proline residues by non-conserved protein side-chains,^{21,22} we are unable to identify such residues in the PLC γ 1-SH3/SLP-76 complex: the two XP binding slots are exclusively occupied by proline residues, which mediate most of the interactions with the SH3 domain. However, the non-core proline at position 189, which is most often leucine or valine in the class II consensus motifs of other SH3 domains (e.g. Src, Grb2, Crk),^{21–23} may contribute to optimal binding *via* hydrophobic interactions with PLC γ 1-SH3 residues Tyr802, Gln805, Trp828, Pro842, Asn844 and Tyr845, in which the pyrrolidine ring of Pro189 stacks against Tyr802 and Pro842. In this regard, replacement of Pro189 by leucine or valine would be expected to disrupt shape complementarity at this site, resulting in decreased affinity.

ITC measurements indicate that Pro193, which does not directly contact the SH3 domain, is nevertheless required for optimal binding, since peptide P4, which lacks this residue, exhibits a threefold increment in K_d compared to peptide P5 (Table 1). As proline restricts the conformational freedom of the preceding residue in a polypeptide chain,²⁴ we hypothesize that Pro193 assists in positioning the side-chain of Arg192 into the compass pocket of PLC γ 1-SH3, thereby indirectly increasing peptide affinity. Indeed, the phage library consensus motif, XPPVPPRPXXTL, includes a proline following the compass arginine.²³ However, Pro193 is not absolutely critical for PLC γ 1-SH3 recognition, as its absence does not totally abrogate

binding. Additionally, PLC γ 1-SH3 has been shown to recognize sequence motifs lacking a corresponding proline in several target molecules besides SLP-76 (see below).

For PLC γ 1-SH3 residues in contact with SLP-76 185–194, sequence alignments with other SH3 domains (not shown) indicate that Gln805, Arg806, Asp808, Gly826 and Gly827, located within the RT and n-Src loops, are the most variable. In the PLC γ 1-SH3/SLP-76 structure, the side-chains of Gln805 and Arg806 exhibit dual conformations, one directed towards the ligand and the other away, implying that these residues probably do not contribute greatly to complex stabilization. Likewise, Gly826 and Gly827 make only a few van der Waals contacts with peptide. On the other hand, Asp808, which appears unique to PLC γ 1-SH3, could contribute to peptide selectivity. Instead of using the highly conserved Glu809 (Asp in some SH3 domains; Figure 4(c)), as do other SH3 domains,^{21,22} PLC γ 1-SH3 employs Asp808 to coordinate the critical Arg192 compass residue of SLP-76.

Structure comparison with other SH3 domains

There exist two PLC γ isozymes devoted to tyrosine-dependent signaling pathways: PLC γ 1 and PLC γ 2.³ Whereas PLC γ 1 is mainly involved in T cell signaling, PLC γ 2 is the predominant PLC γ isoform in B cells. Activation of PLC γ 2 upon antigen binding to the B cell receptor closely parallels that of PLC γ 1 following TCR engagement, and requires the B cell cytosolic adaptor BLNK, which is analogous to SLP-76 in T cells.^{25,26}

PLC γ 1 and PLC γ 2 share the same overall domain organization, including SH3 modules displaying 60% sequence identity. The PLC γ 1-SH3 residues contacting the SLP-76 peptide in the complex structure are highly conserved in PLC γ 2-SH3 (Figure 4(a)), indicating that PLC γ 2 probably interacts with BLNK at a site similar to the one on SLP-76 recognized by PLC γ 1 (¹⁸⁶PPVPPQRP¹⁹³). Sequence analysis of mouse BLNK (Swiss-Prot ID O88504) showed that this adaptor does not contain the consensus motif XPXXPXXR. However, two sequences were detected wherein the basic arginine in SLP-76 is substituted by lysine in BLNK: ¹⁶¹KPQVPPKP¹⁶⁸ and ²⁵²KPATPLKT²⁵⁹. Based on their locations in BLNK relative to that of the PLC γ 1-SH3 binding site in SLP-76, ¹⁶¹KPQVPPKP¹⁶⁸ is the sequence most likely recognized by PLC γ 1-SH3. Significantly, this potential docking site also contains a proline following the putative Lys167 compass residue.

Stimulation of the TCR activates the protein tyrosine kinase Lck and leads to phosphorylation of PLC γ 1.^{1,2} It has further been shown that the SH3 domain of Lck interacts with SLP-76 and that the best peptide candidate (¹⁸⁶PPVPPQRP¹⁹³) is, coincidentally, the same as we have defined here for PLC γ 1-SH3.²⁷ Although Lck-SH3 and PLC γ 1-SH3 bear only ~30% sequence identity, it is apparent from their three-dimensional structures that the two SH3 domains probably bind SLP-76 similarly. Thus, least-squares superposition of the main chain atoms of Lck-SH3 in free form²⁸ onto those of PLC γ 1-SH3 in the PLC γ 1-SH3/SLP-76 complex shows that most side-chains in the ligand-binding sites of these SH3 modules display similar conformations (Figure 4(b)). These include those of SLP-76-contacting residues Tyr802, Trp828, Pro842, Asn844 and Tyr845 (Phe115 in Lck).

The most significant structural difference between PLC γ 1-SH3 and Lck-SH3 is located in the RT loop, where Asp808, which establishes the peptide orientation in the PLC γ 1-SH3/SLP-76 complex by making a salt-bridge with SLP-76 Arg192, is substituted by Gly78 in Lck-SH3 (Figure 4(a)). However, as both flanking residues (Asp77 and Asp79) are negatively charged, these represent good alternative candidates for recognition of Arg192 (Figure 4(c)). Indeed, Asp79 of Lck-SH3 has been proposed to interact with a corresponding arginine (Arg182) in a class II peptide from tyrosine kinase interacting protein (Tip),²⁹ while Src-SH3 uses a corresponding aspartate (Asp99) for salt-bridge formation to arginine-oriented class II peptides

(Figure 4(c)).³⁰ The putative Lck binding motif in Tip also includes a proline following the Arg182 compass residue. Lck-SH3 differs from PLC γ 1-SH3 at two other potential ligand-binding residues: Glu96 (Gly827 in PLC γ 1) and Gly109 (Trp840 in PLC γ 1) (Figure 4(a)). However, in the PLC γ 1/SLP-76 structure, only the carbonyl oxygen atom of Gly827 contributes to the binding site, whereas the Lck Glu96 side-chain points away from this site in the free Lck-SH3 structure (Figure 4(b)). By contrast, replacement of the bulky Trp840 in PLC γ 1 by Gly109 in Lck is expected to result in the loss of several van der Waals contacts to the bound peptide, possibly reducing affinity.

Interaction of PLC γ 1-SH3 with other signaling proteins

In addition to interacting with SLP-76 in the T cell signaling pathway, PLC γ 1-SH3 has been implicated in other functions, including targeting PLC γ 1 to the cytoskeleton,³¹ agonist-induced Ca²⁺ entry,³² and mitogenesis.^{33,34} Sequence analysis of proteins known to interact with PLC γ 1-SH3, in conjunction with the crystal structure of the PLC γ 1-SH3/SLP-76 complex, enables us to identify potential binding sites for this SH3 in several of its natural targets (Table 3). In common with SLP-76, MIST,¹⁷ c-Cbl,^{35,36} SOS1,¹⁸ TNK1³⁷ and dynamin²⁰ contain at least one XPXXPXR motif (group A in Table 3). This motif should assure optimal (or near optimal) binding to PLC γ 1-SH3, as it retains, in addition to the XPXXPXR sequence, a proline immediately following the arginine compass residue. Consistent with this analysis, direct binding of PLC γ 1-SH3 to peptides from TNK1 and dynamin representing putative recognition sites has been demonstrated.^{20,37} Other XPXXPXR sequences in c-Cbl and SOS1 (group B), while lacking proline at the position following the compass arginine, may nevertheless exhibit significant affinity for PLC γ 1-SH3, since elimination of the corresponding proline in SLP-76 did not abolish binding (Table 1). Although both CAIR-1/BAG-3 and SAM 68 interact with PLC γ 1-SH3,^{19,38} neither contains a class II motif (group C), suggesting that PLC γ 1-SH3 may also recognize other motifs, as recently demonstrated for Gads-SH3.³⁹

Data bank accession codes

Coordinates and structure factors for unbound PLC γ 1 and the PLC γ 1/SLP-76 complex have been deposited under accession codes 1YWP and 1YWO, respectively.

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Abbreviations used

PLC γ 1, phospholipase C γ 1; SH2, Src homology 2; SH3, Src homology 3; PH, pleckstrin homology; TCR, T cell receptor; LAT, linker for activation of T cells; ITC, isothermal titration calorimetry; GST, glutathione-S-transferase; r.m.s.d., root-mean-square deviation.

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Figure 1.

Calorimetric titration of PLC γ 1-SH3 with peptide derived from SLP-76. ITC was carried out on a MicroCal VP-ITC titration microcalorimeter as described.⁴⁰ SLP-76 peptides were synthesized and purified by Biosource. Purified PLC γ 1-SH3 was exhaustively dialyzed against PBS (5 mM phosphate buffer (pH 7.2), 136 mM NaCl, 4 mM KCl); the final dialysate was used to prepare SLP-76 peptide solutions. A non-linear, least-squares fitting method was used to determine the thermodynamic parameters. Data acquisition and analysis were performed using the software package Origin. (a) Raw data obtained from 30 automatic injections of 3 μl aliquots of 9.2 M SLP-76 ¹⁸⁴QQPPVPPQRPMALPP¹⁹⁹ peptide solution into 0.206 mM PLC γ 1-SH3 solution in PBS at 3 °C. (b) Non-linear, least-squares fit (continuous line) of the

incremental heat per mole of added ligand (open squares) for the titration in (a). A stoichiometry (n) value of 1.02 and K_d of 11.8 μM with uncertainties of 0.3 and 2%, respectively, were obtained for this experiment.

Figure 2.

(a) Structure of the PLCγ1 SH3 domain with bound SLP-76 peptide. Ribbon diagram of PLCγ1-SH3 in complex with the SLP-76-derived 10-mer ¹⁸⁵QPPVPPQRPM¹⁹⁴. The peptide is shown in ball-and-stick representation. Carbon atoms are gray, nitrogen atoms are blue, oxygen atoms are red, and the sulfur atom is yellow. A bridging water molecule (WAT) is represented by a red ball. Residues of PLCγ1-SH3 forming salt-bridge or hydrogen bond interactions (broken lines) with the ligand are drawn. The view is looking down the β-barrel of the SH3 domain. Secondary structure elements are labeled following the nomenclature for Src-SH3,²² and are colored as follows: 3₁₀ helix, brown; β-strands, magenta; loop regions, cyan. This Figure, together with Figures 3 and 4, were produced with MOLSCRIPT.⁴¹ (b)

Electrostatic potential surface representation of PLC γ 1-SH3 in complex with SLP-76 peptide. Solvent-accessible surfaces are colored according to electrostatic potential, with positively charged regions in blue and negatively charged regions in red. Electrostatic surface potentials were calculated with GRASP.⁴² The peptide is shown in stick representation. Carbon atoms are white, nitrogen atoms are blue, oxygen atoms are red, and the sulfur atom is green.

Figure 3.

Comparison of PLCγ1-SH3 in free and SLP-76-bound forms. (a) Superposition of the polypeptide chain of unbound PLCγ1-SH3 (gold) onto that of PLCγ1-SH3 in the PLCγ1-SH3/SLP-76 complex (cyan). The SLP-76 peptide is drawn in ball-and-stick representation. The arrow points to the region where the two structures deviate most. (b) Close-up view of the rearrangement of the n-Src loop of PLCγ1-SH3 following peptide binding. Glycine residues are represented by their carbonyl oxygen atoms. (c) Adjustments in the PLCγ1-SH3 binding site upon ligation of SLP-76 peptide. Free PLCγ1-SH3 was superposed on SLP-76-bound PLCγ1-SH3. The view is looking down on the binding groove. The SLP-76 peptide and the rest of the SH3 structure are omitted for clarity. Only residues contacting the ligand are shown.

Glycine residues are represented by their carbonyl oxygen atoms. Dual conformations were observed for the side-chains Gln805 and Arg806 in the PLC γ 1-SH3/SLP-76 complex.

Figure 4.

(a) Structure-based sequence alignment of the SH3 domains of PLC γ 1, PLC γ 2 and Lck. Secondary structure elements are denoted by violet arrows (β -strands) and a pink cylinder (3_{10} helix). These, and the loop regions (brown lines), are labeled according to the nomenclature for Src-SH3.²² Residues of PLC γ 1-SH3 making van der Waals contacts with SLP-76 are shaded yellow; residues interacting with SLP-76 through hydrogen bonds are shaded cyan. Asp808, which forms a salt-bridge with the bound peptide, is shaded red. (b) Superposition of PLC γ 1-SH3 in bound form (cyan) onto free Lck-SH3 (green; PDB accession code 1LCK).²⁸ The view is the same as that in Figure 3(c). Residues of Lck-SH3 that potentially contact bound peptide, based on sequence alignment with PLC γ 1-SH3 (a), are shown. (c) Interactions at the SH3 compass pocket. The PLC γ 1/SLP-76 complex (cyan) was superposed onto the Src-SH3/App12 complex (PDB accession code 1QWE)³⁰ and free Lck-SH3. Only interactions with the compass residue of the bound peptide (Arg192 of SLP-76 and Arg9 of App12) are shown. Salt-bridges are drawn as broken lines.

Table 1
Equilibrium dissociation constants for the binding of SLP-76 peptides to PLC γ 1-SH3 at 3 °C

P-I ^a (157–223)	QNSILPAKPFPNNSNSMYIDRPSPGKTPQQPPVPPQRPMAALPPPAGRNHSLPPPQTNHEEPSRSR	K_d (μ M)
Peptide		
P1 (157–168)	QNSIL PAKPF PN	NB
P2 (161–172)	LPAKPF PNNSNM	NB
P3 (175–188)	DRPPSGK TPQQPPV	416 $\pm$ 17
P4 (178–192)	PSGK TPQQPPVPPQR	33.1 $\pm$ 0.4
P5 (181–195)	KTPQQPPVPPQRPMA	11.9 $\pm$ 0.4
P6 (184–199)	QQPPVPPQRPMAALPP	11.8 $\pm$ 0.2
P7 (188–202)	VPPQRPMAALPPPPA	TLQ
P8 (193–207)	PMAAL PPPP AGRNHS	TLQ
P9 (196–212)	ALPPPP AGRNH SPLPPP	TLQ
P10 (203–216)	GRNH SPLPPP QTNH	TLQ
P11 (206–223)	HSPLPPP QTNHEEPSRSR	TLQ
P12 (184–195)	QQPPVPPQRPMA	12.5 $\pm$ 0.8
P13 (186–193)	PPVPPQRP	12.9 $\pm$ 0.8

A DNA fragment encoding the SH3 domain of rat PLC γ 1 (residues 790–851) was amplified by PCR. The forward primer was designed to mutate Cys794 to Ser in order to prevent intermolecular disulfide bond formation during storage of the purified PLC γ 1-SH3 domain; Ser794 is distant from the ligand-binding site of PLC γ 1-SH3 in the crystal structure (see the text). The amplified DNA was cloned into the expression vector pGEX-4T-1(+) (Novagen) and transformed in *Escherichia coli* BL-21(DE3) cells (Novagen). The GST-SH3 fusion protein was expressed by induction with 0.2 mM isopropyl- β -D-thiogalactoside at 30 °C in LB medium containing 100 μ g/ml of ampicillin and 25 μ g/ml of chloramphenicol. Cells were harvested by centrifugation, resuspended in PBS, and disrupted by sonication. The supernatant was applied to a GStrap FF column (Amersham Biosciences) and the GST-SH3 fusion protein purified according to standard protocols. The eluted protein was digested with thrombin protease (Amersham Biosciences) overnight at room temperature. The PLC γ 1-SH3 domain was purified by gel-filtration chromatography in 20 mM Tris-HCl (pH 8.5) with a Superdex 75 HR column (Amersham Biosciences). A final purification step was carried out using a Mono Q anion-exchange column equilibrated in the same buffer; the protein was eluted using a linear NaCl gradient. Protein concentration was measured by absorbance at 280 nm using a calculated extinction coefficient of 20,910 M⁻¹ cm⁻¹.⁴³ Equilibrium dissociation constants (K_d) were obtained by non-linear least-squares fit of the ITC data to a single-site binding model wherein the stoichiometry parameter n ranged from 1.00 to 1.16; NB is no binding. TLQ indicates the affinity is too low for accurate quantification. Bold letters represent possible core binding sites for the PLC γ 1-SH3 domain bearing the consensus motif XPXXP (where X can be any amino acid).

^a The P-I region of SLP-76 refers to residues from 157 to 223¹⁰ and is included for comparison purposes.

Table 2

X-ray crystallographic statistics

	PLCγ1-SH3	PLCγ1-SH3/SLP-76
<i>Data collection statistics</i>		
Resolution (Å) ^a	50.0–1.60 (1.66–1.60)	50.0–1.81 (1.87–1.81)
Space group	<i>P</i> 2 ₁	<i>P</i> 4 ₃ 2 ₁ 2
Cell dimensions (Å, deg.)	<i>a</i> =29.0, <i>b</i> =31.3, <i>c</i> =29.8, <i>β</i> =97.1	<i>a</i> = <i>b</i> =46.7, <i>c</i> =58.1
Unique reflections ^a	6979 (669)	6149 (527)
Completeness (%) ^a	97.5 (94.5)	97.4 (85.8)
<i>R</i> _{merge} (%) ^{a,b}	3.7 (16.8)	9.7 (35.3)
Redundancy ^a	4.1 (4.2)	5.3 (2.7)
<i>I</i> /σ(<i>I</i>) ^a	42.2 (10.5)	21.7 (1.8)
<i>Refinement statistics</i>		
Resolution range (Å)	29.49–1.60	36.42–1.81
<i>R</i> _{work} (%) ^c	14.2	17.1
<i>R</i> _{free} (%) ^c	18.8	23.1
Number of reflections used	6604	5831
Number of reflections in <i>R</i> _{free} set	323	279
Number of non-H protein atoms	513	548
Number of water molecules	120	67
Mean <i>B</i> -factor (Å ²)	17.4	23.9
Main-chain atoms	11.9	20.4
Side-chain atoms	15.2	21.9
Water molecules	33.8	44.2
r.m.s.d from ideality		
Bond lengths (Å)	0.017	0.014
Bond angles (deg.)	1.7	1.4
Ramachandran plot statistics		
Most favored (%)	94.3	92.0
Additionally allowed (%)	5.7	8.0

Unbound PLCγ1-SH3 was crystallized in 36% (w/v) PEG4000, 0.1 M sodium acetate, 0.1 M Tris-HCl (pH 9.0) at room temperature. Co-crystallization with the SLP-76-derived 10-mer ¹⁸⁵QPPVPPQRPM¹⁹⁴ was carried out using a 4:1 molar ratio of peptide to protein. Crystals of the complex were grown in 2.4 M sodium malonate (pH 7.0). Crystals of free PLCγ1-SH3 were cryoprotected by step-wise soaking in solutions of mother liquor containing 2–25% (v/v) glycerol before being flash-cooled. Sodium malonate (3.4 M) was used as a cryoprotectant for the complex crystals. Diffraction data were collected at 100 K on beamline X29 of the Brookhaven National Synchrotron Light Source. The data were processed separately using the program HKL2000.⁴⁴ Phases for the free PLCγ1-SH3 domain were determined by the molecular replacement method using Phaser⁴⁵ with the Gads SH3 domain (PDB code 1OEB)¹⁴ as the search model. The graphics program XtalView⁴⁶ was used to examine the crystal packing and initial electron density map. Refinement was carried out using RefMac 5.0.⁴⁷ The final model comprises residues 790–851, one N-terminal serine from the GST fusion partner, and 120 water molecules. The structure of the PLCγ1-SH3/SLP-76 complex was solved with Phaser⁴⁶ using the free PLCγ1-SH3 structure as the search probe. The final model includes residues 793–848 of the SH3 domain, all ten residues of the SLP-76 peptide, and 67 water molecules. Stereochemical parameters were evaluated with PROCHECK⁴⁸ and MolProbity.⁴⁹

^aValues in parentheses are statistics of the highest resolution shell.

^b $R_{\text{merge}} = \Sigma |I_j - \langle I \rangle| / \Sigma I_j$, where I_j is the intensity of an individual reflection and $\langle I \rangle$ is the average intensity of that reflection.

^c $R_{\text{work}} = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$, where F_c is the calculated structure factor. R_{free} is as for R_{work} but calculated for a randomly selected 5.0% of reflections not included in the refinement.

Table 3
Binding sites for PLC γ 1-SH3 in selected signaling partners

Group	Sequence	Positions	Protein	Reference
A	PPVPPQRP ^a	186–193	SLP-76	This work
XPXXPXR	IPLPPRP	158–165	MIST	17
	PPPPDRP	544–551	c-Cbl	36
	PPVPLRP	1216–1223	SOS1	18
	PPGLPPRP ^a	528–535	TNK1	37
	APPVPSRP	812–819	Dynamin	20
	PPQVPSRP ^a	829–836	Dynamin	20
	LPPVPPRL	493–500	c-Cbl	36
B	XPXXPXR	1203–1210	SOS1	18
	PPPLPPRK	1146–1153	SOS1	18
	PPAIPPRQ	1172–1179	SOS1	18
	APPVPPRQ	1288–1295	SOS1	18
C	GPVGPELP	326–333	CAIR-1/BAG-3	19
	XPXXPXXP	371–378	CAIR-1/BAG-3	19
	APPPPPVP	294–301	SAM 68	38

Sequences of proteins known to bind PLC γ 1-SH3 were compared to the SLP-76 186–193 sequence to identify potential binding sites. Consensus motifs for each group are indicated. Possible critical residues are in bold.

^aDirect interaction of peptides bearing this sequence with PLC γ 1-SH3 has been reported.