

A Synthetic Polyphosphoinositide Headgroup Surrogate in Complex with SHIP2 Provides a Rationale for Drug Discovery

Stephen J. Mills,[†] Camilla Persson,[‡] Gyles Cozier,[†] Mark P. Thomas,[†] Lionel Trésaugues,[‡] Christophe Erneux,^{||} Andrew M. Riley,[†] Pär Nordlund,^{‡,§,⊥} and Barry V. L. Potter^{*,†}

[†]Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K.

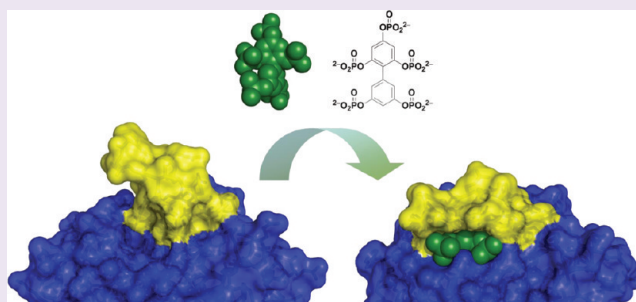
[‡]Structural Genomics Consortium and [§]Division of Biophysics, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm 17177, Sweden

^{||}Institut de Recherche Interdisciplinaire (IRIBHM), Université Libre de Bruxelles, Campus Erasme, Brussels, Belgium

[⊥]School of Biological Sciences, Nanyang Technological University, Singapore 637551

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ABSTRACT: Phosphoinositides regulate many cellular processes, and cellular levels are controlled by kinases and phosphatases. SHIP2 (SH2 (Src homology 2)-domain-containing inositol-phosphatase-2) plays a critical role in phosphoinositide signaling, cleaving the 5-phosphate from phosphatidylinositol 3,4,5-trisphosphate. SHIP2 is thought to be involved in type-2 diabetes and obesity, conditions that could therefore be open to pharmacological modulation of the enzyme. However, rational design of SHIP2 inhibitors has been limited by the absence of a high-resolution structure. Here, we present a 2.1 Å resolution crystal structure of the phosphatase domain of SHIP2 bound to the synthetic ligand biphenyl 2,3',4,5',6-pentakisphosphate (BiPh(2,3',4,5',6)P₅). BiPh(2,3',4,5',6)P₅ is not a SHIP2 substrate but inhibits Ins(1,3,4,5)P₄ hydrolysis with an IC₅₀ of 24.8 ± 3.0 μM, (K_m for Ins(1,3,4,5)P₄ is 215 ± 28 μM). Molecular dynamics simulations suggest that when BiPh(2,3',4,5',6)P₅ binds to SHIP2, a flexible loop folds over and encloses the ligand. Compounds targeting such a closed conformation might therefore deliver SHIP2-specific drugs.



SHIP2 (SH2 (Src homology 2)-domain-containing inositol phosphatase 2) is one member of the Mg²⁺-dependent inositol polyphosphate 5-phosphatase family of proteins that cleave the 5-phosphate of phosphoinositide and inositol polyphosphate substrates. There are 10 known human inositol 5-phosphatase isoenzymes, and direct involvement in human diseases is established for various members of this family.¹ SHIP2 catalyzes the conversion of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) into phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P₂). The water-soluble headgroup inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) is also a substrate for SHIP2, as is phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂), but not Ins(1,4,5)P₃.²

Structurally, SHIP2 is a 155 kDa multidomain protein³ composed of an SH2 domain that plays a key role in binding to other protein partners, the inositol phosphatase domain, a phosphotyrosine binding site (PTB) in a NPXY consensus sequence, proline-rich sequences, a sterile α -motif (SAM), and recently identified ubiquitin binding domain⁴ and phosphorylation sites at Ser132, Thr1254, and Ser1258.⁵ SHIP2 is found in tissues including liver, brain, heart, muscle, and kidney.^{3,6} In the resting state, SHIP2 resides in the cytosol and perinuclear area, but stimulation by external signal molecules effects

recruitment to the plasma membrane where several other proteins may dock to motifs such as the SH2 domain, proline-rich sequences, and the SAM domain that may allow the enzyme to coordinate cell-specific activities.⁷

The most studied cellular substrate of SHIP2, PtdIns(3,4,5)P₃, recruits downstream enzymes such as 3-phosphoinositide dependent kinase-1 and protein kinase B that are directly involved in survival, growth, and development.⁸ Thus, SHIP2 positively regulates the levels of PtdIns(3,4)P₂ and has been proposed in important pathologies in relation to insulin sensitivity⁹ and cancer, for example, in squamous cell carcinoma.¹⁰ Recently, a connection between SHIP2 and release of viral particles was demonstrated.¹¹

Although small molecule inhibitors of SHIP2 have been identified using high-throughput screening,^{12,13} their poor bioavailability precludes consideration as clinical trial candidates. Despite human disease being associated with SHIP2 malfunction, no SHIP2-specific drugs are yet in clinical use, and

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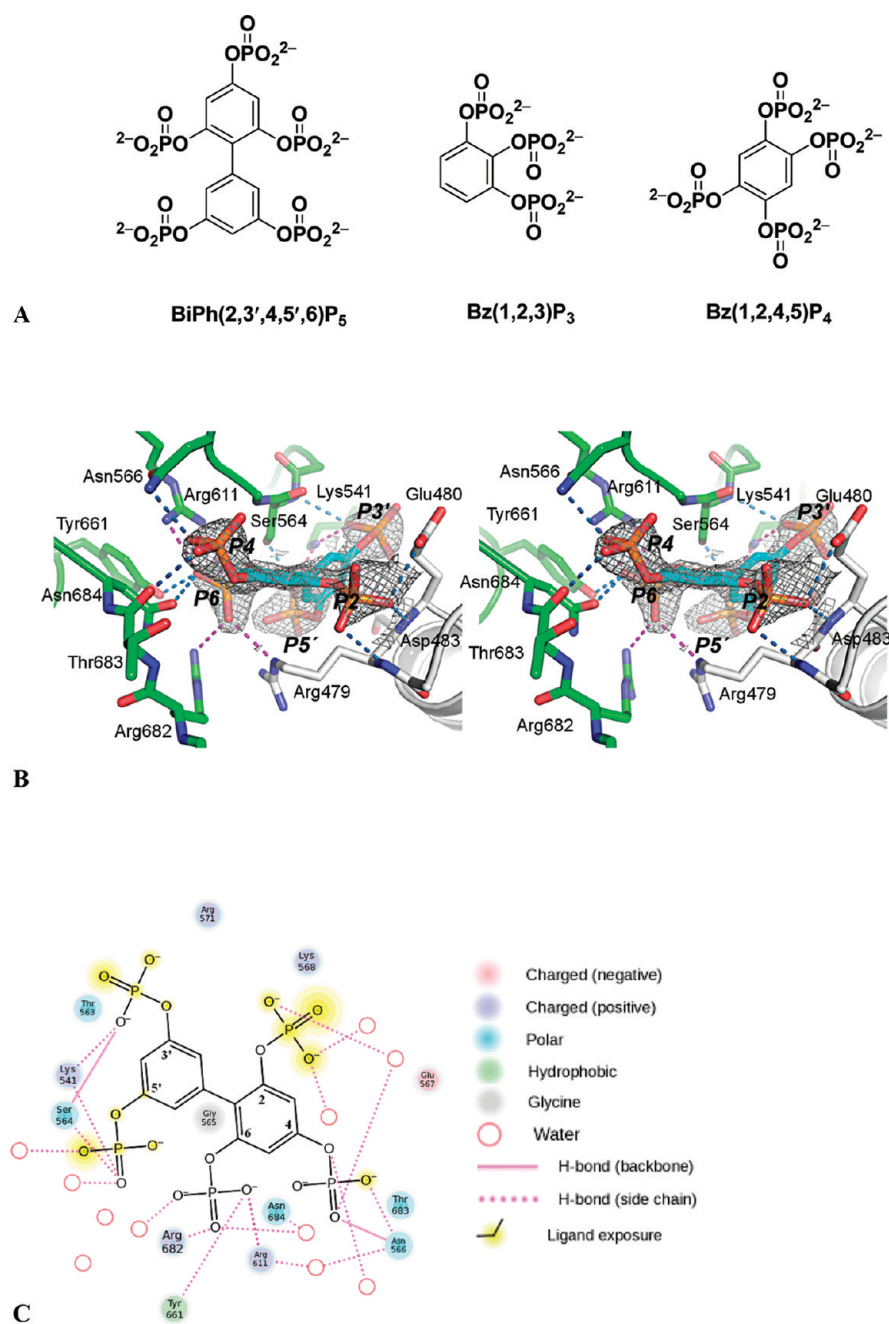


Figure 1. (A) Structures of biphenyl 2,3',4,5',6-pentakisphosphate, benzene 1,2,3-trisphosphate, and benzene 1,2,4,5-tetrakisphosphate. (B) Stereoview of the BiPh(2,3',4,5',6)P₅ binding site. The SHIP2 molecule present in the asymmetric unit and a symmetry-related mate are colored green and white, respectively. BiPh(2,3',4,5',6)P₅ is displayed as a stick model with its carbon atoms colored cyan. BiPh(2,3',4,5',6)P₅ is surrounded by the σ_A -weighted $2F_o - F_c$ simulated annealing omit map contoured at 1σ and shown as a gray mesh. Protein residues and ligand phosphate positions are labeled using regular and italic fonts, respectively. Interactions between protein and ligand atoms are depicted as blue (H-bonds) and purple (salt bridges) dashes. (C) Ligand interaction diagram showing key interactions between BiPh(2,3',4,5',6)P₅ and SHIP2. The H-bonds shown have a maximum length of 2.5 Å. All residues within 4 Å of the ligand are shown. Yellow circles indicate ligand exposure and show the degree to which the highlighted atoms are solvent exposed; the larger the yellow circle, the greater the solvent exposure.

rational design of inhibitors is limited by the absence of any structure of SHIP2 in complex with a ligand.

Previously, we explored co-crystallization of the pleckstrin homology (PH) domain of protein kinase B α (PKB α) with simple synthetic benzene polyphosphates and demonstrated that benzene 1,2,3,4-tetrakisphosphate (Bz(1,2,3,4)P₄) is a good surrogate for Ins(1,3,4,5)P₄.¹⁴ The crystal structure of the Bz(1,2,3,4)P₄-PH domain of PKB α together with the Ins(1,3,4,5)P₄-PH domain PKB α -complex were subsequently

used to develop a docking protocol to identify drug-like inhibitors of PKB PH domain by virtual screening.¹⁵

We also identified earlier a synthetic biphenyl-derived polyphosphate, biphenyl 2,3',4,5',6-pentakisphosphate (BiPh(2,3',4,5',6)P₅) (Figure 1 panel A), as a potent SHIP2 inhibitor.² Here, we report the structure of the phosphatase domain of human SHIP2 (SHIP2-cd) in complex with BiPh(2,3',4,5',6)P₅ at 2.1 Å resolution, together with results

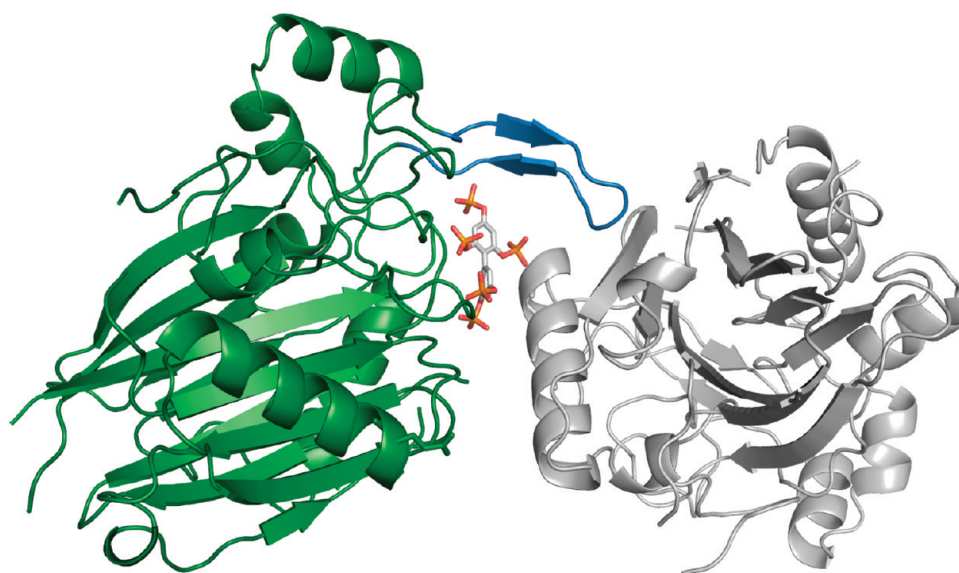


Figure 2. Crystal structure of two SHIP2-cd molecules and one molecule of BiPh(2,3',4,5',6)P₅. The bound BiPh(2,3',4,5',6)P₅ in the catalytic site of SHIP2 (left) has additional interactions with a second, symmetry-related SHIP2 molecule (right). The mobile region from the molecular dynamics simulation is shown in blue.

of molecular dynamics simulations that suggest a rationale for the design of inhibitors of SHIP2.

The crystal structure of the BiPh(2,3',4,5',6)P₅–SHIP2-cd complex contains two SHIP2-cd monomers in the asymmetric unit, but only one monomer accommodates a BiPh(2,3',4,5',6)P₅ molecule, bound in a shallow pocket. This pocket was recently identified as the substrate binding site in the structure of the complex between the phosphatase domain of INPP5B and PtdIns(4)P (Persson, C., *et al.*, unpublished data). Close examination of the bound BiPh(2,3',4,5',6)P₅ (Figure 1, panels B and C) reveals multiple interactions between the 3', 4-, 5', and 6-phosphates of the ligand and both side chain and backbone atoms of SHIP2. Additionally, nine water molecules present in the binding site coordinate the 2-, 4-, 5'- and 6-phosphates, eight of them mediating hydrogen bonds between SHIP2 and BiPh(2,3',4,5',6)P₅ (Figure 1 panel C and Supplementary Table 1). Direct protein–ligand interactions are particularly focused around the 6-phosphate, involving the side chains of Arg611, Asn684, and Tyr661 and a longer range (3.3 Å) interaction with Arg682. These residues all belong to the region designated as the 4-phosphate interacting motif (P4IM) (Persson, C., *et al.*, unpublished data), suggesting that the 6-phosphate of BiPh(2,3',4,5',6)P₅ to some extent mimics the 4-phosphate of the natural substrate or product. The 4-phosphate of BiPh(2,3',4,5',6)P₅ has interactions with the backbone of Thr683 and Asn566, as well as the side chain of Asn566. Both the 3'- and 5'-phosphates of BiPh(2,3',4,5',6)P₅ are connected to Lys541 N ζ through a bidentate interaction, while they also mediate H-bonding with main chain (3'-phosphate) and side chain (5'-phosphate) atoms of Ser564.

The 2-phosphate protrudes out of the active site and interacts with Arg479 (main chain amide nitrogen) and Glu480 (both main chain amide nitrogen and side chain carboxylate) of a symmetry-related SHIP2 molecule. The neighboring molecule of SHIP2 also forms interactions with phosphate groups in position 3', 5', and 6- through the side chains of Asp483 and Arg479. The symmetry-related SHIP2 molecule is positioned above the binding site of the SHIP2 monomer that contains BiPh(2,3',4,5',6)P₅ (Figure 2).

To clarify which structural properties of BiPh(2,3',4,5',6)P₅ might favor its interaction with SHIP2, we compared its potency with the simpler benzene 1,2,3-trisphosphate (Bz(1,2,3)P₃) and benzene 1,2,4,5-tetrakisphosphate (Bz(1,2,4,5)P₄)¹⁶ (Figure 1 panel A). Enzyme activity was assayed by measuring inorganic phosphate released from the substrate Ins(1,3,4,5)P₄ (100 μ M) using the malachite green phosphate assay (BioAssay Systems). None of the inhibitors appears to be a substrate for SHIP2. The percentage inhibition values for the reaction using 100 μ M BiPh(2,3',4,5',6)P₅, Bz(1,2,3)P₃, and Bz(1,2,4,5)P₄ were 76.0 \pm 1.5%, 13.6 \pm 5.2%, and 49.7 \pm 1.3%, respectively. IC₅₀ values obtained using this assay were 24.8 \pm 3.0 μ M for BiPh(2,3',4,5',6)P₅, >1000 μ M for Bz(1,2,3)P₃, and 69.3 \pm 15.4 μ M for Bz(1,2,4,5)P₄. Unfortunately, no complex between any single-ring inhibitor and SHIP2-cd is available from our co-crystallization trials or soaking with apo-SHIP2-cd crystals.

The BiPh(2,3',4,5',6)P₅–SHIP2-cd complex shows interactions with the phosphates of BiPh(2,3',4,5',6)P₅ that are spread over two rings; it is unlikely that a single ring compound could contact the same range of amino acid residues. This is consistent with the IC₅₀ value for Bz(1,2,3)P₃ being >1 mM compared to 24.8 μ M for BiPh(2,3',4,5',6)P₅. However, Bz(1,2,4,5)P₄ not only has an additional phosphate compared to Bz(1,2,3)P₃ but also has “*para*”-phosphates, providing a larger span for active site interactions. These factors are consistent with an IC₅₀ value for Bz(1,2,4,5)P₄ of 69.3 μ M, which is 2–3-fold higher than for BiPh(2,3',4,5',6)P₅ but significantly lower than for Bz(1,2,3)P₃. Finally, it is likely that limited rotation around the C1–C1' bond connecting the two rings allows the phosphate groups in BiPh(2,3',4,5',6)P₅ to optimize their interactions with the binding pocket. This property would confer upon double-ringed aryl polyphosphates a further advantage relative to single-ringed ones.

Crystal structures of two related 5-phosphatases in complex with ligands are currently available: the 5-phosphatase domain of SPSynaptojanin (SPSynaptojanin-cd) in complex with Ins(1,4)P₂¹⁷ and the catalytic domain of INPP5B (INPP5B-cd) in complex with di-C₈–PtdIns(4)P (3MTC; Persson, C., *et*

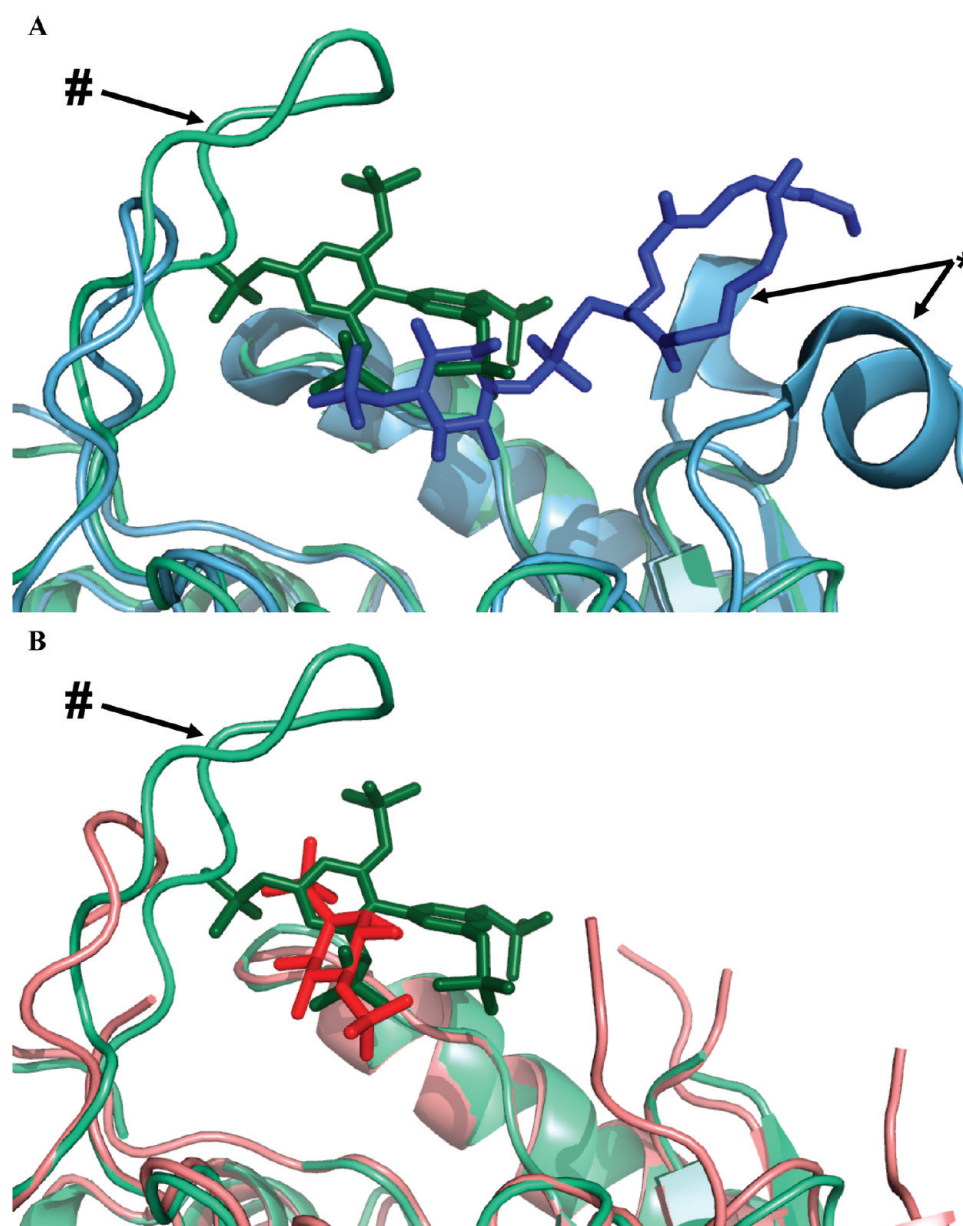


Figure 3. Overlays of the BiPh(2,3',4,5',6)P₅-SHIP2-cd complex (green) with (A) INPP5B-cd-PtdIns(4)P (Persson, C., *et al.* unpublished data) (3MTC) (blue) and (B) SPSynaptojanin-cd-Ins(1,4)P₂¹⁷ (red) complexes. See Supplementary Figure 2 for a model of the SHIP2-di-C₈-PtdIns(3,4,5)P₃-complex. # The elongated loop in SHIP2 that closes over the BiPh(2,3',4,5',6)P₅ during molecular dynamics simulations (see text). * Two loops containing hydrophobic interactions with the fatty acid tails in the INPP5B-cd-PtdIns(4)P structure, which are poorly defined or absent in the SHIP2 and SPSynaptojanin structures.

al., unpublished data). These structures contain the hydrolysis product formed by removal of the 5-phosphate from the substrates Ins(1,4,5)P₃ and di-C₈-PtdIns(4,5)P₂ respectively. Phosphatase domains of SHIP2, INPP5B, and SPSynaptojanin are close homologues, revealed by both sequence conservation (33% identity and 48% similarity between SHIP2 and INPP5B; 27% identity and 44% similarity between SHIP2 and SPSynaptojanin) and structure superposition (rmsd 1.24 Å for 267 superimposed C α between SHIP2 and INPP5B, rmsd 1.19 Å for 266 superimposed C α between SHIP2 and SPSynaptojanin).

Overlays of SPSynaptojanin and INPP5B complexes with the SHIP2-BiPh(2,3',4,5',6)P₅ complex reveal that BiPh(2,3',4,5',6)P₅ binding occurs in the region of SHIP2 expected to accommodate the product (Figure 3). In the SHIP2-cd

complex, the 6-phosphate of BiPh(2,3',4,5',6)P₅ lies at only 2.8 and 3.1 Å from the position occupied by the 4-phosphate of the products in the INPP5B and SPSynaptojanin structures, respectively. Moreover, similar interactions between protein residues and the phosphates mentioned above are conserved. The 3'- and 5'-phosphates of BiPh(2,3',4,5',6)P₅ are located close to, and either side of, the 1-phosphate of PtdIns(4)P (Figure 3 panel A) in the overlay with the INPP5B complex and interact with an equivalent lysine residue (Lys541 in SHIP2 (Figure 1 panel C) and Lys380 in INPP5B). The 4-phosphate of BiPh(2,3',4,5',6)P₅ is located close to the 1-phosphate of Ins(1,4)P₂ in the overlay and has an equivalent backbone interaction (Asn566 in SHIP2 and Tyr704 in SPSynaptojanin). Ligand-protein interactions in the three complexes are summarized in Supplementary Table 1.

A region formed by residues Gly676 to Asn684 in SHIP2 is not conserved in either SPSynaptojanin or INPP5B (Supplementary Figure 1). This zone is located above the putative substrate-binding site and includes residues that contribute to the P4-interacting motif (P4IM) (Persson, C., *et al.*, unpublished data). It adopts a double-stranded antiparallel β -sheet structure and the two strands are separated by a β -turn that contains Pro678. This extension, which is unique to SHIP1 and SHIP2, is almost entirely solvent-exposed and thus is likely to be flexible. This region appears to be structured only in the SHIP2 molecule that binds BiPh(2,3',4,5',6)P₅. However, ligand binding cannot be necessary for the structural organization of this region, because it is defined in a structure of apo-SHIP2 obtained from a crystal belonging to the same space group (Persson, C., *et al.*, unpublished data).

Because the crystal environment is different in the two SHIP2 monomers present in the asymmetric unit, the interactions between the P4IM-containing loop and a symmetry-related molecule could not occur in the SHIP2 molecule whose P4IM-containing loop is disordered. However, in the SHIP2 molecule lacking a structured P4IM-containing loop, the region corresponding to the P4IM-containing loop points toward a zone that also exhibits disordered elements (the loop formed by residues 456 to 460) in a symmetry-related molecule. Thus, the disorder of the loop 456–460 might result in the consecutive destruction of the P4IM-containing loop.

In the structure of INPP5B-cd in complex with di-C₈-PtdIns(4)P, two loops (Asp305 to Pro317 and Thr371 to Asn379) mediate hydrophobic contacts between INPP5B and the fatty acid tails of PtdIns(4)P. Although the first loop is conserved only in SPSynaptojanin, the latter exists in both SPSynaptojanin and SHIP2. However, neither of these loops was modeled in the crystal structures of either SPSynaptojanin or SHIP2 due to their apparent flexibility.

As noted above, BiPh(2,3',4,5',6)P₅ in the crystal structure has several contacts with a second, symmetry-related molecule of SHIP2 in addition to its interactions with the active site. The orientation of the SHIP-specific extension comprising residues 676 to 684 may also be affected by interprotein contacts. Although the double-stranded antiparallel β -sheet structure it adopts suggests that the overall conformation of this region is native, the intermolecular contacts (one H-bond between the backbone carbonyl of Pro678 and the nitrogen amide of Met502 main chain, and a hydrophobic interaction between Pro678 and Val525) are both on the β -turn at the apex of the loop. This could constrain the loop to the orientation seen in the crystal structure. Furthermore, Arg479 in the neighboring SHIP2 molecule interacts with the 6-phosphate of BiPh(2,3',4,5',6)P₅, effectively preventing closure of this loop over the ligand.

Because these factors may perturb the more relevant solution binding mode of BiPh(2,3',4,5',6)P₅, we used molecular dynamics to investigate the active site binding of BiPh(2,3',4,5',6)P₅. When dynamics are performed using the SHIP2-cd–ligand complex (Figure 4) as a starting point, but in the absence of the second SHIP2 molecule, the simulation (see Supporting Information) results in the region composed of residues Ala672 to Asn684 closing over the ligand (see supplementary movie Loop Dynamics) with the expulsion of several water molecules from the substrate binding site. The many polar and positively charged residues in this loop increase the number of potential hydrogen bonds and salt bridges between the ligand phosphate groups and the protein

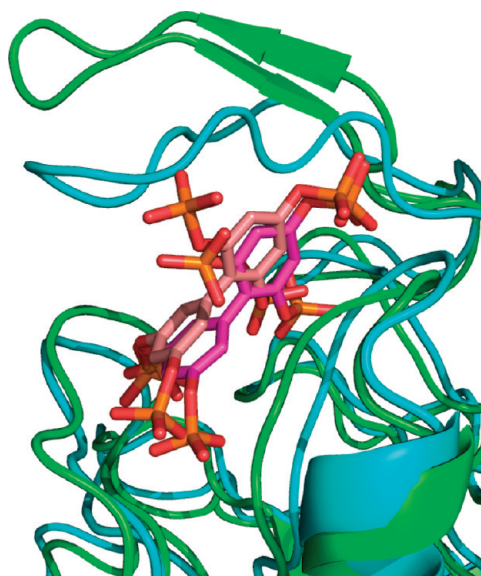


Figure 4. Crystal structure conformation (green (protein) and brown (ligand)) and the post dynamics conformation (blue (protein) and purple (ligand)) of the BiPh(2,3',4,5',6)P₅-SHIP2-cd complex; see Supplementary Loop Dynamics movie).

(Supplementary Figure 2), and the longer range interactions described above for the crystal structure are shorter and therefore stronger after the molecular dynamics. The 2-phosphate, which interacts with the symmetry related molecule in the crystal structure, now forms H-bonds to Arg571, Lys568, and Val681 within the binding site and appears less solvent exposed. Additionally, in the loop-closed structure there are three potential cation- π interactions between Lys451 and Lys677 and the phenyl ring containing the 3'- and 5'-phosphate groups and Lys568 and the ring with the 2-, 4-, and 6-phosphate groups, together with the formation of many more H-bonds.

Thus, using the complex between SHIP2-cd and BiPh(2,3',4,5',6)P₅ as a starting point, but without the neighboring crystallographic molecules of SHIP2, the dynamics simulation may produce a complete and more accurate portrayal of the network of interactions between the two molecules depleted of crystal packing artifacts. The recently identified inhibitor (3-((4-chlorobenzyl)oxy)-N-((1S)-1-phenylethyl))-2-thiophenecarboxamide (AS1949490¹², Supplementary Figure 3) was also synthesized and investigated in co-crystallization studies with SHIP2. Due to its aqueous insolubility it was impossible to co-crystallize this compound with SHIP2-cd. Modeling shows that AS1949490 could bind in the region occupied by BiPh(2,3',4,5',6)P₅ (Supplementary Figure 3), and dynamics (data not shown) show that movement of the loop accommodates the docked AS1949490 in the active site leading to expulsion of aqueous medium and a more hydrophobic environment. We suggest that introducing more electronegative groups into such hydrophobic compounds may provide more specific interactions in a closed-loop binding pocket proposed here.

When a model of a SHIP2:di-C₈-PtdIns(3,4,5)P₃-complex is constructed (Supplementary Figure 4) it can be seen that the diacylglycerol chains exit the active site well away from the flexible loop. Indeed, when dynamics are also carried out on a SHIP2:Ins(1,3,4,5)P₄-complex derived from this model similar loop movement is observed (data not shown). Thus, the

behavior of the flexible loop is dependent upon whether a ligand is present and on the structure of that particular ligand. It can be concluded that the loop can likely adjust to whatever ligand is in the binding site.

In summary, we have determined the first crystal structure of the 5-phosphatase domain of SHIP2 in complex with a ligand in the catalytic site. Although attempts to crystallize SHIP2 with its natural phosphoinositide and inositol polyphosphate substrates and the corresponding hydrolysis products were not successful, a wholly synthetic polyphosphate, BiPh-(2,3',4,5',6)P₅, was effective. The biphenyl framework of BiPh(2,3',4,5',6)P₅ may allow a pattern of phosphate groups to be presented over two aromatic rings that to some extent can rotate relative to one another, allowing optimization of polar interactions with residues in the binding pocket. These data reinforce the emerging use of aryl polyphosphates as surrogates of inositol polyphosphates and phospholipid head groups for co-crystallization studies. Molecular modeling and dynamics studies of the complex suggest that a flexible region unique to SHIP proteins may close over the ligand during binding. Targeting a closed conformation of this loop and the SHIP2-specific residues within it may provide a strategy for the design of small molecule and drug-like inhibitors of SHIP2.

EXPERIMENTAL SECTION

Chemistry. Biphenyl 2,3',4,5',6-pentakisphosphate BiPh-(2,3',4,5',6)P₅ was synthesized according to a previous report.²

Protein Purification. The sequence encoding SHIP2-cd residues 419–832 was subcloned into the vector pNIC-MBP in which an N-terminal 6 × His-tag followed by a TEV protease cleavage site is added. The proteins were expressed in *Escherichia coli* strain BL21(DE3) R3 pRARE. Cultures were grown in TB medium supplemented with 8 g L⁻¹ glycerol, 100 μg mL⁻¹ kanamycin, and 34 μg mL⁻¹ chloramphenicol in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD₆₀₀ reached ~2. Cultures were down-tempered to 18 °C over a period of 1 h before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight. Cells were harvested by centrifugation (4,430 × g, 10 min, 4 °C), and the pellet was resuspended in lysis buffer (100 mM HEPES, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 0.5 mM TCEP, 2000 U Benzonase Merck, Complete EDTA-free protease inhibitor, pH 8.0). Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4 s on, 4 s off), and cell debris was removed by centrifugation (49,000 × g, 20 min, 4 °C). The supernatant was decanted and filtered through a 0.45 μm flask filter. The filtered lysate was loaded onto Ni-charged HiTrap Chelating HP (GE Healthcare) column and washed with IMAC wash1 buffer (IMAC wash1 buffer: 20 mM HEPES, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 0.5 mM TCEP, pH 7.5) followed by IMAC wash2 buffer (20 mM HEPES, 500 mM NaCl, 10% glycerol, 25 mM imidazole, 0.5 mM TCEP, pH 7.5). Bound protein was eluted from the IMAC column with IMAC elution buffer (20 mM HEPES, 500 mM NaCl, 10% glycerol, 500 mM imidazole, 0.5 mM TCEP, pH 7.5) and subsequently loaded onto a HiLoad 16/60 Superdex 75 Prep grade column (GE Healthcare) equilibrated in gel filtration buffer (20 mM HEPES, 300 mM NaCl, 10% glycerol, 0.5 mM TCEP, pH 7.5). The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease in a molar ratio of 30:1 at 4 °C overnight. SHIP2 was purified from tag and protease by passing the reaction mixture over a Ni-charged 1 mL HiTrap Chelating HP column pre-equilibrated with IMAC wash1 buffer. During concentration of the cleaved protein the buffer was changed to gel filtration buffer.

Crystallization; Data Collection, Structure Determination and Refinement; SHIP2 Inhibition assay; and Modeling Methods. Full details are in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

Web-Enhanced Feature

Video file is available.

Accession Codes

Coordinates and structure factors have been deposited in the Protein Data Bank with the code 4A9C.

AUTHOR INFORMATION

Corresponding Author

*E-mail: b.v.l.potter@bath.ac.uk.

Notes

The authors declare no competing financial interest.

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