A new layer of phosphoinositide-mediated allosteric regulation uncovered for SHIP2

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Abstract  
The Src homology 2 containing inositol 5-phosphatase 2 (SHIP2) is a large multi-domain enzyme that catalyzes the dephosphorylation of the phospholipid phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3) to form PI(3,4)P2. PI(3,4,5)P3 is a key lipid second messenger controlling the recruitment of signaling proteins to the plasma membrane, thereby regulating a plethora of cellular events, including proliferation, growth, apoptosis, and cytoskeletal rearrangements. SHIP2, alongside PI3K and PTEN, regulates PI(3,4,5)P3 levels at the plasma membrane and has been heavily implicated in serious diseases such as cancer and type 2 diabetes; however, many aspects of its regulation mechanism remain elusive. We recently reported an activating effect of the SHIP2 C2 domain and here we describe an additional layer of regulation via the pleckstrin homology-related (PHR) domain. We show a phosphoinositide-induced transition to a high activity state of the enzyme that increases phosphatase activity up to 10-15 fold. We further show that PI(3,4)P2 directly interacts with the PHR domain to trigger this allosteric activation. Modeling of the PHR-phosphatase-C2 region of SHIP2 on the membrane suggests no major inter-domain interactions with the PHR domain, but close contacts between the two linkers offer a possible path of allosteric communication. Together, our data show that the PHR domain acts as an allosteric module regulating the catalytic activity of SHIP2 in response to specific phosphoinositide levels in the cell membrane.

KEYWORDS  
allosteric regulation, enzyme kinetics, lipid phosphatase, phosphinositides, SHIP2

Abbreviations: CD, circular dichroism; CRC, colorectal cancer; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LSCC, laryngeal squamous cell carcinoma; NSCLC, non-small-cell lung cancer; PDK1, 3-phosphoinositide-dependent kinase-1; PHR, pleckstrin homology-related; PI(3,4,5)P3, phospholipid phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatase and tensin homolog; SAM, sterile alpha motif; SEC-MALS, size exclusion chromatography coupled to multi-angle light scattering; SHIP2, Src homology 2 containing inositol 5-phosphatase 2; TCEP, tris(2-carboxyethyl)phosphine; UIM, ubiquitin identification motif.

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1  INTRODUCTION

Phosphoinositide membrane lipids are critical to the regulation of many cellular processes such as cytoskeletal reorganization, cellular trafficking, and signal transduction. They carry out various functions including membrane identity, recruitment of proteins to specific membranes and operating as second messengers in major signaling pathways. Their involvement in numerous important cellular processes requires their levels to be tightly regulated, as alterations in the phosphoinositide metabolism have been linked to serious diseases such as cancer and type 2 diabetes. Phosphatidylinositol 3,4,5-trisphosphate (P(3,4,5)P3) acts as a key lipid second messenger of the phosphoinositide 3-kinase (PI3K)/Akt/mTor pathway and recruits signaling proteins including Akt and 3-phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane, thereby controlling important cellular processes such as proliferation, growth, apoptosis, and cytoskeletal rearrangements. Aberrant activation of the PI3K/Akt/mTor pathway due to increased levels of PI(3,4,5)P3 is a frequent cause of cancer and over the years, PI3K and other kinases in the pathway have been targeted for cancer therapy. However, due to frequent occurrence of resistance, new potential targets, such as phosphatases, are now being investigated. The generation of PI(3,4,5)P3 is triggered via stimulation of growth factor and cytokine receptors that activate type I PI3K to produce PI(3,4,5)P3 from PI(4,5)P2. The two main enzymes responsible for PI(3,4,5)P3 degradation are the well-studied tumor suppressor phosphatase and tensin homolog (PTEN), which removes the 3-phosphate and the less-studied SH2-containing inositol 5-phosphatase (SHIP), which dephosphorylates the 5-phosphate to generate PI(3,4)P2.

SHIP belongs to a family of Mg2+-dependent inositol 5′-phosphatases. In humans, there are two SHIP isoforms, SHIP1 and SHIP2, which catalyze the same reaction but have non-redundant roles. SHIP1 is mainly expressed in hematopoietic cells, but can also be found in mesenchymal stem cells and osteoblasts and plays an important role in myeloid homeostasis. SHIP1 exhibits reduced levels or mutational inactivation in various leukemias and lymphomas, suggesting a role as tumor suppressor by negatively regulating the PI3K/Akt pathway. SHIP2, on the other hand, is ubiquitously expressed and has a more ambivalent oncogenic role. It functions as a tumor suppressor in glioblastoma, erythroleukemia, and squamous cell carcinoma, but has an oncogenic role in other types of cancer including breast, colorectal (CRC), laryngeal squamous cell carcinoma (LSCC), and non-small-cell lung cancer (NSCLC), where high SHIP2 levels correlate with poor prognosis. This raises a strong interest to use SHIP2 as a biological marker and a potential new cancer target. Furthermore, increasing evidence points to a key role of SHIP2 in diet-induced obesity and type 2 diabetes, two widespread diseases where the enzyme negatively regulates the insulin pathway. Patients suffering from either or both diseases are at higher risk of developing cancer. More recently, emerging evidence points to a link between SHIP2 and Alzheimer’s disease. The important role of SHIP2 in disease has led to the identification and development of several small molecules capable of modulating the enzyme’s activity.

SHIP1 and SHIP2 are multidomain enzymes that share a very similar primary structure: a N-terminal SH2 domain, a pleckstrin homology-related (PHR) domain, a central 5-phosphatase (5-Ptase) catalytic core followed by a C2 domain, and a proline-rich region containing NPXY motifs (Figure 1A). In addition, SHIP2 has an ubiquitin identification motif (UIM), a sterile alpha motif (SAM) domain located at its C-terminus as well as a putative RhoA-binding domain between the SH2 and PHR domain. The SH2 domain is responsible for targeting the enzyme to phosphorylated cell surface receptors, whereas the PHR and the C2 domains flanking the catalytic 5-Ptase have lipid-binding properties. We previously described structural and mechanistic details of how the C2 domain allosterically activates the phosphatase domain of SHIP2. The PHR domain was first identified in SHIP1 based on chemical shifts from NMR and was found to contribute to the translocation of SHIP1 from the cytosol to the plasma membrane without affecting the enzymatic activity.

Here we investigate the role of the PHR domain in SHIP2. We find that the PHR domain increases the SHIP2 phosphatase activity for the PI(3,4,5)P3 substrate. The enzyme response follows a sigmoidal curve indicating a transition to a high activity state at high substrate concentration. We show that direct binding of the reaction product PI(3,4)P2 to the PHR domain induces this high activity state. Our results further demonstrate that PI(3,4)P2, PI(3,4,5)P3, and PI(4,5)P2 bind with similar affinity and in a competitive manner to the PHR domain, suggesting that they likely are all able to act as positive regulators. We propose a model where both the PHR and C2 domains, flanking the 5-Ptase domain, interact with specific lipids in the membrane, providing optimal positioning toward the PI(3,4,5)P3 substrate as well as allosteric activation of SHIP2.

2  MATERIALS AND METHODS

2.1  Cloning, expression, and purification

The experimental details regarding the cloning, expression, and purification of human SHIP2 Ptase (residues 420-732) and Ptase-C2 (residues 420-878) have been previously described. Human SHIP2 PHR (residues 300-419), PHR-Ptase (residues 300-732) and PHR-Ptase-C2 (residues 300-878) have been previously described.
300-878) were cloned and expressed following the same protocol as the two first constructs and purified with minor modifications to the original protocol. Briefly, the different constructs were purified from sonicated *E. coli* cell lysate that was then centrifuged (30 minutes, 40 000 rpm, 277 K) and the supernatant was applied onto a Ni²⁺ resin. The column was washed, starting with a high salt buffer (20 mM Na, K, phosphate, 500 mM NaCl, 5% glycerol, 2 mM TCEP, pH 7.4) followed by washes, gradually lowering the salt concentration to 100 mM NaCl. The proteins were then eluted using an imidazole gradient (20-500 mM Imidazole). Subsequently, the N-terminal tag was cleaved off by overnight proteolysis with PreScission Protease at 277 K, while the proteins were dialyzed into the appropriate buffers. Next, the protein was purified using a Source 15S column with a NaCl gradient, followed by a reverse Ni column. Finally, all proteins were purified on a size-exclusion Superdex 200 column, except for SHIP2 PHR that was applied to a Superdex 75 column instead. All details regarding the buffers used in the purification steps are summarized in Supporting Information Table S1.

### 2.2 | Kinetics

Activity measurements were performed using a Malachite Green phosphatase activity assay with slight modifications to the previously described one. Proteins were incubated for 2 minutes at 23°C with the chosen substrate (either inositol 1,3,4,5-tetraakisphosphate, IP₄ or phosphatidylinositol 3,4,5-trisphosphate diC₈, PI(3,4,5)P₃-diC₈, Echelon Biosciences) in a reaction buffer containing 20 mM Hepes, 150 mM NaCl, 2 mM MgCl₂, 1 mM TCEP, pH 7 in a total volume of 25 μL. The reaction was quenched by the addition of 5 μL of 0.5 M EDTA, pH 8. Subsequently, 25 μL of the reaction was mixed with 100 μL of Malachite Green solution made based on Harder et al. and Sherwood et al. and incubated for 15 minutes at room temperature and the optical density was measured at 620 nm. Each data point is measured at least in triplicates. The kinetic parameters listed in Table 1 were determined using 100 nM of the different enzyme constructs for the assay when using IP₄ as the substrate and 50 nM when using PI(3,4,5)P₃-diC₈ except for the 5-Ptase construct where a 400 nM
concentration was used. Kinetic data were fitted to the Michaelis–Menten equation \( v = \frac{V_{\text{max}}X}{K_M + X} \), where \( X \) is the substrate concentration and \( K_M \) is the substrate concentration at which the initial rate (\( v \)) is one-half of the maximum velocity (\( V_{\text{max}} \)), or to the Hill equation \( v = \frac{V_{\text{max}}X^h}{K_{\text{half}}^h + X^h} \).

To evaluate the effect of PI(3,4)P_2diC8, at 0 or 150 µM (Figure 2), on the different constructs, we used the following enzyme concentrations: PHR- Ptase (50 nM), PHR- Ptase-C2 (20 nM), Ptase (400 nM), and Ptase-C2 (20 nM). To determine the half-maximal activation concentration (AC50) of PI(3,4)P_2diC8, we carried out a titration curve at a fixed concentration of PI(3,4)P_2diC8 (0.044 mM), \( [\text{PI}(3,4)\text{P}_2\text{diC}_8] \). For activity measurements with vesicles, PI(3,4,5)P_3 vesicles were prepared at a total lipid concentration of 4.5 mM, containing 10% (mol/mol) saturated PI(3,4,5)P_3 (16:0, Avanti Polar Lipids), 30% (mol/mol) dipalmitoylphosphatidylinositol (16:0, Avanti Polar Lipids), 60% (mol/mol) chicken egg phosphatidylinositol (Avanti Polar Lipids), and 250 nM enzyme. The organic solvent was removed by rotary evaporation for 90 minutes at 45°C. The lipid film lipid was subsequently resuspended in 20 mM HEPES, 150 mM NaCl, 5% glycerol, and pH 7. The anisotropy measurements were taken at room temperature on an EnVision Multilabel Reader (Perkin Elmer) and the binding curves and apparent dissociation constants (\( K_{\text{app}} \)) were determined by fitting an IC50 equation \( Y = B0 + (B_{\text{max}} - B0)/(1 + 10^{(\log(X) - \log(K_{\text{app}}))}) \), using the program Prism (GraphPad Software Inc).

### 2.4 CD spectroscopy

Circular dichroism was used to assess the overall content of secondary structure and protein fold stability of the SHIP2 PHR and PHR-Ptase-C2 constructs (Figure S1) following previously published experimental conditions. Circular dichroism was used to assess the overall content of secondary structure and protein fold stability of the SHIP2 PHR and PHR-Ptase-C2 constructs (Figure S1) following previously published experimental conditions.31 Far-UV CD spectra between 260 and 200 nm of protein at 0.25 mg/mL were recorded at 20°C for each sample. To determine the melting temperature of SHIP2 PHR, 5-Ptase, Ptase-C2, and PHR-Ptase-C2, the samples were prepared at a concentration of 0.25 mg/mL in 10 mM Tris, 75 mM NaCl, 5% glycerol, and pH 8. The thermal curves were measured at 216 nm, between 20 and 95°C.

CD spectra and thermal melting plots to compare PHR-Ptase and PHR-Ptase-C2 in the absence and presence of PI(3,4)P_2 were performed in a similar manner with 0.25 mg/mL PHR-Ptase or 0.5 mg/mL PHR-Ptase-C2 in the absence or presence of 150 µM PI(3,4)P_2 in 10 mM Tris, 75 mM NaCl, 5% glycerol, and pH 8.5.

### 2.5 Size exclusion chromatography coupled to multi-angle light scattering

Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) was performed using a

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| Table 1 | Kinetic parameters for SHIP2 constructs |
|---------|-----------------------------------------|
| **PI(3,4,5)P_3** | **IP_4** |
| \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_{\text{half}} \) (µM) | \( h \) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_m \) (µM) |
| Ptase | 1.44 ± 0.06 | 36 ± 6 | – | 4.85 ± 0.26 | 125 ± 23 |
| PHR-Ptase | 5.40 ± 0.90\(^a\) | 133 ± 17\(^a\) | 3.40 ± 0.92\(^a\) | 1.36 ± 0.16 | 107 ± 47 |
| Ptase-C2 | 7.67 ± 0.28 | 76 ± 9 | – | 8.20 ± 0.22 | 83 ± 9 |
| PHR-Ptase-C2 | 14.54 ± 0.71\(^a\) | 82 ± 5\(^a\) | 1.68 ± 0.12\(^a\) | 6.90 ± 0.21 | 76 ± 9 |

Note: Kinetic parameters are extracted by fitting the Michaelis–Menten or Hill\(^a\) equation. Activity assays with PI(3,4,5)P_3diC8 were performed with 50 nM enzyme, except for the 5-Ptase 400 nM was used. Assays with IP_4 were carried out with 100 nM enzyme.

\(^a\)\( K_{\text{half}} \): Substrate concentration resulting in half-maximal activity. For SHIP2 Ptase and Ptase-C2, which follow Michaelis–Menten kinetics, \( K_{\text{half}} = K_v \).
chromatographic system (1200 pump, Agilent Technologies) with a Superdex 200 increase 10/300 GL column, connected to a MALS instrument (DAWNEOS, Wyatt Technology) and, in parallel, to an Optilab rEX refracting index detector (Wyatt Technology). Protein samples (200 μL) with concentrations ranging from 0.5 to 1 mg/mL were loaded onto the size exclusion column equilibrated with 20 mM Hepes pH 7.0, 150 mM NaCl, 5% Glycerol, 1 mM TCEP (SHIP2 constructs without the PHR domain) or 20 mM Hepes pH 7.2, 500 mM NaCl, 5% Glycerol, and 1 mM TCEP (PHR containing constructs). Experiments were performed at RT with a flow rate of 0.5 mL/min. ASTRA software (Wyatt

**FIGURE 2** Ligand-induced allostericity. A-D. Enzyme activities titrating the PI(3,4,5)P3diC8 substrate are shown for PHR-Ptase (50 nM, A), PHR-Ptase-C2 (20 nM, B), Ptase (400 nM, C), and Ptase-C2 (20 nM, D) in the presence or absence of PI(3,4)P2diC8 (150 μM). Curves are fitted to either the Michaelis–Menten or the Hill equation. E and F. Dose-dependent activation of SHIP2 by PI(3,4)P2diC8 are shown relative to the activity in the absence of PI(3,4,5)P3diC8, using a fixed PI(3,4,5)P3diC8 concentration at 44 μM and with PHR-Ptase (50 nM, E) or PHR-Ptase-C2 (20 nM, F). Activation of PHR-Ptase-C2 was fitted to an EC50 dose–response equation (see Methods for details). A-F, Error bars represent SEM from at least three independent measurements.
Technology) was used to calculate the molecular weight (Mw) of the protein species that eluted from the column.

2.6 | Structure model

iTasser\textsuperscript{37} was used to generate the atomic model of the PHR domain based on the amino acid sequence of SHIP2 residues 300-419. Coot\textsuperscript{38} was used to merge together the iTasser generated PHR model with the previously published and experimentally based model of SHIP2 Ptase-C2.\textsuperscript{31} Pymol\textsuperscript{39} was used to illustrate the potential orientation of the SHIP2 protein toward the membrane.

3 | RESULTS

3.1 | The PHR domain induces a sigmoidal behavior and activation of SHIP2

To evaluate the effect of the PHR domain on SHIP2 phosphatase activity, we generated several protein constructs containing or lacking the PHR and C2 domains (Figure 1B). Far-UV circular dichroism (CD) spectra of the PHR and PHR-Ptase-C2 proteins support that this domain is folded and exhibit secondary structure elements (Figure S1A,B). Thermal melting of the PHR domain suggests that by itself it may not be a highly compact domain (Figure S1C), possibly adopting multiple conformations during thermal unfolding.\textsuperscript{40} Furthermore, we observe that the PHR domain does not significantly affect the melting temperature of SHIP2 PHR-Ptase-C2 (Figure S1D,E). When comparing the catalytic responses of the SHIP2 proteins, we observed that the presence of the PHR domain increases maximal phosphatase activity, both when using a soluble PI(3,4,5)P\textsubscript{3} substrate with 8-carbon fatty acid chains (PI(3,4,5)P\textsubscript{3}diC\textsubscript{8}) (Figure 1C) and with long-chain PI(3,4,5)P\textsubscript{3}diC\textsubscript{16} embedded in lipid vesicles (Figure S2). Using soluble PI(3,4,5)P\textsubscript{3}diC\textsubscript{8}, the presence of the PHR domain further induces a sigmoidal behavior while constructs lacking the PHR domain follow Michaelis–Menten kinetics (Figure 1C). In the presence of the C2 domain, which as previously shown also increases SHIP2 activity,\textsuperscript{31} maximal activity (k\textsubscript{cat}) increases \textasciitilde2-fold with the PHR domain, whereas the increase is \textasciitilde4-fold in the absence of the C2 domain (Table 1). The sigmoidal behavior of PHR-Ptase and PHR-Ptase-C2 is indicative of an induced transition to a high activity state as it occurs in cooperative oligomeric systems. The sigmoidal behavior is more pronounced in the absence of the
C2 domain (Figure 1C) as evident from extracted Hill coefficients ($h$) for PHR-Ptase ($h = 3.40 \pm 0.92$) and PHR-Ptase-C2 ($h = 1.68 \pm 0.12$) (Table 1).

Next, we assayed the SHIP2 phosphatase activity using only the inositol 1,3,4,5-tetrakisphosphate (IP$_4$) headgroup as substrate. In this case, all constructs exhibit a catalytic behavior closely following the Michaelis–Menten model and interestingly, the PHR domain now impairs the phosphatase activity, more so in the absence of the C2 domain (Figure 1D). The kinetic parameters show that the PHR domain negatively affects $k_{cat}$ but not $K_m$ (Table 1). Together, these data demonstrate that the PHR domain provides an allosteric and sigmoidal activation of SHIP2, but only if using an intact PI(3,4,5)P$_3$ lipid as substrate, hence also providing selectivity for PI(3,4,5)P$_3$ over IP$_4$.

### 3.2 PHR-mediated sigmoidal behavior occurs via ligand-induced allostericity

Sigmoidal kinetics traditionally indicates cooperativity in oligomeric enzymes, but has also been observed in monomeric enzymes. We show that the purified SHIP2 proteins used in this study behave as monomers in solution (Figure S3), we therefore prefer not to refer to a cooperative effect in SHIP2, since this generally refers to communication between active sites in an oligomer. We wondered whether the PHR-mediated sigmoidal behavior could arise through binding of the substrate and possibly reaction product to the PHR domain followed by intramolecular allosteric activation of the 5-Ptase. To test this, we analyzed the catalytic response in the presence of the product PI(3,4)P$_2$diC8.

We observe that the addition of PI(3,4)P$_2$diC8 changes the response from a sigmoidal to a Michaelis–Menten behavior when the PHR domain is present (Figure 2A,B). The addition of product has no effect on the activity of Ptase-C2 while reducing that of the Ptase (Figure 2C,D). It is unclear why Ptase inhibition occurs in the latter case, but it could reflect a form of substrate and/or product inhibition, potentially due to inefficient product/substrate exchange, which we previously showed is aided by the presence of the C2 domain via coordinating movements of a substrate-binding loop proximal to the active site.

Next, we determined the PI(3,4)P$_2$diC8 concentration required for half-maximal activation (AC50) by titrating PI(3,4)P$_2$diC8 at a fixed substrate concentration (Figure 2E,F). For PHR-Ptase-C2, we obtain an AC50 of $0.15 \pm 0.09$ mM and a fitted maximal 9.6-fold activation.

For PHR-Ptase, the AC50 could not be accurately determined in the usable concentration range of PI(3,4)P$_2$ for this assay. Qualitatively, it appears that the activation of PHR-Ptase occurs at a higher PI(3,4)P$_2$ concentrations (AC50 $\geq 0.15$ mM) with the maximal activation also significantly increased (>15 fold).

We further tested whether the PI(3,4)P$_2$ lipid significantly affects the structure and/or stability of SHIP2 containing the PHR and Ptase domains using CD analysis. While CD spectra are little affected by the presence of PI(3,4)P$_2$diC8 (Figure S4A,D), the Tm increases for PHR-Ptase and decreases for PHR-Ptase-C2 in the presence of PI(3,4)P$_2$ (Figure S4E-H). Together, these data suggest that the sigmoidal behavior described above is allosterically induced by binding of substrate and product to the PHR domain. In addition, it is possible that in the case of PHR-Ptase, stabilization upon substrate and product binding further contributes to reaching a high activity state.

### 3.3 Phosphoinositides directly interact with the SHIP2 PHR domain

To confirm that PHR-mediated activation occurs via a direct interaction with the PHR domain, we performed binding studies using a fluorescence polarization assay. Using a soluble BODIPY labeled PI(3,4,5)P$_3$ probe, we detected a direct interaction with the PHR domain and determined a dissociation constant ($K_d$) of 23.8 $\pm$ 1.6 μM (Figure 3A). The presence of the 5-Ptase and C2 domains lower the affinity for PI(3,4,5)P$_3$ to 30.4 $\pm$ 2.6 μM (PHR-Ptase) and 51.8 $\pm$ 4.4 μM (PHR-Ptase-C2) (Figure 3B,C). Only background binding was observed for constructs lacking the PHR domain (Figure 3D,E). We note that no Mg$^{2+}$ was used in the binding measurements; hence, the 5-Ptase active site is not expected to show significant affinity for its PI(3,4,5)P$_3$ substrate. To determine the lipid-binding specificity of the PHR domain, we performed a competition assay using the unlabeled lipids PI(3,4,5)P$_3$, PI(3,4)P$_2$diC8, PI(3,4)P$_2$diC8, PI(4,5)P$_2$diC8, phosphatidylserine (PSdiC8), and phosphatidylcholine (PCdiC8). As shown in Figure 4, the three phosphoinositides PI(3,4,5)P$_3$, PI(3,4)P$_2$, and PI(4,5)P$_2$ exhibit very similar efficiencies for competing with BODIPY-PI(3,4,5)P$_3$ binding while no significant competition is observed with PCdiC8 and PSdiC8. Together, these results suggest that the phosphoinositides PI(3,4,5)P$_3$, PI(3,4)P$_2$, and PI(4,5)P$_2$ interact specifically and with similar affinities with the PHR domain of SHIP2. Because PI(3,4,5)P$_3$ and PI(4,5)P$_2$ are substrates of SHIP2 (both have a 5-phosphate), we cannot determine how the binding of these lipids to the PHR domain affects the sigmoidal behavior of the PHR-containing constructs (as done with PI(3,4)P$_2$ in Figure 2A,B), but the similar and competitive binding to the PHR domain suggests that they would likely also be able to act as allosteric activators of SHIP2.
3.4 | Structural model of membrane bound SHIP2 PHR-Ptase-C2

With the PHR, 5-Ptase and C2 domains all known to interact with membrane lipids and a short PHR-Ptase linker (5 residues), the orientation of the PHR domain relative to Ptase-C2 and the membrane is restricted. We therefore set out to generate a model of the SHIP2 PHR-Ptase-C2 fragment bound to a membrane surface, taking as a basis our previously reported Ptase-C2 structure. Since no structural information is available for the SHIP2 PHR domain and the coordinates of the reported SHIP1 PHR domain model are not available, we generated a model of the SHIP2 PHR domain using the protein structure prediction server, iTasser. All top models produced by iTasser adopt a PH-like fold without providing a structural template as input. Lipid interactions of the modeled SHIP2 PHR domain were based on the PH domain-IP₃ complex structures of centaurin α1 and Akt (PDB: 3LUJ1 and 1UNQ, respectively) and the membrane-binding surface of the Ptase-C2 model was adopted from. The Ptase-C2 structure positioned on the membrane as in Le Coq et al places the N-terminal Ptase residue (P421) ~34 Å above the membrane surface, which allows for placement of the SHIP2-PHR domain while bound to PI(3,4,5)P₃ embedded in the membrane (C-terminal D419 to P1-PI(3,4,5)P₃ distance: 37 Å), but limits rotational freedom. The final model adopts a tripod-pyramidal shape with all three domains contacting the membrane (Figure 5). The short PHR-Ptase linker does not allow extensive PHR interactions to occur with the Ptase or C2 domains, which is consistent with the lack of thermal stabilization by the PHR domain (Figure S1D,E). Main contacts occur via the two linkers connecting to the Ptase domain, as they lead to two neighboring β-sheets in the Ptase domain.

4 | DISCUSSION

The PI3K pathway continues to be a highly investigated signaling route due to its ongoing pharmaceutical relevance. While the focus has so far primarily been on targeting kinases of the pathway, the results of drug studies are mixed and often show associated toxicity as well as development of resistance; hence, the interest is now shifting to include initially forgotten phosphatases such as the lipid phosphatases SHIP1 and SHIP2. Understanding regulatory features in the SHIP enzymes allows for the design and development...
of allosteric drugs with potentially improved specificity and low toxicity.

We previously showed that the C2 domain of SHIP2 affects the catalytic turnover by coordinating the movements of a substrate-binding loop next to the active site. We now show that there is a second regulatory domain in SHIP2, the PHR domain, that similarly to the C2 domain, has a stimulating effect on the 5-Ptase activity of SHIP2. Although both domains activate SHIP2, they differ in their mechanism of action. While C2-mediated activation occurs via a tight and likely constitutive association with the catalytic 5-Ptase, the PHR domain is likely rather weakly connected to the catalytic module, as supported by the lack of structural stabilization during thermal melting (Figure S1D,E) and modeling of SHIP2 PHR-Ptase-C2 on the membrane (Figure 5).

Furthermore, in contrast to the C2 domain, enzymic activity is enhanced by the PHR domain via a ligand-induced mechanism. We demonstrate that the reaction product PI(3,4)P₂ binds to the PHR domain and increases the turnover rate of SHIP2 via an allosteric mechanism up to 10-fold (PHR-Ptase-C2) or 15-fold (PHR-Ptase). In addition to PI(3,4)P₂, we find that PI(3,4,5)P₃ and PI(4,5)P₂ bind with similar affinity in a competitive manner to the PHR domain (Figure 4), indicating that they might also act as allosteric activators of SHIP2, although we cannot test this due to the presence of a reactive 5-phosphate. PHR-mediated SHIP2 activation only occurs with the intact PI(3,4,5)P₃ lipid as substrate (Figure 1C,D); therefore, the PHR domain also serves to tune the substrate specificity of SHIP2 in favor of the PI(3,4,5)P₃ phospholipid versus the IP₄ headgroup. Together, this suggests that the PHR domain of SHIP2 has two important functions, one in targeting the enzyme to membranes rich in substrate-related lipids, and a second in allosteric activation of SHIP2 to dephosphorylate the PI(3,4,5)P₃ membrane lipid.

On a cautionary note, we point out that our observations are made with truncated SHIP2 proteins. A possibility is that missing SHIP2 regions might be required for full stabilization of the PHR and Ptase domains and PI(3,4)P₂ binding to the PHR domain compensates for this, leading to increased stability and activation of truncated SHIP2 forms. Such an effect might indeed result in the higher activation observed for PHR-Ptase, where PI(3,4)P₂ binding results in thermal stabilization (Figure S4E,F), but does not appear relevant for PHR-Ptase-C2 (Figure S4G,H).

In humans, the PHR regions of SHIP1 and SHIP2 share 43% sequence identity and bind the same phospholipids, although SHIP1 shows a clear preference for PI(3,4,5)P₃. However, SHIP1 shows no activating or sigmoidal behavior upon lipid binding to the PHR domain, although these studies were carried out using IP₄ as substrate that did not display any sigmoidal kinetics or activation with SHIP2 either (Figure 1D). Interestingly, this same study identified a sigmoidal allosteric activation by binding of PI(3,4)P₂ to the SHIP1 C2 domain, a mechanism that does not exist in SHIP2 (Figure 2D). Positive feedback via allosteric activation by reaction products is not a novel feature in the regulation of lipid phosphatases and was previously observed with PI(4,5)P₂ activating PTEN through its N-terminal PI(4,5)P₂-binding motif and PI(5)P activating MTM phosphatases through a divergent PH motif.

A structural model for the SHIP1 PHR domain has previously been reported based on chemical shift-guided homology threading using H,¹⁵N HSQC spectra. However, no experimentally determined structure is available for the SHIP1 or SHIP2 PHR domains. Indeed, the
isolated PHR domains appear to suffer from unfavorable dynamics, which prevented tertiary structure determination by NMR in the case of SHIP1 \(^ {25} \) and likely causes the shallow thermal transition observed for the SHIP2 PHR domain (Figure S1C) as well as its resistance to crystallize. Nevertheless, secondary structure predictions, the NMR analysis of the SHIP1 PHR domain, \(^ {25} \) and the CD analysis of the SHIP2 PHR domain presented here (Figure S1A) strongly support the presence of a folded PH-like domain, and the specific binding of these domains to phosphoinositide lipids further supports this notion \(^ {25} \) (Figures 3 and 4). Notably, the SHIP PHR domains are rather distantly related to other known PH domains, but high sequence and structural variability are known to exist for the PH superfold family. \(^ {49,50} \) Despite the low homology, all top models generated by iTasser \(^ {37} \) adopt a PH domain fold for the PHR domain of SHIP2 using only its sequence as input. Structural sequence alignment of the top model from iTasser suggests the PH domain of phospholipase C-β3 (in iTasser aligned to the pdb 30HM) to be closest related to the SHIP2 PHR domain, with a sequence identity of 15.7%. Using the top iTasser model and the crystal structure of the Ptase-C2 fragment of SHIP2, \(^ {31} \) we present a structural model for the PHR-Ptase-C2 region of SHIP2 bound to a lipid membrane (Figure 5). With all three domains known to interact with membrane lipids and a short 5-residue linker connecting the PHR and 5-Ptase domains, PHR positioning on the membrane is restricted. In our model, the three domains adopt a tripod shape on the membrane with the PHR domain making little contact with the other two domains. The PHR domain is positioned opposite to the active site in the 5-Ptase and even detached from the membrane would be out of reach from the 5-Ptase active site. This model therefore refutes the possibility that PHR-mediated activation of SHIP2 occurs by lipid-binding-induced displacement of the PHR domain from an inhibitory position that occludes the active site. The allosteric activation of SHIP2 via the PHR domain is therefore likely triggered by lipid-induced conformational changes in the PHR domain that are transmitted allosterically to the active site. A conformational change upon \( \text{PI}(3,4,5)P_3 \) binding is in fact observed for the PH domain of Akt. \(^ {51} \) With few inter-domain interactions made by the PHR domain according to our model, a possible path of allosteric communication could occur via the PHR-Ptase linker, which is bound to interact with the Ptase-C2 linker as the two linkers connect to two neighboring β-sheets in the 5-Ptase. Interestingly, we find that the lipid-binding affinity of the PHR domain is ~2-fold lower when the 5-Ptase and C2 domains are present (Figure 3), which is consistent with lipid binding affecting 5-Ptase, C2, and/or Ptase-C2 linker conformations.

In summary, we describe a novel regulatory feature in SHIP2, where binding of its PHR domain to specific phosphoinositide lipids in the cell membrane enhances catalytic activity. This would allow SHIP2 to increase catalytic capacity on membranes with high levels of substrate and/or related phosphoinositides that can be converted into substrate. SHIP1 and SHIP2 are the main enzymes generating \( \text{PI}(3,4)P_2 \) and the increasing importance of the role of \( \text{PI}(3,4)P_2 \) in cells. \(^ {52,53} \) Initially seen as a mere catalytic by-product, puts even more weight on the necessity of a better appreciation on how the \( \text{PI}(3,4,5)P_3/\text{PI}(3,4)P_2 \) ratio is regulated as both phospholipids are associated with severe diseases such as cancer. \(^ {53} \) Our insights presented here might be exploited toward the design of novel strategies to modulate SHIP2 activity and function via its PHR domain.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest in connection with this article.

**AUTHOR CONTRIBUTIONS**

J. Le Coq and D. Lietha designed the research; J. Le Coq, P. López Navajas, B. Rodrigo Martin, and C. Alfonso performed the research; J. Le Coq, C. Alfonso, P. López Navajas, and D. Lietha analyzed the data; J. Le Coq and D. Lietha wrote the paper; D. Lietha provided the funding.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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