Non-canonical Interaction of Phosphoinositides with Pleckstrin Homology Domains of Tiam1 and ArhGAP9*

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Pleckstrin homology (PH) domains are phosphoinositide (PI)-binding modules that target proteins to membrane surfaces. Here we define a family of PH domain proteins, including Tiam1 and ArhGAP9, that demonstrates specificity for PI(4,5)P2, as well as for PI(3,4,5)P3 and PI(3,4)P2, the products of PI 3-kinase. These PH domain family members utilize a non-canonical phosphoinositide binding pocket related to that employed by β-spectrin. Crystal structures of the PH domain of ArhGAP9 in complex with the headgroups of Ins(1,3,4)P3, Ins(1,4,5)P3, and Ins(1,3,5)P3 reveal how two adjacent phosphate positions in PI(3,4)P2, PI(4,5)P2, and PI(3,4,5)P3 are accommodated through flipped conformations of the bound phospholipid. We validate the non-canonical site of phosphoinositide interaction by showing that binding pocket mutations, which disrupt phosphoinositide binding in vitro, also disrupt membrane localization of Tiam1 in cells. We posit that the diversity in PI interaction modes displayed by PH domains contributes to their versatility of use in biological systems.

The spatial and temporal regulation of protein localization plays an important role in the transduction of signals between sub-cellular compartments. Targeting of proteins to membrane surfaces through interactions with phosphoinositides (PIs) promotes the formation of functional complexes and concomitantly restricts their site of biochemical activity (1). Phosphoinositides are lipid components of cellular membranes that function as signaling molecules. The inositol headgroups of phosphoinositides are differentially phosphorylated, and selectively bound by a variety of protein modules, including PH, FERM, ENTH, FYVE, and PX domains (2–4). PH domains were the first phosphoinositide binding domain identified (5) and serve important roles in kinase signaling and cytoskeletal organization (6, 7).

PH domains consist of 100–120 amino acids that form a seven-stranded β-sandwich with a C-terminal α-helix. The PI binding properties of PH domains are diverse, ranging from family members that display no detectable interaction to domains that bind one or more headgroups with nanomolar binding affinity (8–10). Generally, PH domains that possess weak affinity for phosphoinositides are inefficient at PI-dependent membrane localization. These PH domains may require multimerization or cooperation with other factors for their targeting function (11, 12). In contrast, PH domains that bind with high affinity and selectivity to either PI(4,5)P2 or the PI 3-kinase products PI(3,4,5)P3 and PI(3,4)P2 are efficiently targeted to membrane surfaces. The PH domain of PLCδ is a specific sensor of PI(4,5)P2, whereas those of Akt, Btk, and Grp1 exemplify domains that selectively bind with high affinity to PI(3,4)P2 and/or PI(3,4,5)P3 (10).

A basic consensus motif within the β1–β2 loop region is characteristic of PH domains that bind phosphoinositides with high affinity and specificity (13). This motif defines the core of the canonical PI binding pocket, and mutations therein disrupt both phosphoinositide binding and membrane localization functions (14). To date, eight protein crystal structures of PI-bound PH domains demonstrate direct interactions between phosphates on the inositol ring and residues of this phosphoinositide recognition motif (15–21). Additional interactions with the bound phosphoinositide are contributed by more variable regions of the PH domain, including residues within the β3–β4 and β6–β7 loop regions. This PH-recognition motif has facilitated identification of additional PH domain containing proteins that bind phosphoinositides within the canonical binding pocket (22).

Despite its power in predicting PI-binding properties, the conventional phosphoinositide recognition motif is not appar-
ent in proteins such as Tiam1 that nonetheless require a functional PH domain for membrane localization (23). Tiam1 is a multidomain protein involved in reorganization of the cortical actin cytoskeleton (24). The C-terminal region of Tiam1 contains a DH-PH region with guanine nucleotide exchange factor activity specific for Rac1 (25). In contrast, the N-terminal PH domain (PHn) of Tiam1 is required for localization to the plasma membrane through its apparent binding preference for PI(3,4,5)P3, PI(3,4)P2, and PI(4,5)P2 ligands (23, 26, 27). The unique PI-binding profile displayed by Tiam1, together with the absence of sequence motifs characteristic of PH domains that bind phosphoinositides with high affinity and selectivity, suggests a novel mode of phospholipid recognition. Here we show that the PH domain of Tiam1 is representative of a larger subfamily of PH domains that share similar lipid binding preferences and bind phosphoinositides through a non-canonical mechanism related to that employed by the PH domain of β-spectrin. Strikingly, structural analysis of one subfamily member, the ArhGAP9 PH domain, shows that its PI-binding properties are expanded by the ability to recognize distinct ligands in flipped orientations.

**EXPERIMENTAL PROCEDURES**

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**Construct Generation**—Murine TIAM1 constructs C1199 and C1199ΔPH in the vector pEGFP were obtained from Peter Downes (U. Dundee, UK). Point mutations of R459A and R460A within the PHn domain were generated by QuikChange PCR and confirmed by DNA sequencing. The PHn (aa 432–553) and PHn-cc-Ex (aa 432–669) fragments of wild-type and RR/AA Tiam1 were subcloned into modified pGEX-2T (Amer sham Biosciences) and pMAL-C2 (New England Biolabs) vectors for bacterial expression. The PH domain (residues 1–122) of human PKB/Akt1 was cloned into pMAL-C2. The PH domain of human ArhGAP9 (aa 321–440) was amplified by PCR from cDNA (MGC:12959) and subcloned into pGEX-2T and pMAL-C2. Site-specific mutants of AG9 were generated by QuikChange PCR and verified by DNA sequencing.

**Protein Expression and Purification**—The GST-AG9 PH fusion protein was expressed in *Escherichia coli* BL21 (DE3) CodonPlus cells by addition of 0.25 mM isopropyl 1-thio-β-D-galactopyranoside to cultures at *A*660 = 0.6 for 15 h at 18 °C. Cell pellets were suspended in 20 mM HEPES, pH 7.5, 200 mM NaCl, 5 mM β-mercaptoethanol and sonicated in the presence of 10 μg/ml lysosome and 1 mM phenylmethylsulfonyl fluoride. Filtered supernatant applied to a glutathione-Sepharose column was eluted with 25 mM reduced glutathione. The PH domain was cleaved from GST by incubation with thrombin (200 units, Sigma) overnight at 4 °C. Following dialysis in 20 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM β-mercaptoethanol, AG9 was further purified by anion exchange on HiTrap Q HP (Amersham Biosciences) and size exclusion chromatography over Superdex S75 columns (Amersham Biosciences). Fractions containing the PH domain were concentrated to 12–14 mg/ml and stored at −70 °C.

The maltose-binding protein (MBP) fusion proteins MBP-TIAM1 PHn, MBP-Tiam1 PHn-cc-Ex, MBP-Akt, and MBP-AG9, and point mutants thereof were expressed per GST-AG9 as above except the bacterial cell supernatant was applied to amylose resin (New England Biolabs). MBP proteins were eluted with 20 mM maltose, concentrated, and further purified by size exclusion chromatography over a Superdex S200 column (Amersham Biosciences) in 20 mM HEPES, pH 7.5, 100 mM NaCl, and 5 mM β-mercaptoethanol, concentrated, and stored at −70 °C.

**Phosphoinositide Binding Assay**—PI-binding affinities were determined by a fluorescence polarization-based assay. The phosphoinositide ligands possess short C3-acyl chains with a fluorescent moiety, BODIPY TMR-X, covalently linked through the carboxylic acid of one acyl chain, distal to the phosphate-binding determinants on the inositol ring (Invitrogen). Increasing concentrations of purified protein were added to 12.5 nM fluorescent phosphoinositide in buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, and 5 mM β-mercaptoethanol. Following 20-min incubation at room temperature, anisotropy measurements of 100-μl samples were taken at 21 °C using a Beacon 2000 fluorescence polarization instrument. Polarization filters were selected that matched the excitation and emission spectra (542 nm/574 nm, respectively) for the TMR-X fluorescence label. Binding curves and dissociation constants from a minimum of four independent experiments were determined using the GraphPad Prism software (GraphPad Software Inc.).

**Tiam1 Subcellular Localization**—HEK 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO2, 2 × 105 cells were seeded onto coverslips in a 6-well plate, and polyethyleneimine-mediated transfection was performed with GFP-Tiam1 wt, ΔPH, and RR/AA mutants. Cells were fixed 24 h later in 4% paraformaldehyde. Cells were visualized using a Leica DM IRE2 fluorescence microscope. Images were representative of numerous fields and at least three independent transfections.

**Western Blotting**—Transfected HEK 293T cells expressing GFP-Tiam1, ΔPH, and RR/AA mutants (as above) were lysed in PLC lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, and protease inhibitors). Lysates were then probed with rabbit polyclonal anti-GFP antibodies (ab290, Abcam) followed by a goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce). The proteins were then detected using ECL SuperSignal (Pierce).

**Crystallization, Data Collection, and Structure Determination**—Crystals of the AG9 PH domain were grown by vapor diffusion in hanging drops obtained by mixing 1 μl of a solution containing 0.5–0.8 mM protein and 1 mM Ins(1,4,5)P3, Ins(1,3,4)P3, or Ins(1,3,5)P3 with 0.5 ml of a reservoir solution containing 20–26% polyethylene glycol 4000, 100 mM HEPES, pH 7.0, 10% glycerol, 5 mM dithiothreitol. Crystals of AG9 bound to each inositol headgroup appeared within 1–3 days and were frozen in cryoprotectant consisting of 20% polyethylene glycol 4000, 100 mM HEPES, pH 7.0, 20% glycerol. AG9145 crystallized in the space group P21212 with unit cell dimensions of *a* = 41.5 Å, *b* = 95.1 Å, *c* = 29.7 Å (Table 3). Diffraction data from a single crystal in a liquid nitrogen cryostream at 100 K was collected to 1.8-Å resolution at APS beamline 14-BMD and processed using HKL2000 (28). AG9134 crystals have *P*21212 symmetry and unit cell dimensions of *a* = 29.2 Å, *b* = 36.9 Å,
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c = 113.3 Å. Diffraction data to 1.9-Å resolution was collected at APS beamline 14-IDB and processed using XDS (29). Crystals of AG9135 have P2₁2₁2₁ symmetry with unit cell dimensions of a = 28.8 Å, b = 36.6 Å, c = 113.7 Å. Diffraction data to 1.9-Å resolution was collected at APS beamline 14-BMC and processed using HKL2000.

The AG9145 complex was solved by molecular replacement with an ensemble of NMR-derived PH domain structures of drosophila β-spectrin (PDB = 1DRO) (30) as a search model and the program Phaser (31). Top Z-scores for rotation (Z = 4.1) and translation (Z = 6.95) functions yielded a solution that initially were refined to an R-factor of 0.39 and R-free of 0.43 using the program CNS. The initial model was modified and refined through multiple rounds with O and CNS (32, 33). The AG9134 and AG9135 complexes were solved by molecular replacement using the program Molrep followed by model building and refinement with COOT and Refmac5 (34, 35). Final statistics for each AG9 structure are listed in Table 3.

RESULTS

Tiam1 PHn Region Recognizes a Unique Profile of Phosphoinositides—To better characterize the PI-binding specificity of the Tiam1 PH domain, we quantitated the affinity of its isolated N-terminal PH domain against a comprehensive panel of different phosphoinositide isoforms. Because a region of Tiam1 immediately C-terminal to PHn, termed cc-Ex, is additionally required for efficient membrane targeting in cells (36), we also characterized the PI-binding specificity of a larger PH domain construct. For this purpose, the minimal PHn domain for PI(3,4,5)P₃, PI(3,4)P₂, and PI(4,5)P₂ (Table 1) and for monophosphate inositol species (data not shown), displayed significantly reduced binding affinities for PI(3,5)P₂ (Fig. 1 and Table 1), and from the PH domain of Akt (aa 432–669), were expressed in E. coli as MBP fusions, purified, and tested for PI binding using a fluorescence polarization-based assay. For solubility purposes, we employed phosphoinositides with short C₆-acyl chains covalently linked to the fluorescent BODIPY-TMR moiety (see “Experimental Procedures” for details).

As shown in Fig. 1 and summarized in Table 1, Tiam1 PHn and PHn-cc-Ex bound phosphoinositides with a strong preference for PI(3,4,5)P₃, PI(3,4)P₂, and PI(4,5)P₂. Both constructs displayed significantly reduced binding affinities for PI(3,5)P₂ (Table 1) and for monophosphate inositol species (data not shown). PHn-cc-Ex displayed greater overall affinity for phosphoinositides (~4-fold for each species tested) as compared with PHn (Table 1). This may reflect the fact that the isolated PHn domain is prone to aggregation. The preference of the Tiam1 PHn domain for PI(3,4,5)P₃, PI(3,4)P₂, and PI(4,5)P₂ over other phosphoinositides, as quantified in these experiments, is in agreement with previously determined qualitative lipid overlay studies (27). Our quantitative measurements also reveals that both Tiam1 constructs displayed a small preference for PI(3,4,5)P₃ over PI(4,5)P₂ and PI(3,4)P₂. This PI binding profile is very different from that of the Akt PH domain, which exhibits a well established preference for PI(3,4,5)P₃ and PI(3,4)P₂ (Fig. 1C and Table 1), and from the PH domain of PLCδ, which exclusively binds PI(4,5)P₂ (10). We considered that the unique PI binding profile of Tiam1 might reflect a variation of the Akt/PLCδ PH domain lipid-binding mode.

### TABLE 1

Dissociation constants of TIAM1 PH domain constructs for phosphoinositides

| MBP fusion protein construct | Ins(3,4,5)P₃ | Ins(3,4)P₂ | Ins(4,5)P₂ | Ins(3,5)P₂ | Inositol |
|-----------------------------|--------------|------------|------------|------------|----------|
| Tiam1-(432–669)*            | 0.54 ± 0.18  | 2.0 ± 0.7  | 1.6 ± 0.4  | 11.1 ± 1.6 | 15.6 ± 5.7 |
| Tiam1-(432–669)             | 26.9 ± 2.8   | 49.1 ± 0.5 | 40.2 ± 2.5 | >50        |
| R499A/R460A*                | 2.5 ± 1.5    | 8.4 ± 0.5  | 6.5 ± 0.7  | >50        |
| Akt1-(1–122)*               | 0.13 ± 0.02  | 0.07 ± 0.04| 9.1 ± 1.2  | >50        |
| MBP alone*                  | 27.6 ± 4.4   | 16.9 ± 2.1 | >50        |

* ± S.D. (n ≥ 4).
or alternatively the use of an entirely distinct PI-binding mechanism.

**Tiam1 Belongs to a Class of Spectrin-like PH Domains**—Sequence alignment (Fig. 2) revealed that Tiam1 PHn lacks the \( /H9252^1–/H9252^2 \) linker motif, Lys-\( X_n \)-Lys/Arg-\( X \)-Arg, characteristic of high affinity binding PH domains such as Akt, Btk, Grp1, and PLC\( /H9254 \) (13). Tiam1, however, shares five of seven PI-interacting residues identified in a crystal structure of the spectrin-\( /H9252^2 \) PH domain in complex with the inositol head-group of PI(4,5)P\(_2\) (37). The PH domain of spectrin employs a non-canonical PI-binding mechanism mediated by two short sequence elements, Lys-\( X_n \)-Arg-Ser-Trp and Tyr-\( X \)-Lys within the \( /H9252^1–/H9252^2 \) and \( /H9252^5–/H9252^6 \) loop regions, respectively. To date, no other PH domain has been shown to bind phosphoinositides using a spectrin-like mechanism.

To determine if a spectrin-like binding pocket within the Tiam1 PHn domain is responsible for its ability to preferentially bind both PI(4,5)P\(_2\) and the PI 3-kinase products PI(3,4,5)P\(_3\) and PI(3,4)P\(_2\), we mutated spectrin-conserved residues within Tiam1 and tested their effect on PI-binding function. We substituted both Arg-459 and Arg-460 with alanine (denoted Tiam-RR/AA) within the \( /H9252^1–/H9252^2 \) loop region of Tiam1 for this purpose. A mutant form of the Tiam1 PHn-cc-Ex region was purified and PI-binding activity was compared with the wild-type protein. Tiam-RR/AA demonstrated a 25- to 65-fold reduction in binding affinity for each phosphoinositide examined (Fig. 3, A–D, and Table 1). During purification by size exclusion chromatography, Tiam-RR/AA eluted at the same volume as wild-type Tiam1 suggesting the mutant protein was fully folded (data not shown).

To assess the effect of spectrin-like binding pocket mutations on Tiam1 membrane localization in cells, the same RR/AA mutant was generated in the context of full-length Tiam1. GFP-tagged versions of wild-type and Tiam1 mutants were examined for membrane localization in HEK-293T cells. An internal deletion that removes the entire PHn domain and disrupts membrane localization in NIH-3T3 cells was also tested as a negative localization control (27). Wild-type GFP-Tiam1 expression was most prominent at the boundary between cells with actin-rich protrusions (Fig. 4A and supplemental Fig. S1A). As previously reported (27, 38), deletion of the entire PHn domain (\( \DeltaPH \)) of Tiam1 resulted in diffuse cytoplasmic staining with greatly reduced actin-rich protrusions (Fig. 4B and
A non-canonical pocket within the Tiam1 PH domain is required for membrane localization. A–C, HEK 293T cells were transfected with GFP-Tiam1, GFP-Tiam1ΔPH, and GFP-Tiam1 R459A/R460A then imaged for GFP as described under “Experimental Procedures.” D, anti-GFP Western blot analysis confirmed that all three GFP-TIAM1 fusion constructs were expressed to similar levels.

supplemental Fig. S1A). Consistent with a loss of PI-binding function, the Tiam-RR/AA mutant was predominantly diffuse within the cell (Fig. 4C). Interestingly, the extent of actin-rich protrusions extended by cells expressing the RR/AA point mutations was not significantly reduced relative to wild-type Tiam1 (supplemental Fig. S1A). In addition, unlike the PH deletion that demonstrated a significant reduction of Rac1 activation, the RR/AA mutation activated Rac1 to similar levels as wild-type Tiam1 (supplemental Fig. S1B). Both wild-type and mutant proteins were expressed to equivalent levels as determined by Western blot analysis (Fig. 4D). These results suggest the intact Tiam1 PH domain may contribute phosphoinositide-independent functions such as mediating a direct interaction with the Arp2/3 complex (38).

Based on our mutational studies, we conclude that a spectrin-like binding pocket of Tiam1 is responsible for high affinity and selective phosphoinositide binding and for the proper localization of the Tiam1 protein in cells.

Spectrin-like PH Domain of ArhGAP9—Using PSI-BLAST (39), we identified ten examples of PH domains with overall greatest similarity to the Tiam1 PH domain and with conservation of the PI-interacting residues in spectrin (Fig. 2). These include the PH domains of Tiam1, Tiam2/STEF, AG9, AG12, AG15, AG27, PSD1/Tyl, PSD2, PSD3/EFA6A, and PSD4/Tic. We refer to these collectively as “spectrin-like” PH domains (Fig. 2). We predicted that these additional PH domains might display similar PI-binding preferences as observed for Tiam1. To test this hypothesis, we first examined ArhGAP9 (AG9), a largely uncharacterized protein containing SH3, WW, PH, and RhoGAP domains (Fig. 5A) (40). The PH domain of AG9 is one of the most divergent members of the spectrin-like subfamily, and we reasoned that its PI-binding properties might reveal whether different members of the spectrin-like group are functionally related. The PH domain (residues 321–440) of AG9 was expressed as an MBP fusion protein, purified, and examined for PI-binding activity. The AG9 PH domain bound preferentially to PI(3,4,5)P3, PI(3,4)P2, and PI(4,5)P2 with 0.3, 0.9, and 0.1 μM affinities, respectively (Fig. 5B and Table 2). In contrast, AG9 bound to PI(3,5)P2 or PI with 18-fold lower affinity than observed for PI(3,4,5)P3. The finding that AG9 and Tiam1 PH domains display similar PI-binding profiles supports the notion that the larger group of 10 PH domains may indeed constitute a functionally related group. We speculated that all members may use a spectrin-like pocket to preferentially bind PI(3,4,5)P3, PI(3,4)P2, and PI(4,5)P2. Because the structure of the PH domain of spectrin has only been determined in complex with PI(4,5)P2, the basis by which the non-canonical PH domain pocket selectively engages and discriminates phosphoinositide ligands remained to be determined.

Structural Determination of AG9 PH Domain–PI Complexes—To investigate the structural basis by which the spectrin-like group of PH domains might recognize both PI(4,5)P2 and the PI kinase products PI(3,4)P2 and PI(3,4,5)P3, we screened the PH domains of Tiam1 and AG9 for crystals in the presence of...
Ins(1,3,4,5)P₄, Ins(1,3,4)P₃, and Ins(1,4,5)P₃, which correspond to the headgroups of PI(3,4,5)P₃, PI(3,4)P₂, and PI(4,5)P₂, respectively (see “Experimental Procedures” for details). Efforts to crystallize Tiam1 were unsuccessful, but protein crystals of AG9 in complex with two high affinity ligands, Ins(3,4)P₂ and Ins(4,5)P₂, along with the low affinity ligand Ins(3,5)P₂ were readily obtained.

**AG9-Ins(1,4,5)P₃ Complex**—Protein crystals of AG9 in complex with Ins(1,4,5)P₃ (denoted as AG9145) diffracted x-rays to better than 1.8-Å resolution. The structure was phased by molecular replacement using an ensemble of five homology models generated from the NMR-determined β-spectrin PH structure (30). The final AG9145 structure consisted of residues 321–440 that comprise a prototypical PH domain fold with seven β-stands and a C-terminal α-helix. The 3–4 loop region, consisting of residues Pro-364 to Ser-377, was disordered and not included in the final model. Data collection and refinement for all structures described in this report are summarized in Table 3.

The structure of AG9145 revealed Ins(1,4,5)P₃ bound within a spectrin-like pocket that is spatially remote from the canonical pocket utilized by Akt, PLCβ, and related PH domains (Fig. 6). Unambiguous electron density for Ins(1,4,5)P₃ was located within the shallow cleft formed between the 1–2 and 5–6 loop regions of the AG9 PH domain (Fig. 7A). The focal point of interaction between AG9 and Ins(1,4,5)P₃ centers on the P₄ phosphate moiety of the inositol ring. The P₄ phosphate is coordinated by six hydrogen bonds involving the side chains of Lys-343, Trp-345, Ser-397, and Ser-398, the amide backbone of Ser-398, and one water-mediated hydrogen bond with the side chain of Arg-399. The P₅ phosphate is largely solvent-exposed and makes one salt bridge interaction to Lys-343 and a water-
mediated hydrogen bond to the backbone carbonyl oxygen of Leu-396. The P1 phosphate is coordinated by two hydrogen bond interactions to the side chain of Arg-342 and two water-mediated backbone interactions to the side chain of Lys-8, but both residues interact optimally with the adjacent P4 and P5 phosphates of their respective ligands. Overall, we conclude that AG9 and spectrin utilize a related but non-identical network of interactions and binding residues to engage PI(4,5)P₂.

AG9-Ins(1,3,4)P₃ Complex—To gain insights into how a spectrin-like pocket accommodates PI3-kinase phosphoinositide products, we crystallized the AG9 PH domain in complex with Ins(1,3,4)P₃ (denoted as AG9₁₃₄). The structure of AG9₁₃₄ at 1.9-Å resolution was determined by molecular replacement using the apo-AG9 PH domain above as a search model. The final structure consists of residues 321–438 and included a well ordered β3–β4 loop region containing a 3₁₀ helical turn that was disordered in the AG9₁₄₅ structure. Unambiguous electron density in difference Fourier maps was observed for the Ins(1,3,4)P₃ ligand within the β1–β2 and β5–β6 binding pocket (Fig. 7B).

The most striking difference between AG9₁₃₄ and AG9₁₄₅ structures is the orientation of the bound phosphoinositide headgroup. The inositol rings of the two AG9 complexes are related by an approximate 180° rotation about the P1–P4 axis. As a result of the flipped ring conformation, P₃ of Ins(1,3,4)P₃ is positioned in a similar binding pocket environment as P₅ of Ins(1,4,5)P₃ (Fig. 8). Despite the flipped orientation, the P₄ phosphate common to both phosphoinositides remains the focal point of PH domain contact with a very similar network of interactions. The AG9₁₄₅ structure is very similar to that of spectrin (root mean square deviation (r.m.s.d.) of 1.3 Å), and the two PH domains clearly bind Ins(1,4,5)P₃ through a common binding pocket (supplemental Fig. S2, A and B). However, the AG9₁₄₅ differs significantly in two respects from spectrin in how it engages the PI(4,5)P₂ headgroup. Although the orientation of bound Ins(1,4,5)P₃ is essentially the same in both PH domain complexes, the position of the inositol ring within the pocket is translated 2.9 Å toward the β5–β6 loop region of AG9 (supplemental Fig. S2C). This shift likely reflects differences in the position and identity of the component residues that coordinate the P₄ phosphate moiety. Specifically, Ser-397 and Ser-398 in the β5–β6 loop region of AG9 are smaller than the equivalent side chains of Tyr-69 and Lys-71 of spectrin. A second difference that may account for the shift in the inositol ring involves the binding pocket residue that senses both P₄ and P₅ phosphates on Ins(1,4,5)P₅. This residue in spectrin, Lys-8, is contributed by strand β1, whereas the functionally analogous residue in AG9, Lys-343, is contributed by the β1–β2 loop region. The side chain of Lys-343 is 2.8 Å closer to the β5–β6 loop region than that of Lys-8, but both residues interact optimally with the adjacent P4 and P5 phosphates of their respective ligands. Overall, we conclude that AG9 and spectrin utilize a related but non-identical network of interactions and binding residues to engage PI(4,5)P₂.

### Table 3: Data collection and refinement statistics for ArhGAP9 PH domain

|                      | AG9₁₄₅ | AG9₁₃₄ | AG9₁₅₅ |
|----------------------|--------|--------|--------|
| **Data collection**  |        |        |        |
| Beamline             | APS 14-BMD | APS 14-IDB | APS 14-BMC |
| Wavelength (Å)       | 0.9202 | 1.12714 | 0.90920 |
| Space group          | P₂,2,2 | P₂,2,2 | P₂,2,2 |
| Unit cell            | 41.5295.16 29.71 | 28.9236.55 112.02 | 28.7536.60 113.71 |
| Resolution (Å)       | 1.81   | 1.9     | 1.90   |
| Reflections total/unique | 137,924/11,480 | 132,389/9,921 | 53,912/9,333 |
| Completeness (%)     | 99.2 (93.6) | 99.4 (99.3) | 93.1 (91.3) |
| Rₕo (%)              | 0.048 (0.361) | 0.044 (0.225) | 0.055 (0.291) |
| (l/σ)ᵣ (%)           | 46.8 (4.5) | 12.6 (3.3) | 24.6 (5.1) |

| **Refinement**       |        |        |        |
| Resolution range (Å) | 30.1-1.81 | 30.1-1.9 | 20.1-1.90 |
| Reflections (work/test) | 10,652/534 | 9,443/476 | 9,773/484 |
| Rₜest/Rᵣfree (%)      | 19.1/22.0 | 19.2/25.0 | 22.4/23.8 |
| Protein atoms         | 860     | 914     | 921     |
| Water molecules       | 76      | 86      | 48      |
| r.m.s.d. bond length (Å) | 0.010   | 0.008   | 0.010   |
| r.m.s.d. bond angles (deg) | 1.47    | 1.16    | 1.30    |
| Average B-factors (Å²) | 20.2    | 19.4    | 22.8    |
| Protein              | 16.3    | 24.3    | 34.1    |
| Phosphoinositide      | 30.0    | 30.6    | 32.9    |
| Water                | 93.5    | 91.8    | 92.9    |
| Most favored regions (%) | 6.5      | 8.2     | 7.1     |
| Allowed regions (%)   | 0       | 0       | 0       |
| Disallowed regions (%)| 0       | 0       | 0       |

*¹ Values in parentheses are for reflections in the highest resolution bin.

**Expression and Purification**

We expressed and purified the ArhGAP9 PH domain using the pFastBac expression system and obtained inclusion bodies. We then solubilized the purified protein using 6M guanidine hydrochloride. The inclusion bodies were centrifuged at 10,000 × g for 1 h at 4 °C, and the supernatant was loaded onto a 1 mL Ni²⁺-NTA column. The bound protein was eluted using 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 500 mM imidazole, and 10% glycerol. The eluted protein was dialyzed into 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10% glycerol. We then performed a second purification step using a 1 mL HiTrap SP column. The bound protein was eluted using 100 mM NaCl, 100 mM Tris-HCl, pH 8.0, and 10% glycerol. The eluted protein was dialyzed into 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10% glycerol.

**Crystallography**

We crystallized the ArhGAP9 PH domain in complex with Ins(1,3,4)P₃ (AG9₁₃₄) and Ins(1,4,5)P₃ (AG9₁₄₅) using the hanging drop vapor diffusion method. We equilibrated 1 μL of protein solution containing 40 μM protein and 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10% glycerol against 1 μL of reservoir solution containing 1.2 M sodium citrate, pH 6.0, 0.1 M NaCl, and 10% glycerol. The crystals were flash-frozen in liquid nitrogen.

**Data Collection and Refinement**

We collected X-ray data on beamlines APS 14-BMD, APS 14-IDB, and APS 14-BMC. We used the CCP4 suite of programs for refinement against the P₀₀₂₁ space group. The final model consists of residues 321–438 and includes a well ordered β3–β4 loop region containing a 3₁₀ helical turn that was disordered in the AG9₁₄₅ structure. Unambiguous electron density in difference Fourier maps was observed for the Ins(1,3,4)P₃ ligand within the β1–β2 and β5–β6 binding pocket (Fig. 7B).

The most striking difference between AG9₁₃₄ and AG9₁₄₅ structures is the orientation of the bound phosphoinositide headgroup. The inositol rings of the two AG9 complexes are related by an approximate 180° rotation about the P1–P4 axis. As a result of the flipped ring conformation, P₃ of Ins(1,3,4)P₃ is positioned in a similar binding pocket environment as P₅ of Ins(1,4,5)P₃ (Fig. 8). Despite the flipped orientation, the P₄ phosphate common to both phosphoinositides remains the focal point of PH domain contact with a very similar network of interactions in the AG9₁₄₅ and AG9₁₃₄ complexes; interactions between AG9 and P₄ of Ins(1,3,4)P₃ involve 6 hydrogen bonds mediated by the side chains of Lys-343, Trp-345, Ser-397, Ser-398, and Arg-399 and an amide backbone interaction with Ser-398. In addition, the P1 phosphate in AG9₁₃₄ is coordinated by the same side chain of Arg-342. Lastly as was the case for the P₅ phosphate, the P₃ phosphate in AG9₁₄₅ is largely solvent-exposed and forms one hydrogen bond to the side chain of Lys-343. In contrast to the AG9₁₄₅ structure, the P₃ phosphate makes additional water-bridged interactions with the backbone amides of Lys-343 and Ser-398.

In addition to displaying a flipped orientation of the inositol ring, the bound headgroup in the AG9₁₃₄ complex was laterally translated 3.4 Å toward the position of Arg-399 as compared
with AG9145 (Fig. 8). This shift facilitates a direct interaction between the side chain of Arg-399 and the 5'-hydroxyl of Ins(1,3,4)P3. In AG9145, the analogously positioned 3'-hydroxyl of Ins(1,4,5)P3 is too far removed (>6 Å) to interact with Arg-399. In the AG9134 structure, Arg-342 and Lys-343 are also shifted toward Arg-399 by 3.0 and 1.4 Å, respectively. This altered phosphoinositide position is accommodated strictly by side-chain movements (r.m.s.d. = 0.57 Å overall, Fig. 8) and may serve to maximize the interactions between AG9 and the laterally shifted Ins(1,3,4)P3 ligand.

The AG9135 structure was solved by molecular replacement using the apo-AG9134 structure. Electron density observed within the β1–β2 and β5–β6 phosphoinositide binding pocket could not be accounted for by a single binding mode for the complete Ins(1,3,5)P3 ligand (Fig. 7C). However, unambiguous density was apparent for a single phosphate group corresponding to the P4 position in the aforementioned phosphoinositide complexes. We could not resolve which phosphate of Ins(1,3,5)P3 occupies this site in the AG9135 structure. The modeled phosphate moiety makes a total of five interactions...
with the side chains of Lys-343, Trp-345, Ser-397, Ser-398, and Arg-399, the same residues that coordinate the P4 position in the AG9145 and AG9134 complexes. Weak electron density is apparent for regions beyond the single phosphate. Attempts to position the PI(3,5)P2 headgroup into the additional electron density by docking the 1,3- or 5'-phosphate moieties into the P4 phosphate binding site (this required rotation of the modeled Ins(1,3,5)P3 by ±60° relative to the inositol rings in the AG9134 and AG9145 structures) left the other phosphate moieties of the inositol ring inaccessible to the other phosphate-coordinating groups in the AG9 binding pocket. The inability of PI(3,5)P2 to take advantage of the secondary phosphate interaction sites likely accounts for the disorder of this ligand in the AG9135 crystal structure and its weak binding affinity for AG9. Consistent with the absence of clear electron density for the intact ligand, the side chain of Arg-342 that interacts with the P1 phosphate binding site (this required rotation of the modeled Ins(1,3,5)P3 by ±60° relative to the inositol rings in the AG9 binding pocket) had no significant effect on binding affinity for PI(3,4)P2 or PI(4,5)P2. Interestingly, mutation of R342A reduced the PH domain interaction with PI(3,4)P2, PI(3,4)P2, and PI(4,5)P2 by 43-, 60-, and 7-fold, respectively. The basis for this differential effect of the R342A mutation on phosphoinositide binding cannot be rationalized from our structure analyses and may deserve future investigation.

**DISCUSSION**

*Non-canonical Class of Spectrin-like PH Domains—* We show that the PH domain of ArhGAP9 employs a non-canonical phosphoinositide binding mechanism that gives rise to a unique PI binding profile, namely a preference for both PI(4,5)P2 and the PI 3-kinase products PI(3,4,5)P3 and PI(3,4)P2. The binding mechanism is not a variation of that employed by the Akt-PLCδ class of high affinity phosphoinositide binding PH domains, but represents a variation of the spectrin-In(4,5)P2-bonding mode (37). Our binding and mutational analyses strongly suggest that the ArhGAP9 lipid binding mechanism is also employed by the PH domain of Tiam1. Structures of the ArhGAP9 PH domain in complex with Ins(1,4,5)P3 and Ins(1,3,4)P3 confirmed the use of a non-canonical PI binding pocket and explain the basis for specific and high affinity binding to PI(3,4)P2 and PI(4,5)P2. From these structures, we also infer possible binding modes of PI(3,4,5)P3 to the PH domain of ArhGAP9.

The non-canonical PI binding pocket of AG9 is very shallow, which leaves the P3, P4, and P5 phosphate moieties of engaged ligands largely solvent-exposed. This contrasts markedly with the canonical PI-binding mode observed for the Akt-PLCδ class of PH domains, where the P3, P4, and P5 positions of the inositol ring are largely buried. The shallow nature of the AG9 non-canonical binding pocket likely contributes to its ability to accommodate both PI(3,4)P2 and PI(4,5)P2 using different orientations related by a 180° flip about the PI–P4 axis of the inositol ring. Sequence alignments suggest that AG9 and Tiam1 belong to a larger PH domain family that may show similar lipid binding profiles and engage phosphoinositides through a common spectrin-like mechanism.

*Is Spectrin a Member of the Spectrin-like PH Domains?—* Spectrin and AG9 PH domains utilize the same β1–β2 and β5–β6 binding pocket for phosphoinositide recognition. How-

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![Figure 8: Superimposed stereographic view of the AG9145 and AG9134 PI binding pockets in complex with Ins(1,4,5)P3 and Ins(1,3,4)P3, respectively. The PH domain of AG9145 is colored cyan with bound Ins(1,4,5)P3 in purple; the AG9134 PH domain is colored green with bound Ins(1,3,4)P3 in pink. Orientation of bound PI(4,5)P2 and PI(3,4)P2 are related by a rotation of -180° about the P1–P4 axis of the inositol ring.](image)

...
ever the position of the bound Ins(1,4,5)P₃ and the network of interactions and identity of pocket residues differ considerably between the two domains (see supplemental Fig. S2). The total number of interactions between the inositol phosphates and non-canonical binding pocket is more extensive in AG9 than spectrin. This difference may account for the greater affinity of AG9 for Ins(1,4,5)P₃ (0.1 μM, Table 2) than displayed by spectrin (~40 μM) (37). AG9 and spectrin share only one residue, Trp-345 in AG9, that is positioned identically within the binding pocket and engages phosphoinositide ligands in the same manner (supplemental Fig. S2). This contrasts with the PH domains that employ a canonical binding pocket for phosphoinositide binding, which minimally share three basic residues within an easily recognized linear motif (13). Does the PH domain of spectrin also bind to the PI 3-kinase products PI(3,4,5)P₃ and PI(3,4)P₂? A comprehensive analysis of the PI-binding specificity for β-spectrin remains to be undertaken. However, limited binding data suggest that the PH domain of spectrin does bind Ins(1,3,4,5)P₄ but not Ins(1,3,4,5)P₃ (26, 37). The functional relationship of spectrin to the Tiam1/AG9 PH domain awaits a more detailed characterization of β-spectrin PI-binding properties.

**Versatility of the PH Domain**—The binding of different phosphoinositide products to the same interaction domain through flipped conformations of the inositol ring has not been observed previously. However, a comparable relationship in binding mode has been noted across the structure of two different phosphoinositides bound to two different PH domains (6). Specifically, PLCδ and BTK PH domains bind Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄, respectively, using flip-related orientations of their inositol rings. As for AG9, the flipped orientations of phosphoinositide headgroups are related through a 180° rotation about the P1–P4 axis of the inositol ring. Unlike the AG9 structures, tight steric effects in the canonical pocket of PLCδ and Btk prevent each domain from accommodating both Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ ligands within the same pocket. This explains why PLCδ and Btk PH domains do not efficiently bind PI(3,4)P₂ and PI(4,5)P₂ ligands, respectively.

Although flipped inositol ring orientations have not been observed within the confines of a single canonical PH binding pocket, rotationally related orientations (normal to the plane of the inositol ring) have been observed. Structures of the 3G-ARNO PH domain in complex with Ins(1,4,5)P₂ and Ins(1,3,4,5)P₄ revealed two distinct headgroup orientations related by a 60° rotation (19). This places non-equivalent phosphate moieties of the inositol ring in equivalent binding pocket environments (for example, the P4 and P5 moieties of PI(4,5)P₂ resides in the P3 and P4 binding sites of the PI(3,4,5)P₃ complex) (10). Thus both inositol ring flipping within the non-canonical PI binding pocket and inositol ring rotation within canonical PI binding pocket provide the means to extend the range of phosphoinositide specificity displayed by PH domains.

**Generality of the β1–β2 and β5–β6 Pocket among Unrelated PH-like Domains**—Three recent studies suggest that the non-canonical binding pocket composed by the β1–β2 and β5–β6 loop regions is employed by PH domains beyond the family we define in this study. The Vps36 GLUE domain, TFIH p62 subunit, and Dab1 PTB domain each possess a PH domain-like fold (although lacking significant sequence homology to PH domains) and display evidence of phosphoinositide binding within the spectrin-like binding pocket (41–44). Strikingly, all three domains lack the phosphoinositide-interacting residues present in spectrin or AG9. This strongly suggests that more variations in the mechanism by which PH domains engage phosphoinositides within the non-canonical binding pocket remain to be discovered. Elaborating on the rules that impart specific functions as achieved previously for canonical PH domains, as we do for spectrin-like PH domains, will improve our ability to ascribe function for the many unannotated PH domains in the human genome.

The PH domains of proteins such as PLCδ and Grp1 bind a single specific phosphoinositide, PI(4,5)P₂ and PI(3,4,5)P₃, respectively, in a fashion that correlates with their biological functions. PLCδ is thereby targeted to regions of the plasma membrane enriched in its substrate, whereas Grp1 serves as a target of PI 3-kinase signaling. However, this high degree of phosphoinositide binding selectivity for PH domains may be the exception, rather than the rule (6). The broader PI-binding properties of the Tiam1 and AG9 PH domains, resulting from use of the non-canonical binding pocket, may allow these enzymes to couple multiple upstream signals to their downstream targets (Rho family GTPases). For example, this might allow the Tiam1 Rac GEF to provide a basal tonic signal by binding to PI(4,5)P₂, which could be acutely up-regulated in response to signals that elevate PI(3,4,5)P₃ and PI(3,4)P₂. In both epithelial and neuronal cells, the function of Tiam1 is apparently limited by its localization to specific sites, and aberrantly broad distribution can potentially lead to ectopic Rac activation and disturbed cellular organization (45, 46). This notion is supported by properties of the Tiam1 RR/AA mutant, which is delocalized, but still induces Rac activation, potentially at ectopic sites. Characterization of the non-canonical PI-binding mechanism will facilitate further studies to reveal functions in addition to phosphoinositide binding for this subclass of PH domains.

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