Engineering the Phosphoinositide-binding Profile of a Class I Pleckstrin Homology Domain

Pleckstrin homology (PH) domains are protein modules that bind with varying degrees of affinity and specificity membrane phosphoinositides. Previously we have shown that although the PH domains of the Ras GTPase-activating proteins GAP1m and GAP1IP4BP are 63% identical at the amino acid level they possess distinct phosphoinositide-binding profiles. The GAP1m PH domain binds phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3), whereas the domain from GAP1IP4BP binds PtdIns(3,4,5)P3 and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) equally well. These phosphoinositide specificities are translated into distinct subcellular localizations. GAP1m is cytosolic and undergoes a rapid PtdIns(3,4,5)P3-dependent association with the plasma membrane following growth factor stimulation. In contrast, GAP1IP4BP is constitutively associated, in a PtdIns(4,5)P2-dependent manner, with the plasma membrane (Cozier, G. E., Lockyer, P. J., Reynolds, J. S., Kupzig, S., Bottomley, J. R., Millard, T., Banting, G., and Cullen, P. J. (2000) J. Biol. Chem. 275, 28261–28268). In the present study, we have used molecular modeling to identify residues in the GAP1IP4BP PH domain predicted to be required for high affinity binding to PtdIns(4,5)P2. This has allowed the isolation of a mutant, GAP1IP4BP(K591T), which while retaining high affinity for PtdIns(3,4,5)P3 has a 6-fold reduction in its affinity for PtdIns(4,5)P2. Importantly, GAP1IP4BP(K591T) is predominantly localized to the cytosol and undergoes a PtdIns(4,5)P2-dependent association with the plasma membrane following growth factor stimulation. We have therefore engineered the phosphoinositide-binding profile of the GAP1IP4BP PH domain, thereby emphasizing that subtle changes in PH domain structure can have a pronounced effect on phosphoinositide binding and the subcellular localization of GAP1IP4BP.

Pleckstrin homology (PH) domains are small β-sandwich protein modules of ~120 residues that occur once or, more rarely, several times in a protein sequence (1–4). Of those PH domains examined nearly all bind phosphoinositides present in cell membranes although with varying degrees of specificity and affinity. In the vast majority of PH domains, phosphoinositide binding is weak and quite nonspecific (1–4). However in around 10% of cases the phosphoinositide binding is strong and highly specific. For example, the PH domain of phospholipase C-δ1 (PLC-δ1) specifically binds phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2)(5, 6). In this case, the PH domain interaction with PtdIns(4,5)P2 results in the targeting of PLC-δ1 to the plasma membrane (7). In contrast the PH domain from Brutons tyrosine kinase (Btk) specifically binds phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3)(8, 9), an interaction that results in the dynamic, plasma membrane association of cytosolic Btk following the receptor-mediated production of PtdIns(3,4,5)P3(10).

Several PH domain structures have now been solved (11–34). The core of each domain is a β-sandwich composed of two nearly orthogonal β-sheets, β1-β4 and β5-β7, that are connected by six loop regions. Three of these, β1/β2, β3/β4, and β6/β7, have been termed the variable loops, as they display hypervariable sequences in PH domain alignments. These loops close off one corner of the β-sandwich, whereas an amphipathic carboxyl-terminal α-helix closes off the opposite open corner. To date the structure of six PH domains have been solved in complex with the inositol phosphate head group of their cognate phosphoinositide (18, 19, 25, 27, 28, 32). These studies have shown that although PH domains share a common fold, they have evolved their sequence and structure to provide a wide range of different phosphoinositide specificities and affinities.

We have described the phosphoinositide-binding profile of the PH domains from the Ras GTPase-activating proteins GAP1m and GAP1IP4BP (35). Whereas the PH domain of GAP1m specifically binds PtdIns(3,4,5)P3, the corresponding domain from GAP1IP4BP binds PtdIns(3,4,5)P3 and PtdIns(4,5)P2 equally well (35). These phosphoinositide specificities are translated into distinct subcellular distribution of these proteins (36). GAP1m is a cytosolic protein that undergoes a rapid association with the plasma membrane upon the agonist-stimulated production of PtdIns(3,4,5)P3 (37). In contrast, GAP1IP4BP is constitutively associated with the plasma membrane as a result of its PH domain-binding PtdIns(4,5)P2 (35, 36).

In the present study, we have used molecular modeling to generate predicted structures for the PtdIns(4,5)P2 and/or PtdIns(3,4,5)P3 binding pockets within the PH domains of GAP1m and GAP1IP4BP (38). These models were analyzed to identify residues in the GAP1IP4BP PH domain predicted to be required for high affinity binding to PtdIns(4,5)P2. Three possible residues, lysine 591, arginine 604, and lysine 616, were identified each of which was individually mutated into the corresponding residue from GAP1m. Analysis of the phos-
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FIG. 1. Sequence alignment of the PH domains from GAP1IP4BP, GAP1m, ARNO, and Btk. The alignment is based on sequence comparison and the known secondary structure of Btk. β-Sheets and α-helical regions are in bold, and the secondary structure features conserved in PH domains are labeled β1-β7 and α1. Basic residues present in GAP1m are labeled.

EXPERIMENTAL PROCEDURES

Molecular Modeling of the GAP1IP4BP and GAP1m PH Domains—All analysis and modeling of the PH domains was performed using the program packages QuantaCHARMm and InsightII. The alignment of PL-3 and Btk was structure-based, with the GAP1IP4BP and GAP1m PH domains being aligned by sequence, using Quanta and then refined by hand where necessary. The modeling was performed as described previously (38), with GAP1IP4BP and GAP1m being modeled independently on the structure of Btk in complex with Ins(1,3,4,5)P4 (50 °C at 4°C).

Site-directed Mutagenesis of the GAP1IP4BP PH Domain—The mutations were introduced into GAP1IP4BP using the QuikChange™ (Stratagene) and pCI-neo-GAP1IP4BP as template. Primers used were as follows: Lys-591T, 5′-AGGCCCAAGGACGGCCGCTTGGTGGATGAAA-3′; Lys-591Trc, 5′-CTTTACCCCAAAAGGCTCCGTCCTGTGGCCCTT-3′; Arg-604C, 5′-AAGGATGTTGTGTGTAACCGATATGTTCTT-3′; Arg-604Crc, 5′-GAATTCATGGTTGGTCAAACAAAACCATCTCT-3′; Lys-616P, 5′-ACCTCAGACCCAGGGGCTGGCGCAGTGTGC-3′; Lys-616Pα, 5′-GAGGGGCTGGCGCAGTGTGCAGTGTGC-3′. In each case the underlined codon describes the particular mutation, and α1-α3 designates reverse complement. For prokaryotic expression of the site-directed mutants, the corresponding cDNAs were fragmented and ligated into the corresponding sites within pGEX4T-2 (Amersham Biosciences).

Expression and Purification of GAP1IP4BP and GAP1IP4BP Mutants—Both wild-type and mutagenic GST-GAP1IP4BP fusion proteins were isolated as follows. An overnight 20 ml culture was used to inoculate each of 4 × 2.5 liter conical flasks of 500 ml of LB containing ampicillin (50 μg/ml). The cells were cultured at 25 °C until an A660 of 0.5. Protein expression was then induced by the addition of 0.1 mM isopropyl-1-thio-

β-D-galactopyranoside followed by an overnight incubation at 15 °C prior to harvesting the bacteria by centrifugation (3000 × g for 10 min at 4°C). All of the subsequent steps were carried out at 4°C. The bacterial pellet was gently resuspended in 40 ml of PBS buffer (pH 7.2) containing EDTA (1 mM), EGTA (1 mM), β-mercaptoethanol (1 mM), and Triton X-100 (1%) (v/v) and sonicated for four periods of 30 s with 30 s on ice between each sonication. Any cell debris was removed by centrifugation (38000 × g for 30 min). The supernatant was removed, and 2 ml of a 1:1 suspension of glutathione-agarose beads (washed and pre-swollen with several volumes of PBS buffer) was added and incubated on a rotating wheel for 1 h at 4°C. The beads were pelleted by centrifugation and washed sequentially with 3 × 20 ml of PBS containing 0.1% (v/v) Triton X-100. The beads were added to a column and washed with a further 3 × 20 ml of PBS. The protein was cleaved from the GST tag while bound to the column using thrombin at room temperature overnight. The free protein was then washed off the column with PBS.

Phosphoinositide Binding as Determined Using a Protein-Lipid Overlay Assay—To assess the phosphoinositide binding properties of each GAP1IP4BP mutant, a protein-lipid overlay assay was performed using the GST fusion proteins. Briefly, 1 μl of lipid solution containing 1–450 pmol of phospholipids dissolved in a mixture of chloroform/methanol/water (1:2:0.8 by volume) was spotted on to Hybond-C extra membrane and allowed to dry at room temperature. The membrane was incubated with blocking solution (50 ml Tris-HCl buffer (pH 7.5) containing 3% (v/v) bovine serum albumin, NaCl (150 mM), and Tween 20 (0.1% (v/v) final) for 1 h at room temperature. The membrane was then incubated, with gentle rocking, overnight at 4°C with 0.5 μg/ml of the relevant protein in blocking solution. The membranes were washed 4 × 15 min in washing solution (50 ml Tris-HCl buffer (pH 7.5) containing NaCl (150 mM) and Tween 20 (0.1% (v/v) final) and then incubated for 1 h with 1:1000 dilution of anti-GAP1IP4BP monoclonal antibody. The membranes were washed as before to prevent being incubated for 1 h with 1:1000 dilution of anti-mouse horseradish peroxidase conjugate. Finally, the membranes were washed as before, and the bound protein was detected by enhanced chemiluminescence.

RESULTS

Molecular Modeling and Site-directed Mutagenesis of the GAP1IP4BP PH Domain—Previously we have shown that, although the PH domains from GAP1m and GAP1IP4BP are ~63% identical at the amino acid level, they have distinct phosphoinositide-binding profiles (35). Whereas the GAP1m PH domain...
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GAP1IP4BP and GAP1m PH domains. We specifically examined the distribution of the basic residues to be compared between the basic residues in the binding of phosphoinositides to PH domains (1, 2), these model structures allowed an examination of the distribution of basic residues that were present in the GAP1m PH domain but were absent from the GAP1m PH domain. This analysis revealed that within the GAP1IP4BP PH domain six residues, lysine 591, arginine 604, lysine 616, lysine 641, lysine 666, and lysine 681, are present that are equivalent to non-basic residues in the GAP1m PH domain (threonine 619, cysteine 632, proline 644, asparagine 689, asparagine 694, and asparagine 709, respectively) (Fig. 2). Of the basic residues present in the GAP1IP4BP PH domain, lysine 641, lysine 666, and lysine 681 are located away from the phosphoinositide-binding site on the opposite corner of the PH domain structure and so are not in a position to directly interact with the bound phosphoinositide or are unlikely to interact with the membrane surface (Fig. 2). Only residues lysine 591, arginine 604, and lysine 616 are located in, or around, the proposed phosphoinositide-binding site in the model GAP1IP4BP PH domain (Fig. 3). Lysine 591 is on the β1/β2-loop in a basic run of residues, arginine 590, lysine 591, and arginine 592. In Btk the equivalent residues are all lysines, and the first of the three, lysine 17 (equivalent to arginine 590 in GAP1IP4BP), was shown in the crystal structure of Btk in complex with Ins(1,3,4,5)P4 to directly interact with the 3-phosphate (25). Therefore, lysine 591 of GAP1IP4BP is located in the correct area for potential interactions with the bound lipid. Arginine 604 and lysine 616 are located on the opposite side of the binding site. Lysine 616 is on the β3/β4-loop, which is important in the PH domain of PLC-δ1 for binding PtdIns(4,5)P2 (19). Based solely on the model structure, lysine 616 appears close enough for direct interaction with the bound lipid (Fig. 3). In contrast, arginine 604, which is located on the β2/β3 loop, may not interact directly with the bound phosphoinositide as it is located too far from the binding site. However, it should not be overlooked that this residue may interact with the negatively charged plasma membrane surface.

To study the role of these residues in the binding of PtdIns(4,5)P2 to the PH domain of GAP1IP4BP, we mutated each residue to the equivalent residue found in GAP1m, threonine 619, cysteine 632, and proline 644, thereby generating the GAP1IP4BP mutants, GAP1IP4BP-(K591T), -(R604C), and -(K616P).

**Effect of Mutations on the Binding Affinity of GAP1IP4BP for PtdIns(4,5)P2 and PtdIns(3,4,5)P3**

GST fusion proteins of wild-type GAP1IP4BP and the various mutant proteins bound to high concentrations of the lipids were reduced a clear difference in their position in relation to the phosphoinositide-binding site.

**Fig. 2. Schematic representation of the GAP1IP4BP and GAP1m model structures.** The conserved structural features of the GAP1IP4BP (A) and the GAP1m PH domains (B) are shown as ribbons and labeled β1-β7 and α1. The model structures show Ins(1,3,4,5)P4, equivalent to the inositol head group of PtdIns(3,4,5)P3, as a stick structure positioned in an equivalent orientation as observed in the Btk/Ins(1,3,4,5)P4 crystal structure (25). The basic residues present in GAP1IP4BP and the equivalent residues in GAP1m are shown as stick structures to highlight their position in relation to the phosphoinositide-binding site.
residues plays an important role in the high affinity binding to PtdIns(4,5)P2.

**Examining the Subcellular Localization of the GAP1IP4BP Mutants**—To observe the effect of the altered phosphoinositide-binding profile on the subcellular distribution of GAP1IP4BP, we initially transiently transfected HEK293 cells with each mutant, examining the subcellular localization by indirect immunofluorescence (Fig. 5). Consistent with our previous observations (35, 37), wild-type GAP1IP4BP displayed a strong plasma membrane localization with very little cytosolic staining (Fig. 5). Similarly, GAP1IP4BP-(K616P) was predominantly associated with the plasma membrane, again with very little detectable cytosolic staining (Fig. 5). In contrast, like the PtdIns(3,4,5)P3-specific binding proteins GAP1m and ARNO (36, 40), both GAP1IP4BP-(K591T) and -(R604C) were predominantly localized to the cytosol, although a minor residual plasma membrane localization was also observed (Fig. 5). These data highlight a correlation between the affinity of these GAP1IP4BP mutants for PtdIns(4,5)P2 and their resultant ability to associate with the plasma membrane in serum-starved cells. Thus, whereas the GAP1IP4BP-(K616P) retains its ability to bind with high affinity PtdIns(4,5)P2 and to associate with the plasma membrane, the GAP1IP4BP-(K591T) and -(R604C) mutants have a significantly reduced affinity for PtdIns(4,5)P2 and are predominantly localized to the cytosol.

To examine the effect of PtdIns(3,4,5)P3 production on the subcellular localization of the GAP1IP4BP mutants we transiently co-transfected HEK293 cells with the GAP1IP4BP mutants and an expression construct encoding for p110CAAX. This is a constitutively active phosphatidylinositol 3-kinase catalytic subunit that induces an elevation in plasma membrane PtdIns(3,4,5)P3. Under these conditions, as has been shown previously (36, 40), both GAP1m and ARNO became associated with the plasma membrane (Fig. 5). Interestingly, GAP1IP4BP-(K591T) and -(R604C) also became associated with the plasma membrane under these conditions, an association that was dependent upon the catalytic activity of the p110CAAX as incubation with the phosphatidylinositol 3-kinase inhibitor wortmannin (100 nM for 30 min) resulted in each mutant retaining their cytosolic localization (data not shown). These data show that the reduction in affinity for PtdIns(3,4,5)P3 observed with these mutants, ~2- and 6-fold for GAP1IP4BP-(K591T) and -(R604C), respectively, does not affect their ability to detect the elevated plasma membrane PtdIns(3,4,5)P3 observed in HeLa cells transiently transfected with p110CAAX.

**Effect of Growth Factor Stimulation on the Subcellular Localization of the GAP1IP4BP Mutants**—Using p110CAAX causes a high unphysiological elevation in the levels of plasma membrane PtdIns(3,4,5)P3. To test the effect of more physiological levels of PtdIns(3,4,5)P3 on subcellular localization prior to, and after, stimulation with epidermal growth factor. In wortmannin-treated, serum-starved PC12 cells GAP1m and ARNO were localized to the cytoplasm whereas GAP1IP4BP was constitutively associated with the plasma membrane (Fig. 6). Consistent with the data from HEK293 cells (Fig. 5), whereas GAP1IP4BP-(K616P) was associated with the plasma membrane, both GAP1IP4BP-(K591T) and -(R604C) were predominantly localized to the cytosol although, as with the HEK293 cells, some residual plasma membrane association was also observed (Fig. 6). Following a 2-min stimulation with 100 ng/ml of EGF, GAP1IP4BP-(K591T) and -(R604C) became associated with the plasma membrane (Fig. 6). A similar EGF-induced plasma membrane association was also observed for GAP1m and ARNO (Fig. 6) (36, 40). The EGF-induced plasma membrane association of GAP1IP4BP-(K591T) and -(R604C) required the ability of the growth factor to activate phosphatidylinositol 3-kinase as membrane association was inhibited by pre-incubation with 100 nM wortmannin (data not shown). These data therefore demonstrate that the affinity of the GAP1IP4BP-(K591T) and -(R604C) mutants for PtdIns(3,4,5)P3 are sufficiently high to sense the changes in the plasma membrane level of this phosphoinositide following growth factor stimulation. Thus, the lowering of the affinity for PtdIns(4,5)P2 observed in the GAP1IP4BP-(K591T) and -(R604C) mutants results in the generation of GAP1IP4BP mutants whose dynamic subcellular localization is regulated by PtdIns(3,4,5)P3 in a similar manner to that of GAP1m.

**DISCUSSION**

Previously we have described that the PH domains of the Ras GTPase-activating proteins GAP1m and GAP1IP4BP possess dis-
FIG. 4. Comparison of the affinity of wild-type (WT) GAP1IP4BP and the GAP1IP4BP mutants, GAP1IP4BP-(K591T), -(R604C), and -(K616P) for PtdIns(4,5)P2 and PtdIns(3,4,5)P3. The ability of wild-type GAP1IP4BP (A), GAP1IP4BP-(K591T) (B), GAP1IP4BP-(R604C) (C), and GAP1IP4BP-(K616P) (D) to bind PtdIns(4,5)P2 and PtdIns(3,4,5)P3 was analyzed using a protein-lipid overlay assay. Decreasing amounts of the relevant phosphoinositide were spotted onto a nitrocellulose membrane, which was then incubated with the purified proteins. The membranes were washed, and proteins bound to the membrane by virtue of their interaction with lipid were detected using specific antibodies. A representative of at least three separate experiments is shown.

The data were obtained by measuring the total pixel intensity for each spot and are an average of three separate experiments.
Distinct phosphoinositide-binding profiles (35). Whereas the GAP1m PH domain is specific for PtdIns(3,4,5)P3, the PH domain from GAP1IP4BP binds equally well to PtdIns(3,4,5)P3 and PtdIns(4,5)P2 (35). This difference in phosphoinositide specificity is manifested in the distinct subcellular localization of these proteins (36). GAP1m is a cytosolic protein that undergoes a rapid plasma membrane association upon the agonist-stimulated production of PtdIns(3,4,5)P3 (37). In contrast, GAP1IP4BP is constitutively plasma membrane-associated as a direct result of its PH domain-binding PtdIns(4,5)P2 (35, 37). In the

**Fig. 5.** The subcellular localization of wild-type GAP1IP4BP, GAP1m, ARNO, and the various site-directed mutants of GAP1IP4BP in HeLa cells with and without co-transfection of p110CAAX. Expression vectors encoding GFP-GAP1IP4BP, GFP-GAP1m, and GFP-ARNO and the various GAP1IP4BP mutants were transiently co-transfected with or without p110CAAX into HeLa cells. 24 h after transfection the cells were serum-starved for 2 h and fixed, and GAP1IP4BP, GAP1m, and ARNO were detected by confocal microscopy. The GAP1IP4BP mutants were detected by indirect immunofluorescence using GAP1IP4BP-specific antiserum as described under "Experimental Procedures." The white arrows highlight the residual plasma membrane association observed with the GAP1IP4BP-(R604C) and -(K591T) mutants. It should also be noted that the GAP1IP4BP-(R604C) had an increased presence in the nucleus of serum-starved cells. This was, however, absent from cells co-transfected with p110CAAX. Image analysis was performed by measuring the intensities according to pixel brightness along cell transects on the depicted cells. Similar data were observed in an additional 10 cells for each construct.
current study we have used molecular modeling to identify residues within the GAP1IP4BP PH domain that are required for high affinity binding to PtdIns(4,5)P2. We have highlighted three residues, lysine 591, arginine 604, and lysine 616, as potentially providing the necessary interactions for PtdIns(4,5)P2 binding. By mutating each residues into the equivalent residue in GAP1m, we have shown that whereas the GAP1IP4BP-(K616P) mutant binds PtdIns(4,5)P2 and PtdIns(3,4,5)P3 with affinities comparable with wild-type, GAP1IP4BP-(R604C) has a 6-fold reduction in its affinity for PtdIns(4,5)P2 and

![Image of subcellular localization of wild-type GAP1IP4BP, GAP1m, ARNO, and the GAP1IP4BP mutants in PC12 cells treated with either 100 nM wortmannin or 100 ng/ml EGF. Expression constructs encoding GFP-GAP1IP4BP, GAP1m, and ARNO, and the various GAP1IP4BP mutants were transiently transfected into PC12 cells. 24 h after transfection cells were serum-starved for 2 h prior to incubation with either 100 nM wortmannin (37°C for 30 min) or 100 ng/ml EGF (37°C for 2 min). Wild-type GAP1IP4BP, GAP1m, and ARNO were then fixed, mounted, and detected by confocal microscopy. The various GAP1IP4BP mutants were fixed and detected by indirect immunofluorescence as described under “Experimental Procedures.” Again residual plasma membrane association was observed with the GAP1IP4BP-(R604C) and -(K591T) mutants, and GAP1IP4BP-(R604C) had an increased presence in the nucleus of serum-starved, wortmannin-treated cells. Image analysis was performed by measuring the intensities according to pixel brightness along cell transsects on the depicted cells. Similar data were observed in an additional 10 cells for each construct.

![Figure 6](https://example.com/figure6.png)
PtdIns(3,4,5)P₃. In contrast, GAP₁¹⁴⁸R⁻(K951T) has an affinity for PtdIns(4,5)P₂ that is reduced 6-fold, whereas the affinity for PtdIns(3,4,5)P₃ is reduced only 2-fold. Through the isolation of GAP₁¹⁴⁸R⁻(K951T) we have therefore engineered a PH domain that displays a phosphoinositide-binding profile reminiscent of that observed for the corresponding domain from GAP₁.

Such a conclusion is further supported by our examination of the subcellular localization of the GAP₁¹⁴⁸R⁻ mutants. Whereas GAP₁¹⁴⁸R⁻(K616P) retains the constitutive plasma membrane association observed for wild-type GAP₁¹⁴⁸R⁻, the GAP₁¹⁴⁸R⁻(R604C) and (K951T) mutants are no longer capable of associating with the plasma membrane. Rather, consistent with their reduced affinity for PtdIns(4,5)P₂, these proteins are like GAP₁ in being located within the cytosol. Importantly, following growth factor stimulation GAP₁¹⁴⁸R⁻(K951T) becomes associated in a PtdIns(3,4,5)P₃-dependent manner with the plasma membrane. These data emphasize that subtle changes in the phosphoinositide-binding profile of the GAP₁¹⁴⁸R⁻ PH domain can have a pronounced effect on the subcellular distribution and regulation of this protein.

Apart from the study described here, the dramatic effects that subtle changes can have on phosphoinositide binding is most apparent when considering the diglycine verses triglycine forms of Grp1 (39, 41), a member of the cytohesin family of ADP-ribosylation factor nucleotide exchange factors. Here, the addition of a single glycine residue to the PH domain can have a significant effect on the phosphoinositide-binding specificity; the triglycine form shows much less discrimination between PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ than the diglycine form. How the extra glycine causes the increase in affinity for PtdIns(4,5)P₂ is difficult to comprehend. One possibility is that the third glycine opens up the binding pocket, allowing the PtdIns(4,5)P₂ to enter in an orientation, possibly more akin to the PtdIns(4,5)P₂-binding site in the PH domain of PLC-δ₁ (19), that is sterically hindered in the diglycine form (42, 43). It is tempting to extrapolate this idea and speculate that although PtdIns(3,4,5)P₃ may bind to the GAP₁¹⁴⁸R⁻ PH domain in a similar orientation to that observed in Btk (25), the binding of PtdIns(4,5)P₂ may be similar to that observed in PLC-δ₁ whereby the PtdIns(4,5)P₂ molecule is rotated 180° through the P1/P4 axis of the inositol ring (19). This could lead to lysine 591 having a significant role in the binding of PtdIns(4,5)P₂, but because of the inositol head group orientation may not play such a significant role in PtdIns(3,4,5)P₃ binding.

In summary therefore, through the use of molecular models we have successfully predicted residues that allow the PH domain from GAP₁¹⁴⁸R⁻ to bind PtdIns(4,5)P₂ with high affinity. We have demonstrated that a single targeted site-directed mutant, GAP₁¹⁴⁸R⁻(K951T), while retaining its ability to bind PtdIns(3,4,5)P₃ with high affinity, has a specific reduction in its affinity for PtdIns(4,5)P₂. Furthermore, this subtle alteration in phosphoinositide binding is translated into a dynamic change in the regulation of the subcellular localization of the mutant protein following growth factor stimulation.

Acknowledgments—We thank the Medical Research Council for providing Infrastructure Award G450006 to establish the School of Medical Sciences Cell Imaging Facility and Mark Jeppson and Alan Leard for assistance.

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